NIH Public Access
Author Manuscript
J Neurosci. Author manuscript; available in PMC 2009 November 20.

Published in final edited form as:
J Neurosci. 2009 May 20; 29(20): 6700–6709. doi:10.1523/JNEUROSCI.0233-09.2009.

NIH-PA Author Manuscript

A retrograde neuronal survival response: Target-derived neurotrophins regulate MEF2D and bcl-w

Maria F. Pazyra-Murphy[1,3], **Aymeric Hans**[1,2,3], **Stephanie L. Courchesne**[1], **Christoph Karch**[1,4], **Katharina E. Cosker**[1], **Heather M. Heerssen**[1,5], **Fiona L. Watson**[1,6], **Taekyung Kim**[7], **Michael E. Greenberg**[7], and **Rosalind A. Segal**[1,8]

[1] Department of Neurobiology, Harvard Medical School, and Departments of Cancer Biology and Pediatric Oncology, Dana-Farber Cancer Institute, Washington and Lee University, Lexington, VA

[6] Department of Biology and Neuroscience Program, Washington and Lee University, Lexington, VA

[7] Department of Neurobiology, Children's Hospital and Harvard Medical School

SUMMARY

Survival and maturation of dorsal root ganglia sensory neurons during development depends on target-derived neurotrophins. These target-derived signals must be transmitted across long distances to alter gene expression. Here we address the possibility that long-range retrograde signals initiated by target-derived neurotrophins activate a specialized transcriptional program. The transcription factor MEF2D is expressed in sensory neurons; we show that expression of this factor is induced in response to target-derived neurotrophins that stimulate the distal axons. We demonstrate that MEF2D regulates expression of an anti-apoptotic *bcl-2* family member, *bcl-w*. Expression of *mef2d* and *bcl-w* is stimulated in response to activation of a Trk-dependent ERK5/Mef2 pathway, and our data indicate that this pathway promotes sensory neuron survival. We find that *mef2d* and *bcl-w* are members of a larger set of retrograde response genes, which are preferentially induced by neurotrophin stimulation of distal axons. Thus activation of an ERK5/MEF2D transcriptional program establishes and maintains the cellular constituents of functional sensory circuits.

Keywords

Neurotrophin; signal transduction; survival; Sensory Neurons; Transcription; Dorsal Root Ganglion [Drg]

INTRODUCTION

Nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) can elicit a wide variety of cellular responses, including proliferation, migration, survival, axonal outgrowth, synapse formation and plasticity (Segal, 2003; Chao et al., 2006; Reichardt, 2006). Some of this diversity reflects the two classes of neurotrophin receptors, Trk receptor tyrosine kinases and p75NTR (Casaccia-Bonnefil et al., 1999; Patapoutian and Reichardt, 2001), as Trks and

8To whom correspondence should be addressed: Rosalind Segal Dana-Farber Cancer Institute 44 Binney Street Boston, MA 02115 617-632-4737, E-mail: Rosalind_segal@dfci.harvard.edu.
[2]Current Address: French Food Safety Agency (AFSSA), LERPE 14430 Dozule', France
[3]These two authors contributed equally
[4]Current Address: Newton, MA
[5]Current Address: Helix Medical Communications, San Mateo, CA

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

p75NTR induce distinct responses (Yano and Chao, 2000; Lee et al., 2001; Roux and Barker, 2002; Lu et al., 2005; Hempstead, 2006). However, cellular localization of Trk receptor activation could further specify responses (Ginty and Segal, 2002; Segal, 2003). Neurotrophins were first identified as target-derived trophic factors that bind receptors on axon terminals (Levi-Montalcini, 1968) and elicit both local and long-range effects. Rapid responses initiated at axon terminals by neurotrophins influence direction and extent of axonal growth through kinases, phosphatases and G-proteins that quickly modulate cell morphology or intracellular trafficking (Segal, 2003; Reichardt, 2006). Axon terminal Trk receptors also stimulate long-term changes (Lom and Cohen-Cory, 1999) that depend on transcriptional programs involving factors such as CREB, c-fos, FOXO, NFAT, and MEF2 (Segal et al., 1992; Finkbeiner et al., 1997; Graef et al., 2003; Shalizi et al., 2003; Gan et al., 2005; Zhou and Snider, 2006). These long-term responses to distal axon neurotrophin stimulation require retrograde signaling from the axon terminal to the cell body. Location of neurotrophin stimulation is important for rapid local responses, for example, neurotrophin stimulation reorients axonal growth towards a trophic factor (McCaig et al., 2000; Markus et al., 2002; Zhou and Snider, 2006). However, it is less clear whether location of neurotrophin stimulation also specifies long-term transcriptional responses.

Here we consider whether transcriptional responses to neurotrophins depend on location of stimulation. NGF in the skin promotes survival, axonal growth and differentiation of nociceptive neurons of dorsal root ganglia (Chen et al., 1999), while BDNF promotes survival and differentiation of dorsal root ganglia neurons that innervate specialized touch receptors (Carroll et al., 1998; Watanabe et al., 2000; Gonzalez-Martinez et al., 2005). Therefore, to identify transcriptional programs that utilize spatial information, we focus on survival of developing dorsal root ganglion neurons in response to NGF and BDNF stimulation of distal axons. We show the transcription factor MEF2D and the anti-apoptotic Bcl-2 family member Bcl-w (Bcl-2l2) are expressed in sensory neurons and are regulated by target-derived neurotrophins and retrograde signaling pathways. *Bcl-w* and *mef2d* are members of a set of retrograde response genes preferentially induced by neurotrophin stimulation of distal axons compared to neurotrophin stimulation of cell bodies. We demonstrate that neurotrophin-induced expression of *bcl-w* is regulated by ERK5 and MEF2D. Thus, target-derived neurotrophins differentially activate a MEF2 transcriptional program that regulates sensory neuron survival.

Materials and Methods

Cell cultures

Compartmented chamber cultures (Campenot cultures) were prepared as described previously (Heerssen et al., 2004). Briefly, dorsal root ganglia (DRGs) from embryonic day 15 (E15) rats were dissected and plated in the center compartment of a Teflon divider (Camp10, Tyler Research, Canada) (Campenot, 1982). Cultures were maintained in media consisting of DMEM with 5% horse serum, 1% penicillin-streptomycin, and 0.3 μM cytosine arabinoside (AraC) at 37°C, 7.5% CO_2; neurotrophins were added to the cell body compartment at 10 ng/ml BDNF (Peprotech) and 10 ng/ml NGF (Peprotech) and to the axon compartment at a concentration of 100 ng/ml BDNF and 100 ng/ml NGF (100 ng/ml NGF+BDNF) for 3 days. On Day 4, media was replaced and the 0.3 μM AraC omitted. On Day 6, neurotrophins were removed from the cell body compartment and reduced to 1 ng/ml in axon compartments for 3–4 days (unless otherwise specified). For each experiment, vehicle control (100 ng/ml BSA in PBS) or neurotrophins (100 ng/ml NGF+BDNF in vehicle, NGF or BDNF individually) were applied to distal axon or cell body compartments for the indicated time.

Mass cultures consisting of 2.5×10^5 DRG neurons were grown on laminin coated p35 culture dishes for 2 days in neurotrophin-enriched (100 ng/ml NGF+BDNF) media with 0.3 μM AraC,

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

followed by 3 days in 10 ng/ml neurotrophins without AraC. Mass cultures were changed to serum and neurotrophin-free media for 2 hours and then stimulated for the indicated time with neurotrophins (100 ng/ml NGF+BDNF) or vehicle control.

Luciferase Assays

COS cells were maintained in DMEM containing 10% fetal calf serum and 1% penicillin-streptomycin at 37°C, 5% CO_2. Firefly luciferase reporter plasmids were transfected into COS cells or DRG neurons using FuGENE 6 (Roche) or by nucleofection (Amaxa Biosystems), respectively, together with TK-pRL, which expresses Renilla luciferase and serves as an internal control. Firefly and Renilla luciferase activity was assessed 72 hours after cell transfection. The results shown represent the average of 8 independent experiments, with three replicates each.

Plasmids

3xMRE-Luc, MEF2-VP16 plasmids were described previously (Flavell et al., 2006). plenti-hU6BX RNAi plasmids and TK-pRL were from Cellogenetics (Baltimore, MD) and Promega (Madison, WI), respectively. The *bcl-w* plasmid was the generous gift of Susan Cory (The Walter and Eliza Hall Institute of Medical Research). To generate the *bcl-w* promoter construct, we cloned the 2.5kB upstream of the *bcl-w* transcription starting site into the pGL3-basic luciferase plasmid. The dominant negative MEK5 (S311A, T315A) and constitutively active MEK5 (S311D, T315D) were obtained from S. Gutkind (NIH). The siRNA against MEF2D was designed and inserted in a lentiviral system as described previously (Flavell et al., 2006).

Reagents

We used the following antibodies: MEF2D (1:2500 for Western blot and 1:500 for immunostaining; BD Transduction Labs), Bcl-w (1:500; Chemicon), GFP (1:1000; Roche), pan-actin (1:1000; Cell Signaling), phospho-ERK5 (1:1000; Cell Signaling), ERK5 (1:500; Cell Signaling), phospho-ERK1/2 (1:1000; Cell Signaling), HA (1:1000; Cell Signaling), Tubulin (1:500; Sigma), phospho-Mef2 (1:500; Abcam); secondary antibodies conjugated to HRP (1:10,000; BioRad), Alexa-488 and Alexa-546 (1:1000; Molecular Probes). MEF2D 6066 (1:1000 for Western blot and 1:100 for immunostaining) was developed in the Greenberg lab, and generated by injecting antigen (a fragment of MEF2D protein, amino acids 292-514; see Han et al., 1995) into rabbit. Characterization and specificity of this antibody are shown in Supplemental Figure 2.

Drug treatment

Distal axons and cell bodies of DRG neurons grown in compartmented cultures were treated either with 200 nM K252A (Calbiochem), 10 μM UO126 (Calbiochem), 50 μM LY294002 (Calbiochem), or DMSO vehicle control for 30 minutes before neurotrophin stimulation.

Real-time quantitative RT-PCR

RNA was harvested from DRG neurons in compartmented chamber cultures or in mass cultures using the RNAqueous-4PCR kit (Ambion). For *in vivo* analysis, DRG neurons from embryonic day 18 (E18) rats were dissected and RNA was extracted using TRIzol (Invitrogen). Reverse transcription was performed using the cDNA archive kit (Applied Biosystems) according to manufacturer's specifications. Quantitative real-time RT PCR was performed using Taqman Gene expression assays (Applied Biosystems) to assess the expression of *c-fos* (Rn02105452_s1), *bcl-w* (Rn00821025_s1), *alsin* (Mm00511865_m1), *igf-1* (Rn00710306_m1), *decorin* (Rn01503161_m1), *enpep* (Rn00573861_m1) and *mef2d* (Rn00578329_m1). Data were normalized by the expression level of *gapdh* RNA for each sample (Applied Biosystems). Significance was calculated by z-test.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Protein analysis

Cells were lysed in a non-ionic detergent. Protein lysates from 8–10 compartmented cultures were pooled and equal amounts of protein were separated by 7% SDS-PAGE, analyzed by immunoblot and visualized using the SuperSignal chemiluminescent substrate kit (Pierce).

Immunostaining

For in vitro analysis, compartmented chamber cultures were fixed in 4% paraformaldehyde (PFA) for 20 minutes, permeabilized in 0.1% TritonX for 10 minutes and blocked in 5% normal goat serum and 0.1% TritonX for 60 minutes. Cultures were incubated overnight at 4°C in MEF2D (1:500) followed by incubation in goat anti-mouse Alexa 546 (1:1000) for one hour at room temperature.

For in vivo analysis, MEF2D positive cells were counted from post-natal day 0 (P0) *bcl-w* +/− or *bcl-w* −/− animals. Animals were sacraficed and fixed in 4% PFA, cyropreserved in 30% sucrose, and 14 μm slices were prepared. Tissue was subjected to antigen retrieval by 10 mM sodium citrate buffer for 15 minutes at 95°C, followed by 30 minutes cooling at room temperature. Tissue sections were permeabilized in 0.5% TritonX for 10 minutes and blocked in 5% normal goat serum and 0.1% TritonX for 60 minutes. Sections were incubated overnight at 4°C in MEF2D 6066 (1:100) followed by incubation in goat anti-rabbit Alexa 488 (1:1000) for one hour at room temperature. Three animals of each genotype were used, and 3–6 dorsal root ganglia from each lumbar and cervical region were counted. Significance was calculated by Student's two-tailed t-test.

siRNA knockdown

For infection of mass cultures or compartmented cultures with siRNA lentivirus constructs, neurons at 3 days and 5 days, respectively, were treated with either control Sh6L3-GFP siRNA or Mef2D-GFP siRNA with polybrene (4 ug/ml) for 48 hours. After infection, media was changed to DMEM only for 2 hours and then cells were stimulated with 100 ng/ml NGF+BDNF or vehicle control for 2 hours. Neurons were harvested and protein levels of MEF2D were analyzed by Western blot. RNA was extracted from cell bodies of compartmented chamber cultures using TRIzol (Invitrogen). Quantitative real-time RT PCR was performed using Taqman Gene expression assays (Applied Biosystems) to assess the expression of *c-fos, bcl-w* and *mef2d*. Data were normalized by the expression level of *gapdh* RNA for each sample (Applied Biosystems). Significance was calculated by z-test.

Survival assay

DRG neurons grown in compartmented cultures for 6 days were transferred to serum-free media or to serum-free media supplemented with 100 ng/ml NGF+BDNF in the distal axon or cell body compartments only. Inhibitors were added to the cell body and distal axon compartments as indicated. We exchanged the media every 12 hours to replenish inhibitors and to guard against leakage between compartments. After 72 hours, cultures were fixed in 4% paraformaldehyde and apoptotic cells were visualized using the DeadEnd Fluorometric TdT-mediated dUTP nick end labeling (TUNEL) system kit (Promega), using biotinylated dUTP (5 μM) and Cy3-conjugated avidin (1:1,000), and counterstained for 1 minute with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1,000). Each data point represents the mean of five fields per culture from 6–12 cultures in 2–6 different experiments scored in a blind fashion. Significance was calculated by Student's t-test. For in vivo analysis, condensed nuclei using DAPI (1:1000) were counted from post-natal day 0 (P0) *bcl-w* +/+ or *bcl-w* −/− animals. Three animals of each genotype were used, and 3–6 dorsal root ganglia from each lumbar and cervical region were counted. Significance was calculated by Student's two-tailed t-test.

NIH-PA Author Manuscript

Animal use

Timed pregnant rats were purchased from Charles River. *Bcl-w* −/− mice were a generous gift from Grant MacGregor (UC Irvine) (Ross et al., 1998). All experimental procedures were done in accordance with the National Institutes of Health (NIH) guidelines and were approved by the Dana-Farber Cancer Institutional Animal Care and Use Committee.

RESULTS

MEF2D expression is regulated by neurotrophin stimulation of axons, and is important for survival of sensory neurons

Neurotrophin stimulation of distal axons in sensory neurons has previously been shown to increase expression levels of the transcription factors *c-fos* and *c-jun* (Watson et al., 1999). These transcription factors are rapidly induced by neurotrophin stimulation of either cell bodies or distal axons, and so are unlikely to be critical for spatially selective responses to neurotrophin stimulation. MEF2D is a transcription factor that can also be regulated by neurotrophin stimulation (Liu et al., 2003; Shalizi et al., 2003). Therefore we asked whether this transcription factor is expressed by sensory neurons at the developmental time period when they are sensitive to neurotrophin levels. As shown in Figures 1A and 1B, MEF2D is expressed by dorsal root ganglia in late embryonic stages (E18- birth) *in vivo*. We used quantitative RT-PCR to ask whether *mef2d* levels are regulated by neurotrophin stimulation of distal axons. Using sensory neurons grown in compartmented cultures, we find that the level of *mef2d* mRNA is increased within 2 hours after selective neurotrophin stimulation of distal axons (Figure 1C). In contrast, when neurotrophins are applied instead to cell bodies of sensory neurons grown in compartmented cultures, *mef2d* mRNA levels do not increase. We next asked whether this spatial selectivity is maintained when sensory neurons are stimulated with NGF or BDNF alone rather than a combination of these neurotrophins. Each of the individual neurotrophins can induce *mef2d* when applied to the distal axons, but not when applied to the cell bodies (Figure 1D). While either NGF or BDNF alone can induce this response, there is less variability when the two neurotrophins are used in combination. Therefore in subsequent experiments we use a combination of the two neurotrophins to stimulate cultured neurons.

To determine whether the changes in *mef2d* mRNA are likely to have functional consequences, we asked whether neurotrophin stimulation also affects the levels of MEF2D protein. As shown in Figure 1E, there is a consistent increase in MEF2D protein in response to neurotrophin stimulation of the distal axons. Furthermore, MEF2D protein is localized to nuclei both before and after neurotrophin stimulation of sensory neurons (Supplemental Figure 1). Together these data indicate that *mef2d* mRNA and MEF2D protein are expressed in developing sensory neurons and regulated by neurotrophin stimulation of distal axons, and that the protein is appropriately expressed to participate in responses to target-derived neurotrophins.

MEF2 family members have been implicated in diverse processes in the nervous system, including survival, proliferation and synaptic regulation (Mao et al., 1999; Liu et al., 2003; Heidenreich and Linseman, 2004; Shalizi and Bonni, 2005; Flavell et al., 2006; Shalizi et al., 2006). To determine whether neurotrophin stimulation of *mef2d* might participate in neurotrophin-dependent survival of sensory neurons, we reduced the level of MEF2D using *mef2d* RNAi expressed in lentivirus (Figure 2A), and then tested the ability of neurotrophins to promote survival in compartmented cultures. After three days in neurotrophin-free media, the majority of control sensory neurons undergo apoptosis, as assessed by TUNEL staining. Neurotrophin stimulation of either cell bodies or distal axons maintains neuronal survival (Figure 2B,C). In cultures where MEF2D has been reduced by lentivirus RNAi, neurotrophin stimulation of the distal axons is unable to prevent apoptosis (Figure 2B,C). However, neurotrophin stimulation of cell bodies does rescue the survival of some, although not all,

NIH-PA Author Manuscript

sensory neurons. These data indicate that MEF2D is required for neurotrophin-dependent survival of developing sensory neurons, particularly when neuronal survival depends on retrograde signaling mechanisms.

The ERK5/MEF2D pathway regulates *bcl-w* and promotes neuronal survival

We previously found that ERK5 is involved in retrograde signaling and plays an important role in neurotrophin-mediated survival (Watson et al., 2001). Consistent with previous studies, activated ERK5 can phosphorylate, hence activate MEF2 (Figure 3A) (Kasler et al., 2000; Seyfried et al., 2005). Stimulation of sensory neurons by neurotrophins, applied either to cell bodies (Figure 3B) or to distal axons (Figure 3C), increases MEF2 phosphorylation by 17 ± 6 % and 20 ± 8 %, respectively. To determine whether ERK5, like its substrate MEF2D, has a prominent role in the survival of neurons that depend on retrograde signaling mechanisms, we tested the effects of an ERK5 dominant negative construct (DN-ERK5). As shown in Figure 3D, this construct is able to prevent ERK5 activation. When tested in compartmented cultures, this dominant inhibitory construct decreases sensory neuronal survival as assessed by an increase in apoptosis (Figure 3E). Furthermore, like *mef2d* RNAi, DN-ERK5 has a greater effect on neuronal survival when neurons are supported by neurotrophin stimulation of distal axons compared to survival when neurons are supported by neurotrophin stimulation of cell bodies. Thus, ERK5 and MEF2D both contribute to neuronal survival responses initiated by target-derived neurotrophins. These data suggest that an ERK5/MEF2D pathway is needed for retrograde signaling events that promote survival.

As MEF2D is a transcription factor, we postulate that the ERK5/MEF2D pathway stimulates expression of anti-apoptotic components, such as members of the bcl-2 family. As shown in Figure 4A, the anti-apoptotic gene *bcl-w* is upregulated by neurotrophin stimulation of distal axons by NGF+BDNF, NGF alone, or BDNF alone, and not by cell body stimulation. Similarly, when we analyzed Bcl-w protein levels after cell body or distal axon neurotrophin stimulation, western blotting revealed a significant increase in Bcl-w protein levels (32% ± 5%) upon distal axon stimulation, with no increase observed with cell body stimulation (Figure 4B). The pattern of induction observed for *bcl-w* resembles that shown for *mef2d*, in that expression is selectively induced by neurotrophin stimulation of distal axons. We therefore tested induction of *mef2d* and *bcl-w* following pharmacologic and genetic interventions that prevent activation of the ERK5 pathway, which is needed for retrograde survival responses. Induction of *c-fos* serves as a positive control in all experiments, as *c-fos* is induced in response to neurotrophin stimulation of either cell bodies or of distal axons. Both *bcl-w* and *mef2d* induction require Trk activation, and so are prevented by K252A, an inhibitor of Trk kinase activity; Trk kinase activity is also required for induction of *c-fos* (Figure 4C). Like *c-fos* induction, induction of *bcl-w* and *mef2d* are prevented by UO126, a pharmacologic agent that inhibits MEK1 and MEK5 and thus prevents activation of both ERK1/2 and ERK5 (Figure 4D). In contrast, DN-MEK5, which inhibits activation of ERK5 but not ERK1/2, prevents induction of *bcl-w* and *mef2d* but does not affect *c-fos.* We postulate that ERK5 acts by regulating MEF2D activity. Therefore, we asked whether inhibition of MEF2D has the same effect on gene expression as DN-MEK5. As shown, *mef2d* RNAi prevents induction of *bcl-w*, but it does not affect induction of *c-fos* (Figure 4E). Together these data indicate that distal axon stimulation by neurotrophins regulates expression of *bcl-w* by a pathway that involves Trk/ERK5 and MEF2D, and that the mechanisms regulating *bcl-w* and *mef2d* expression diverge from pathways that regulate *c-fos*.

To explore the relationship between retrograde signals and neurotrophin-dependent responses originating from activated receptors in the plasma membrane of the cell soma, we asked whether simultaneous neurotrophin stimulation of distal axons and cell bodies of dorsal root ganglion neurons grown in mass culture induces these retrograde response genes. Global

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

stimulation leads to dramatic upregulation of *c-fos*; in contrast, *mef2d* and *bcl-w* are only induced by localized stimulation of distal axons (Figure 4F). We find that neurotrophin stimulation of cell bodies only increases *mef2d* and *bcl-w* mRNA levels when combined with a second intervention that can activate ERK5 (Turjanski et al., 2007), such as viral infection or mechanical stress (Supplemental Figure 4), not when combined with neurotrophin stimulation of distal axons (Figure 4F). Of note, when cultures were maintained for 5–7 days before testing as in Figure 4F, the extent of *mef2d* and *bcl-w* induction was lower than that seen in our usual paradigm wherein cultures are maintained for 8 days before stimulation. These data suggest that induction of *mef2d* and *bclw* may be more robust following neurotrophin stimulation of distal axons in neurons with longer processes.

As increases in *bcl-w* and *mef2d* in response to neurotrophin stimulation occur at the mRNA level, and induction of *bcl-w* depends on the transcription factor MEF2D, we postulate that induction of these retrograde response genes is mediated (at least in part) at the transcriptional level. To test this directly, we prepared a *bcl-w* promoter luciferase construct by fusing the 2.5 kB of upstream sequence from the mouse *bcl-w* promoter to luciferase. As shown (Figure 5A), this construct contains one of three conserved consensus MEF2D binding sites upstream of the start site. When expressed in sensory neurons, the *bcl-w*-luciferase construct is induced by neurotrophin stimulation of distal axons (Figure 5B). To determine whether this regulation depends on an ERK5/MEF2D pathway, we analyzed the response of the *bcl-w*-luciferase construct to constitutive activation of ERK5 or MEF2D in COS cells. Co-expression of constitutively active MEK5 and wild type ERK5 increases expression of *bcl-w*-luciferase (Figure 5C). However, no induction is seen when kinase-inactive MEK5 is expressed together with ERK5. As controls, we assessed the response of a basal promoter, and an MRE-containing (MEF2 Response Element) promoter. While activated MEK5/ERK5 induces expression of *bcl-w*-luciferase and MRE-luciferase, it does not induce expression of basal constructs. To determine whether *bcl-w* transcription is also mediated by the ERK5 substrate MEF2, we expressed a constitutively active MEF2 construct (VP16-MEF2) together with *bcl-w*-luciferase and Renilla luciferase control (Figure 5D). Constitutively active MEF2 stimulates *bcl-w*-luciferase and the MRE luciferase, but does not induce expression of a basic luciferase construct. Together these studies indicate that *bcl-w* transcription can be induced by the ERK5/MEF2D pathway, and that relevant response elements are contained in the 2.5 kB region upstream of the *bcl-w* start site. This may include other promoter elements; for example two sites that resemble half of a CRE binding motif are also present in the bcl-w promoter region (−9871 to−9867 and 2048 to 2052, TGACG).

Bcl-w, and other retrograde response genes, promote neuronal survival

Bcl-w has not previously been shown to play a role in dorsal root ganglia neuron survival. To determine whether Bcl-w is important for survival of these spinal sensory neurons, we analyzed dorsal root ganglia neurons from *bcl-w* −/− mice. These mice are viable, but males are infertile due to apoptosis in the developing testes (Print et al., 1998; Ross et al., 1998). Levels of apoptosis were analyzed *in vivo* in DRGs from P0 *bcl-w* −/− and wild type littermates. Cervical and lumbar sections were stained with DAPI and assessed for condensed nuclei as a measure of apoptosis (Figure 6A). DRGs from either the cervical or lumbar regions of *bcl-w* −/− mice exhibited significantly higher levels of dying cells compared to wild type (Figure 6B).

As MEF2D regulates *bcl-w* and thereby promotes survival, we predict that MEF2D-expressing cells would show decreased survival in the absence of Bcl-w. Therefore, we asked whether the percentage of sensory neurons that express MEF2D is decreased in *bcl-w* −/− mice. As shown, 30–40% of cervical and lumbar neurons express MEF2D in control neonatal animals. The MEF2D positive cells include neurons that are of various sizes, indicating that this transcription factor is expressed by multiple types of sensory neurons. In *bcl-w* −/− animals, the pool of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

MEF2D expressing neurons is reduced by 23% (Percentage of DRG neurons that are MEF2D positive is 37% in control and 28% in *bcl-w* −/− mice; Supplemental Figure 3). Thus, *in vivo*, bcl-w promotes the survival of DRG neurons, and particularly affects MEF2D-expressing cells.

We then assessed the survival of dorsal root ganglia sensory neurons from *bcl-w* −/− mice in compartmented culture conditions. These neurons were grown for one week with neurotrophins present in the distal axon compartment, at which point Bcl-w is expressed in wild type cultures. As shown in Figure 6C, Bcl-w is important for survival of dorsal root ganglia neurons. Indeed, survival of *bclw* −/− sensory cells is compromised (although not eliminated) at three days with, or without, neurotrophins. We were surprised to find that *bcl-w* −/− neurons exhibit reduced survival during neurotrophin deprivation, and under conditions where they are sustained by neurotrophins applied to the cell bodies or distal axons. This differs from the results seen when ERK5 or MEF2D activity is inhibited, in that sensory neurons that depend on target-derived neurotrophins are particularly sensitive to inhibition of ERK5 or MEF2D. Therefore, we asked whether ERK5/MEF2D might regulate additional retrograde response genes as well as *bcl-w*. Other retrograde response genes might contribute to the differential effect of *mef2d* RNAi and DN ERK5 on the survival of neurons supported by neurotrophin stimulation of distal axons or cell bodies. We used quantitative RT-PCR to examine a panel of candidate genes that were induced by neurotrophin stimulation of distal axons in Affymetrix gene arrays, and we identified several genes that are preferentially induced by retrograde signaling (Figure 6D). The genes identified as retrograde response genes include *igf-1* and *alsin*, which encode proteins that have been implicated in promoting neuronal survival (Boillee and Cleveland, 2004; Bondy and Cheng, 2004; Davila et al., 2007). Taken together these studies indicate that neurotrophin stimulation of distal axons activates ERK5/MEF2D to initiate a transcriptional program that includes the anti-apoptotic gene *bcl-w* as well as a set of additional retrograde response genes. Thus the ERK5/MEF2 pathway, and the resultant gene expression program, is important for neuronal responses to target-derived neurotrophins.

Discussion

The mechanisms by which target-derived neurotrophic factors initiate and propagate survival signals from nerve terminals to a remote neuronal cell body have been the focus of investigation over many years (Hamburger and Levi-Montalcini, 1949; Hendry et al., 1974; Purves, 1986; Korsching, 1993; Campenot, 1994; Ahn et al., 2000; Neet and Campenot, 2001; Heerssen and Segal, 2002; Segal, 2003; Moises et al., 2007). Here, we address the role of transcriptional regulation in mediating responses to target-derived survival signals. We show that selective neurotrophin stimulation of distal axons of sensory neurons results in increased expression of the transcription factor MEF2D. Activation and expression of MEF2D requires the ERK5 MAP kinase. Both ERK5 and MEF2D promote survival of developing sensory neurons; one mechanism for this effect is induction of the anti-apoptotic gene *bcl-w*. An intriguing feature of this system is that this ERK5/MEF2D survival pathway is predominantly important for the survival of neurons that depend exclusively on neurotrophin support applied to distal axons. Thus, we have identified a transcriptional program that enables neurons to survive once they have contacted a correct target and are incorporated into a functional neural circuit.

Much of the recent work on long-range signals has focused on the mechanisms by which a signal initiated by an activated receptor at the nerve terminal travels retrogradely to a remote cell body to initiate transcriptional changes (Ginty and Segal, 2002). Abundant evidence indicates that neurotrophin binding to Trk receptors initiates both activation and endocytosis of the receptor (Figure 7A), and the resultant signaling endosomes, containing the ligand-receptor complex, travel by dynein-dependent transport (Figure 7B) (Grimes et al., 1996; Riccio et al., 1997; Senger and Campenot, 1997; Tsui-Pierchala and Ginty, 1999; Watson et al., 1999; Howe et al., 2001; Neet and Campenot, 2001; Watson et al., 2001; Weible, 2nd et

al., 2001; Delcroix et al., 2003; Heerssen et al., 2004). These specialized signaling endosomes activate a variety of second messenger signaling cascades as they are transported through the axon to the cell soma, including MAP kinases, as well as PI3 kinase and Akt (Kuruvilla et al., 2000; Watson et al., 2001; Delcroix et al., 2003; Wu et al., 2001). The ensuing activation of transcription factors, such as CREB, NFAT, and AP-1 mediate the necessary changes in gene expression in response to target-derived neurotrophins (Riccio et al., 1997; Riccio et al., 1999; Watson et al., 1999; Graef et al., 2003). In this way, dynein-dependent transport eliminates the barrier of distance, and allows transcriptional changes in response to target-derived neurotrophins.

Several lines of evidence suggest that transport of signaling endosomes also provides a mechanism by which a neuron can distinguish the location of neurotrophin stimulation, and so adjust the nature of its response accordingly. Retinal ganglion cells exhibit increased dendritic arborisation in response to BDNF applied at the axon terminals, while dendritic arborisation is decreased by BDNF stimulation of dendrites within the retina, indicating that the biological response to neurotrophins varies based on location of stimulation (Lom et al., 2002). Here, we provide evidence of additional differential responses and of the underlying mechanisms that account for these spatial distinctions. Our studies have outlined a long-distance signaling pathway that requires activation of ERK5 and MEF2D to initiate a spatially distinct transcriptional program (Figure 7C, D). This program promotes expression of retrograde response genes, which we define as a set of genes selectively induced by neurotrophin stimulation of distal axons. The retrograde response gene set includes *bcl-w* and *mef2d,* as well as *igf-1* and *alsin* (Figure 7E). These retrograde response genes in aggregate represent an ERK5-regulated expression program that may contribute to distinct biological responses to neurotrophins depending on the location of stimulation.

Gene expression programs regulated by ERK5 or other MAPK cascades are specified by the distinct ERK involved, the kinetics of pathway activation and repression, the subcellular localization of the pathway components, and cross-talk among distinct kinase cascades (Raman et al., 2007). Each of these features may contribute to the selective induction of retrograde response genes by distal axon stimulation, with little or no induction by cell body or global stimulation. It is possible that cell body stimulation initiates both the ERK5/MEF2 pathway and a signaling pathway that counteracts ERK5-dependent gene induction (Figure 7F). As cell body stimulation simultaneously activates the ERK5 and ERK1/2 pathways (Watson et al., 2001), the lack of induction of retrograde response genes may reflect the balance of timing or activity of these distinct MAPKs. Under conditions of heightened ERK1/2 activity, such as cell body stimulation, the effects of ERK5 might be limited. However, in the face of heightened ERK5 activity, as occurs during distal axon stimulation or in response to cell body stimulation following virus infection or other stress signals, activation of ERK5 may override negative effects of ERK1/2 and so lead to retrograde response gene induction. This and other mechanistic explanations for the location selectivity of retrograde response gene induction will require extensive additional investigation and modeling to evaluate how the kinetics and localization of MAPK activation are integrated to produce a specific transcriptional response.

Our studies have focused on pathways that are required for long-range signaling that promote neuronal survival. We found that ERK5 is particularly important for survival in neurons that depend on target-derived neurotrophins. Neuronal survival is compromised by inhibition of ERK5 activity, and this effect is most dramatic when neurons are supported solely by neurotrophin stimulation of axons. Likewise, MEF2D has a greater role in survival in neurons that depend exclusively on neurotrophins stimulation of the axons. Our data indicate that the anti-apoptotic molecule *bcl-w*, which is regulated by ERK5 and MEF2D, is important for neuronal survival. However, as survival of Bcl-w −/− sensory neurons is compromised irrespective of the location of neurotrophin stimulation, we postulate that ERK5 and MEF2D

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

regulate additional, anti-apoptotic retrograde response genes. Intriguingly, *igf-I* and *alsin* are among the putative retrograde response genes (Figure 6E). IGF-I promotes neuronal survival by binding to IGF-IR and activating PI3 kinase and Akt (Dudek et al., 1997; Bondy and Cheng, 2004), while the *alsin* gene is mutated in ALS2, an early onset motor neuron degenerative disease (Yang et al., 2001; Eymard-Pierre et al., 2002; Rowland, 2005). Alsin protein is a vesicle-associated molecule that has been implicated in the endocytosis and trafficking of neurotrophin receptors (Devon et al., 2006) and so is potentially important for long-range survival pathways. Thus, we suggest that the retrograde response genes in aggregate encode components needed for the prolonged survival and functioning of mature neurons with long axons.

Induction of MEF2D by target-derived neurotrophins promotes survival of sensory neurons appropriately connected in a functional circuit, and this is mediated in part by increased transcription of *bcl-w*. While our studies have focused on the roles played by ERK5 and MEF2D in neuronal survival in response to target-derived neurotrophins, it is likely that retrograde signaling and retrograde response genes also function in other spatially distinctive responses to neurotrophins. Interestingly, MEF2 transcription factors have been implicated in regulating synaptic connections. Recent studies have shown that a MEF2-dependent transcription program is critical for establishing synaptic morphology and for regulating synapse number (Shalizi and Bonni, 2005; Flavell et al., 2006; Shalizi et al., 2006). Therefore, increased expression of MEF2D in response to neurotrophin stimulation of distal axons may eliminate supernumerary synapses, while preserving neuronal survival. In this way, target-derived neurotrophins may play a role in maintaining the cellular constituents of newly established functional circuits, while simultaneously refining the synaptic connections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Grant MacGregor (UC Irvine), Susan Corey (The Walter and Eliza Hall Institute of Medical Research), and Steve Gutkind (NIH) for the generous gifts of reagents, we thank Todd Golub for advice. We thank Erin Berry, Kelly Dakin, Parizad Bilimoria and Athar Malik for technical assistance. This work was supported by grants from the NIH (RAS, MEG).

References

Ahn S, Riccio A, Ginty DD. Spatial considerations for stimulus-dependent transcription in neurons. Annu Rev Physiol 2000;62:803–823. [PubMed: 10845112]

Boillee S, Cleveland DW. Gene therapy for ALS delivers. Trends Neurosci 2004;27:235–238. [PubMed: 15111001]

Bondy CA, Cheng CM. Signaling by insulin-like growth factor 1 in brain. Eur J Pharmacol 2004;490:25–31. [PubMed: 15094071]

Campenot RB. Development of sympathetic neurons in compartmentalized cultures. II. Local control of neurite survival by nerve growth factor. Developmental Biology 1982;93:13–21. [PubMed: 7128928]

Campenot RB. NGF and the local control of nerve terminal growth. [Review]. Journal of Neurobiology 1994;25:599–611. [PubMed: 8071664]

Carroll P, Lewin GR, Koltzenburg M, Toyka KV, Thoenen H. A role for BDNF in mechanosensation. Nat Neurosci 1998;1:42–46. [PubMed: 10195107]

Casaccia-Bonnefil P, Gu C, Khursigara G, Chao MV. p75 neurotrophin receptor as a modulator of survival and death decisions. Microsc Res Tech 1999;45:217–224. [PubMed: 10383114]

Chao MV, Rajagopal R, Lee FS. Neurotrophin signalling in health and disease. Clin Sci (Lond) 2006;110:167–173. [PubMed: 16411893]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Chen WP, Chang YC, Hsieh ST. Trophic interactions between sensory nerves and their targets. J Biomed Sci 1999;6:79–85. [PubMed: 10087438]

Curran T, Morgan JI. Superinduction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepines. Science 1985;229:1265–1268. [PubMed: 4035354]

Davila D, Piriz J, Trejo JL, Nunez A, Torres-Aleman I. Insulin and insulin-like growth factor I signalling in neurons. Front Biosci 2007;12:3194–3202. [PubMed: 17485293]

Delcroix JD, Valletta JS, Wu C, Hunt SJ, Kowal AS, Mobley WC. NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron 2003;39:69–84. [PubMed: 12848933]

Devon RS, Orban PC, Gerrow K, Barbieri MA, Schwab C, Cao LP, Helm JR, Bissada N, Cruz-Aguado R, Davidson TL, Witmer J, Metzler M, Lam CK, Tetzlaff W, Simpson EM, McCaffery JM, El-Husseini AE, Leavitt BR, Hayden MR. Als2-deficient mice exhibit disturbances in endosome trafficking associated with motor behavioral abnormalities. Proc Natl Acad Sci U S A 2006;103:9595–9600. [PubMed: 16769894]

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661–665. [PubMed: 9005851]

Eymard-Pierre E, Lesca G, Dollet S, Santorelli FM, di Capua M, Bertini E, Boespflug-Tanguy O. Infantile-onset ascending hereditary spastic paralysis is associated with mutations in the alsin gene. Am J Hum Genet 2002;71:518–527. [PubMed: 12145748]

Finkbeiner S, Tavazoie SF, Maloratsky A, Jacobs KM, Harris KM, Greenberg ME. CREB: a major mediator of neuronal neurotrophin responses. Neuron 1997;19:1031–1047. [PubMed: 9390517]

Flavell SW, Cowan CW, Kim TK, Greer PL, Lin Y, Paradis S, Griffith EC, Hu LS, Chen C, Greenberg ME. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science 2006;311:1008–1012. [PubMed: 16484497]

Gan L, Zheng W, Chabot JG, Unterman TG, Quirion R. Nuclear/cytoplasmic shuttling of the transcription factor FoxO1 is regulated by neurotrophic factors. J Neurochem 2005;93:1209–1219. [PubMed: 15934941]

Ginty DD, Segal RA. Retrograde neurotrophin signaling: Trk-ing along the axon. Curr Opin Neurobiol 2002;12:268–274. [PubMed: 12049932]

Gonzalez-Martinez T, Farinas I, Del Valle ME, Feito J, Germana G, Cobo J, Vega JA. BDNF, but not NT-4, is necessary for normal development of Meissner corpuscles. Neurosci Lett 2005;377:12–15. [PubMed: 15722178]

Graef IA, Wang F, Charron F, Chen L, Neilson J, Tessier-Lavigne M, Crabtree GR. Neurotrophins and netrins require calcineurin/NFAT signaling to stimulate outgrowth of embryonic axons. Cell 2003;113:657–670. [PubMed: 12787506]

Grimes ML, Zhou J, Beattie EC, Yuen EC, Hall DE, Valletta JS, Topp KS, LaVail JH, Bunnett NW, Mobley WC. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J Neurosci 1996;16:7950–7964. [PubMed: 8987823]

Hamburger V, Levi-Montalcini R. Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J Exp Zool 1949;111:457–501. [PubMed: 18142378]

Han TH, Prywes R. Regulatory role of MEF2D in serum induction of the c-jun promoter. Mol Cell Biol 1995;15 (6):2907–15. [PubMed: 7760790]

Heerssen HM, Segal RA. Location, location, location: a spatial view of neurotrophin signal transduction. Trends Neurosci 2002;25:160–165. [PubMed: 11852149]

Heerssen HM, Pazyra MF, Segal RA. Dynein motors transport activated Trks to promote survival of target-dependent neurons. Nat Neurosci 2004;7:596–604. [PubMed: 15122257]

Heidenreich KA, Linseman DA. Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. Mol Neurobiol 2004;29:155–166. [PubMed: 15126683]

Hempstead BL. Dissecting the diverse actions of pro- and mature neurotrophins. Curr Alzheimer Res 2006;3:19–24. [PubMed: 16472198]

Hendry IA, Stach R, Herrup K. Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. Brain Res 1974;82:117–128. [PubMed: 4140012]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

Howe CL, Valletta JS, Rusnak AS, Mobley WC. NGF Signaling from Clathrin-Coated Vesicles. Evidence that Signaling Endosomes Serve as a Platform for the Ras-MAPK Pathway. Neuron 2001;32:801–814. [PubMed: 11738027]

Kasler HG, Victoria J, Duramad O, Winoto A. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol Cell Biol 2000;20:8382–8389. [PubMed: 11046135]

Korsching S. The neurotrophic factor concept: a reexamination. J Neurosci 1993;13:2739–2748. [PubMed: 8331370]

Kuruvilla R, Ye H, Ginty DD. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 2000;27:499–512. [PubMed: 11055433]

Lee FS, Kim AH, Khursigara G, Chao MV. The uniqueness of being a neurotrophin receptor. Curr Opin Neurobiol 2001;11:281–286. [PubMed: 11399425]

Levi-Montalcini R, Angeletti PU. Nerve Growth Factor. Physiol Rev 1968;48:534–569. [PubMed: 4875350]

Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci U S A 2003;100:8532–8537. [PubMed: 12826611]

Lom B, Cohen-Cory S. Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization in vivo. J Neurosci 1999;19:9928–9938. [PubMed: 10559401]

Lom B, Cogen J, Sanchez AL, Vu T, Cohen-Cory S. Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. J Neurosci 2002;22:7639–7649. [PubMed: 12196587]

Lu B, Pang PT, Woo NH. The yin and yang of neurotrophin action. Nat Rev Neurosci 2005;6:603–614. [PubMed: 16062169]

Mao Z, Bonni A, Xia F, Nadal-Vincens M, Greenberg M. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science 1999;286:785–790. [PubMed: 10531066]

Markus A, Zhong J, Snider WD. Raf and akt mediate distinct aspects of sensory axon growth. Neuron 2002;35:65–76. [PubMed: 12123609]

McCaig CD, Sangster L, Stewart R. Neurotrophins enhance electric field-directed growth cone guidance and directed nerve branching. Dev Dyn 2000;217:299–308. [PubMed: 10741424]

Miller FD, Kaplan DR. On Trk for Retrograde Signaling. Neuron 2001;32:767–770. [PubMed: 11738023]

Moises T, Dreier A, Flohr S, Esser M, Brauers E, Reiss K, Merken D, Weis J, Kruttgen A. Tracking TrkA's trafficking: NGF receptor trafficking controls NGF receptor signaling. Mol Neurobiol 2007;35:151–159. [PubMed: 17917104]

Neet KE, Campenot RB. Receptor binding, internalization, and retrograde transport of neurotrophic factors. Cell Mol Life Sci 2001;58:1021–1035. [PubMed: 11529495]

Patapoutian A, Reichardt LF. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 2001;11:272–280. [PubMed: 11399424]

Print CG, Loveland KL, Gibson L, Meehan T, Stylianou A, Wreford N, de Kretser D, Metcalf D, Kontgen F, Adams JM, Cory S. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. Proc Natl Acad Sci U S A 1998;95:12424–12431. [PubMed: 9770502]

Purves D. The trophic theory of neural connections. Trend in Neuroscience 1986;9:486–489.

Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. Oncogene 2007;14;26 (22):3100–12. [PubMed: 17496909]

Reichardt LF. Neurotrophin-regulated signalling pathways. Philos Trans R Soc Lond B Biol Sci 2006;361:1545–1564. [PubMed: 16939974]

Riccio A, Pierchala BA, Ciarallo CL, Ginty DD. An NGF-Trka-Mediated Retrograde Signal to Transcription Factor CREB In Sympathetic Neurons. Science 1997;277:1097–1100. [PubMed: 9262478]

Riccio A, Ahn S, Davenport C, Blendy J, Ginty D. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 1999;286:2358–2361. [PubMed: 10600750]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Ross AJ, Waymire KG, Moss JE, Parlow AF, Skinner MK, Russell LD, MacGregor GR. Testicular degeneration in Bclw-deficient mice. Nat Genet 1998;18:251–256. [PubMed: 9500547]

Roux PP, Barker PA. Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol 2002;67:203–233. [PubMed: 12169297]

Rowland LP. Primary lateral sclerosis, hereditary spastic paraplegia, and mutations in the alsin gene: historical background for the first International Conference. Amyotroph Lateral Scler Other Motor Neuron Disord 2005;6:67–76. [PubMed: 16036429]

Segal RA. Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci 2003;26:299–330. [PubMed: 12598680]

Segal RA, Takahashi H, McKay RD. Changes in neurotrophin responsiveness during the development of cerebellar granule neurons. Neuron 1992;9:1041–1052. [PubMed: 1463606]

Senger DL, Campenot RB. Rapid Retrograde Tyrosine Phosphorylation Of TrkA and Other Proteins In Rat Sympathetic Neurons In Compartmented Cultures. Journal of Cell Biology 1997;138:411–421. [PubMed: 9230082]

Seyfried J, Wang X, Kharebava G, Tournier C. A novel mitogen-activated protein kinase docking site in the N terminus of MEK5alpha organizes the components of the extracellular signal-regulated kinase 5 signaling pathway. Mol Cell Biol 2005;25:9820–9828. [PubMed: 16260599]

Shalizi A, Lehtinen M, Gaudilliere B, Donovan N, Han J, Konishi Y, Bonni A. Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern. J Neurosci 2003;23:7326–7336. [PubMed: 12917366]

Shalizi A, Gaudilliere B, Yuan Z, Stegmuller J, Shirogane T, Ge Q, Tan Y, Schulman B, Harper JW, Bonni A. A calcium-regulated MEF2 sumoylation switch controls postsynaptic differentiation. Science 2006;311:1012–1017. [PubMed: 16484498]

Shalizi AK, Bonni A. Brawn for Brains: The Role of MEF2 Proteins in the Developing Nervous System. Curr Top Dev Biol 2005;69:239–266. [PubMed: 16243602]

Tsui-Pierchala BA, Ginty DD. Characterization of an NGF-P-trkA retrograde-signaling complex and age- dependent regulation of TrkA phosphorylation in sympathetic neurons. J Neurosci 1999;19:8207–8218. [PubMed: 10493722]

Turjanski AG, Vaque JP, Gutkind JS. MAP kinases and the control of nuclear events. Oncogene 2007;14;26(22):3240–53. [PubMed: 17496919]

Watanabe M, Endo Y, Kimoto K, Katoh-Semba R, Arakawa Y. Functional regulation of tactile sense by brain-derived neurotrophic factor in adult rats during acute inflammation. Neuroscience 2000;97:171–175. [PubMed: 10771348]

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 2001a;4:981–988. [PubMed: 11544482]

Watson FL, Heerssen HM, Moheban DB, Lin MZ, Sauvageot CM, Bhattacharyya A, Pomeroy SL, Segal RA. Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. J Neurosci 1999;19:7889–7900. [PubMed: 10479691]

Weible MW 2nd, Bartlett SE, Reynolds AJ, Hendry IA. Prolonged recycling of internalized neurotrophins in the nerve terminal. Cytometry 2001;43:182–188. [PubMed: 11170104]

Wu C, Lai CF, Mobley WC. Nerve growth factor activates persistent Rap1 signaling in endosomes. J Neurosci 2001;21:5406–5416. [PubMed: 11466412]

Yang Y, Hentati A, Deng HX, Dabbagh O, Sasaki T, Hirano M, Hung WY, Ouahchi K, Yan J, Azim AC, Cole N, Gascon G, Yagmour A, Ben-Hamida M, Pericak-Vance M, Hentati F, Siddique T. The gene encoding alsin, a protein with three guanine-nucleotide exchange factor domains, is mutated in a form of recessive amyotrophic lateral sclerosis. Nat Genet 2001;29:160–165. [PubMed: 11586297]

Yano H, Chao MV. Neurotrophin receptor structure and interactions. Pharm Acta Helv 2000;74:253–260. [PubMed: 10812966]

Zhou FQ, Snider WD. Intracellular control of developmental and regenerative axon growth. Philos Trans R Soc Lond B Biol Sci 2006;361:1575–1592. [PubMed: 16939976]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

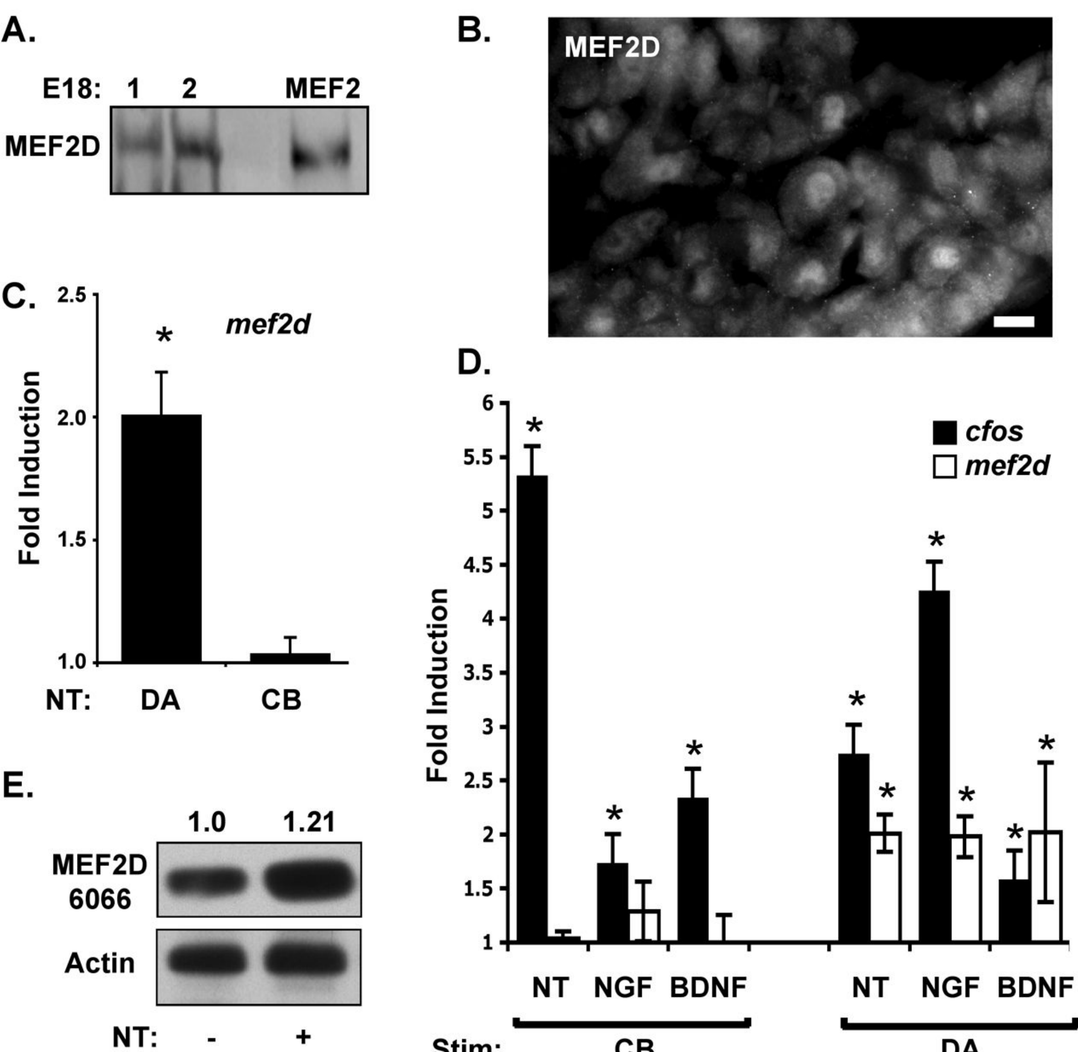


Figure 1. Neurotrophins induce *mef2d* expression in DRG neurons in response to distal axon stimulation

(A) MEF2D protein is detected *in vivo* in dorsal root ganglia at E18 (Lanes 1 and 2 represent two separate animals). As a positive control, lysates of neurons over-expressing MEF2VP16 were blotted for MEF2D. (B) MEF2D immunostaining of dorsal root ganglia at P0 *in vivo.* Scale bar = 10 μm (C) Expression of *mef2d* mRNA in DRG neurons in response to neurotrophin (NT) stimulation. DRG neurons were treated with neurotrophins (100 ng/ml NGF+BDNF) for 2 hours either on distal axons (DA) or on cell bodies (CB). *Mef2D* mRNA expression is specifically induced by distal axon stimulation and not by cell body stimulation. Expression is compared to DRG neurons treated with vehicle (100 ng/ml BSA). Results represent the mean ± SEM of 8 experiments, *P<0.05. (D) *Mef2d* mRNA expression is upregulated upon stimulation of distal axons with NGF alone, BDNF alone or NGF+BDNF (NT). Induction of *cfos* mRNA is shown as a positive control. (E) MEF2D protein levels were analyzed by Western blotting before and after NT stimulation for 2 hours. Normalized relative band density of MEF2D/actin reveal an increase of 21% ± 7% in response to distal axon stimulation.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

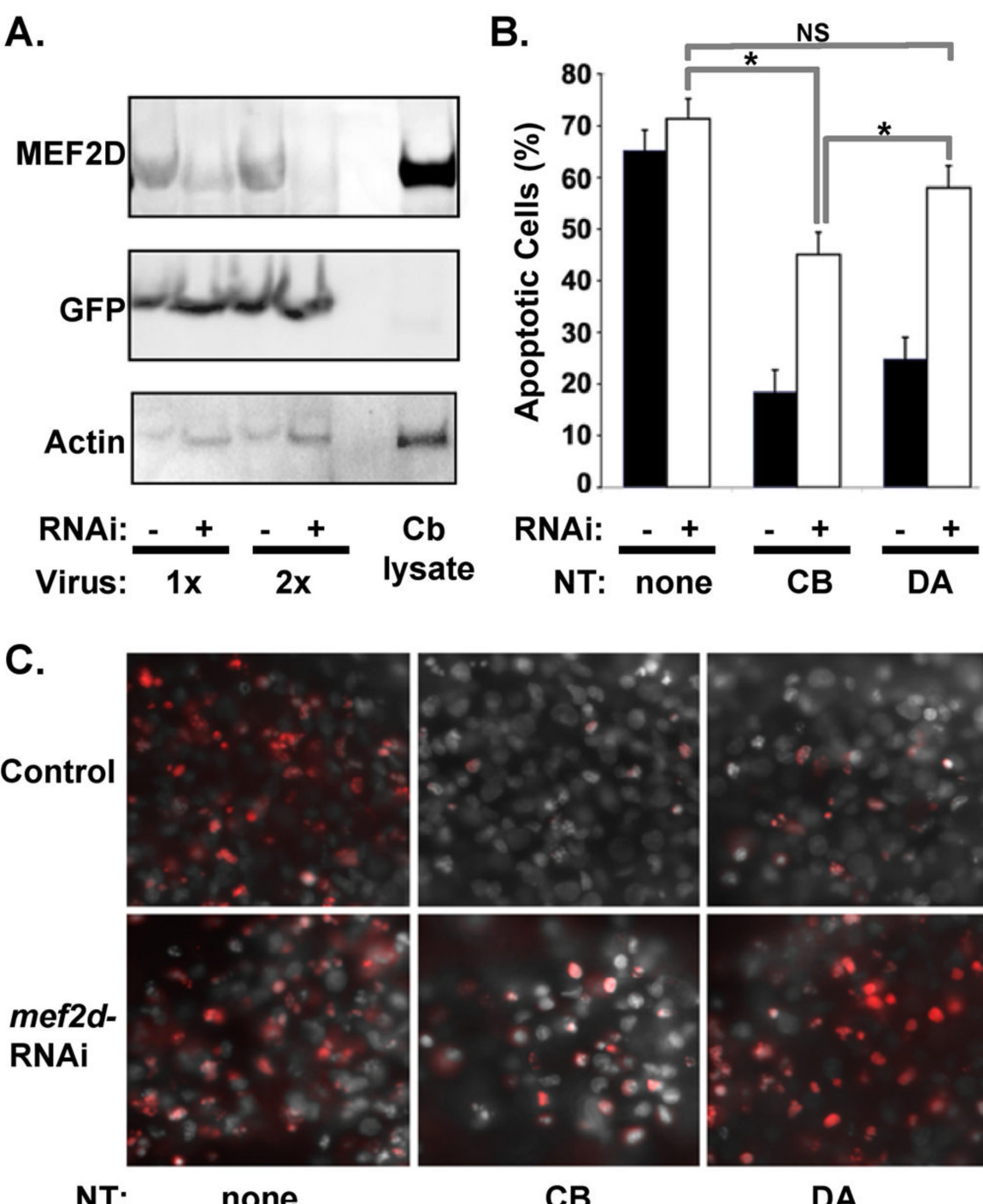


Figure 2. MEF2D is required for survival responses to neurotrophins

(A) DRG neurons in culture were infected with lentivirus expressing *mef2d*-GFP RNAi and cell lysates analyzed by Western blotting with indicated antibodies. *Mef2d* RNAi (+) results in decreased expression of MEF2D protein levels while a control lentivirus (−) does not. Cerebellar lysate (Cb lysate) was run as a positive control. (B) Apoptosis of DRG neurons was analyzed by TUNEL staining. *Mef2d* RNAi reduces survival of DRG neurons in response to neurotrophins when applied to cell bodies and blocks survival when applied to distal axons. Results represent the mean ± SEM of 5 experiments, $^{*}P<0.05$. (C) Representative images of DRG neuron cell bodies grown in culture and stimulated with neurotrophins or vehicle control on cell bodies (CB) or on distal axons (DA). Cells with (*mef2d* RNAi) or without (control

RNAi) *mef2d* reduction are shown. Panels show TUNEL staining (red) and DAPI-stained nuclei in control (top) and in neurons expressing *mef2d* RNAi (bottom).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

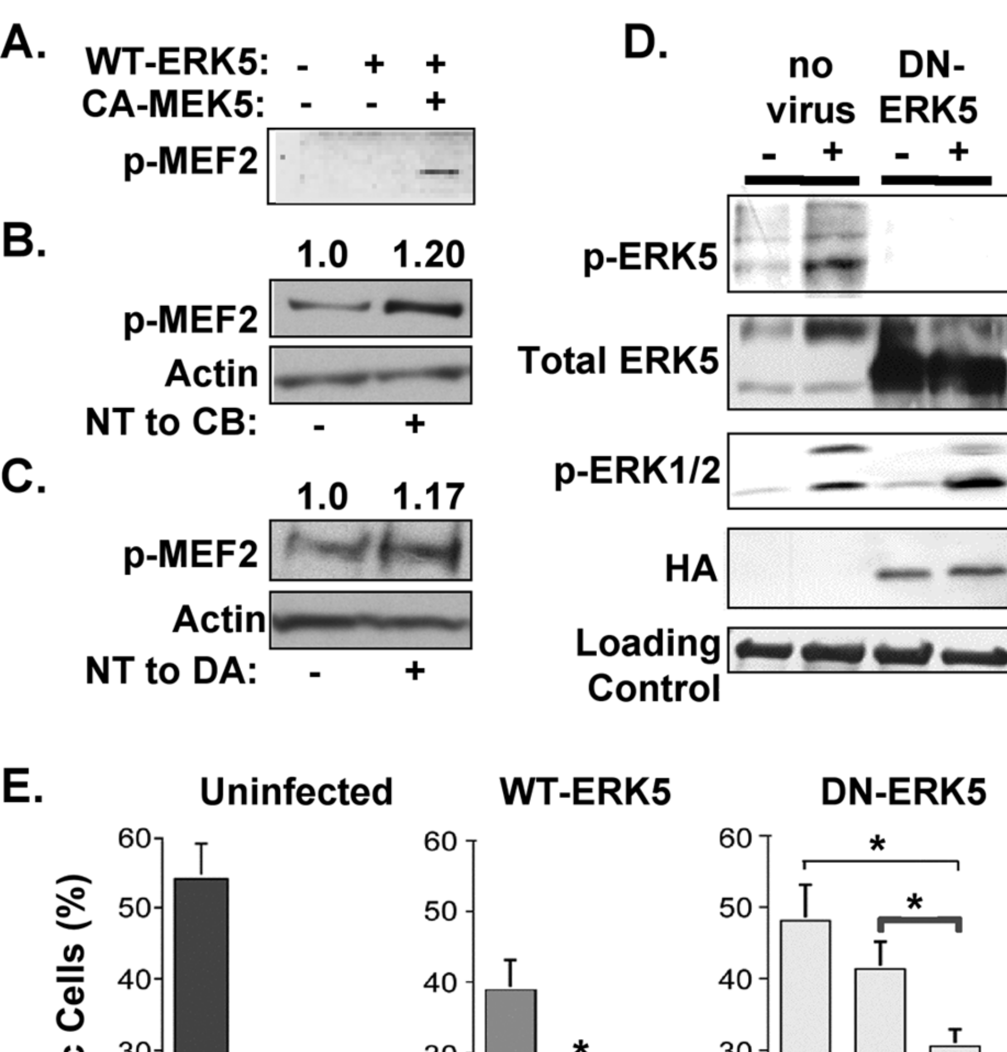


Figure 3. ERK5 regulates MEF2D, and promotes survival in response to target derived neurotrophins

(A) MEF2D is phosphorylated in response to ERK5 activation. COS cells were transfected with CA-MEK5 and WT-ERK5, or WT-ERK5 alone and lysates were blotted with an antibody to phospho-MEF2 (p-MEF2). (B) MEF2D is phosphorylated in response to cell body stimulation (20% ± 8% increase, $P<0.05$) for 20 minutes. Lysates were blotted with an antibody to phospho-MEF2 and with an antibody to pan-actin as a loading control. (C) MEF2D is phosphorylated in response to distal axon stimulation (17% ± 6% increase, $P<0.05$) for 2 hours. Lysates were blotted with an antibody to phospho-MEF2 and with an antibody to pan-actin as a loading control. (D) Neurons were infected with an adenovirus that expresses HA-tagged

ERK5-dominant negative form (DN-ERK5), then treated with neurotrophins for 30 minutes. Compared to uninfected neurons, expression of DN-ERK5 prevents ERK5 phosphorylation without inhibiting ERK1/2 activation. (E) ERK5 supports neuronal survival induced by target-derived neurotrophins. Cell apoptosis was measured by TUNEL staining in uninfected neurons, and in neurons infected with WT-ERK5 or DN-ERK5 for two days. DN-ERK5 blocks survival of neurons that depend on target-derived neurotrophins, whereas DN-ERK5 has a lesser effect on survival supported by cell body stimulation. All data shown are means ± SEM, * $P \leq 0.05$.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

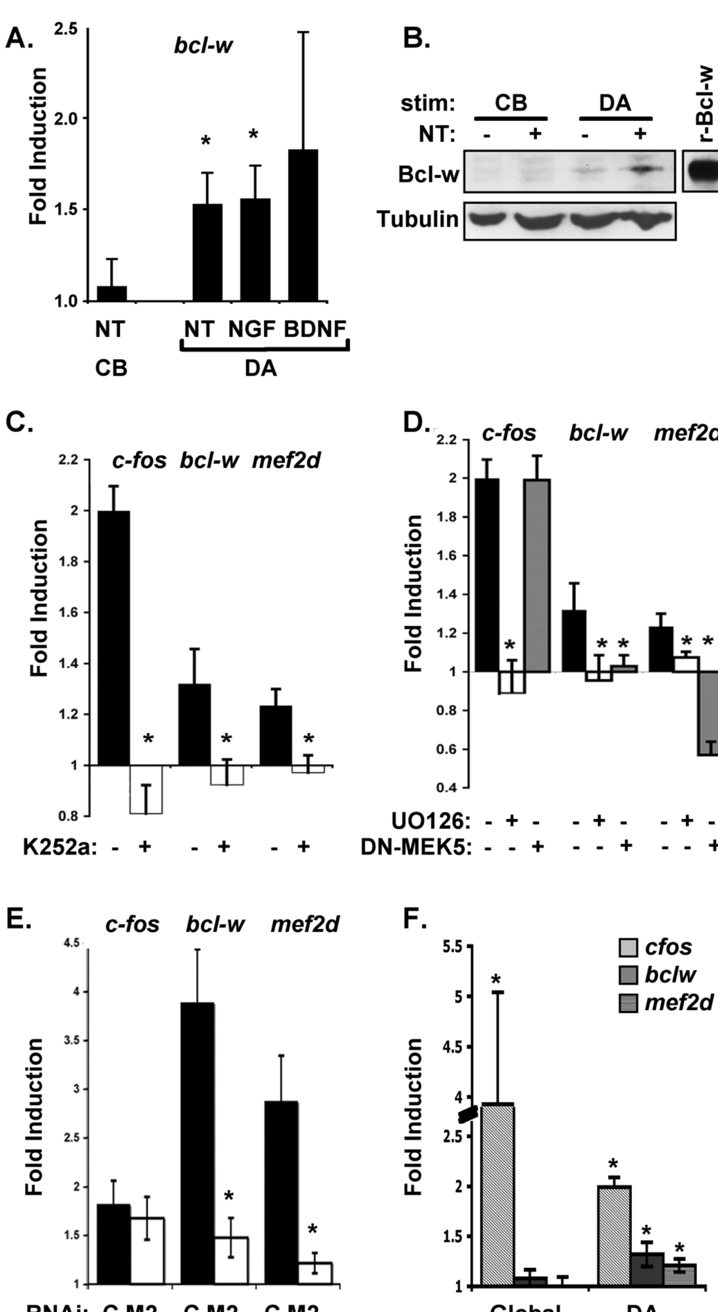


Figure 4. Target-derived neurotrophins induce *bcl-w* and *mef2d* expression in compartmented cultures by a Trk-dependent ERK5/MEF2 pathway

(A) Expression of *bcl-w* mRNA in response to neurotrophin stimulation. DRG neurons were treated with neurotrophin (NT=100 ng/ml NGF+BDNF) for 2 hours either on distal axons (DA) or on cell bodies (CB). *Bcl-w* mRNA expression is specifically induced by distal axon stimulation and not by cell body stimulation. *Bcl-w* mRNA is also upregulated upon distal axon stimulation with NGF or BDNF alone. All data show mean ± SEM, *P<0.05. (B) Compartmented cultures were stimulated at cell bodies (CB) or distal axons (DA) for 8 hours with neurotrophins. Cell body lysates were analyzed by Western blotting for Bcl-w (recombinant-Bclw was the positive control). DA stimulation leads to an increase in Bcl-w

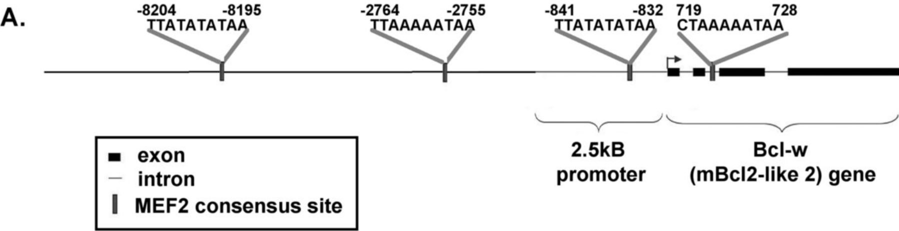
protein levels (32% ± 5%). (C) The Trk kinase inhibitor K252a or vehicle control was applied to cell bodies and distal axons at 200 nM, a concentration that inhibits phosphorylation of Trk receptors in these neurons. Neurons in compartmented cultures were globally treated or not with K252a and distal axons (DA) were stimulated with neurotrophins. K252a prevents induction of *c-fos*, *bcl-w* and *mef2d* by target-derived neurotrophins. (D) The Erk kinase inhibitor UO126 or vehicle control was applied to cell bodies and distal axons at 50 nM, a concentration that inhibits phosphorylation of ERK kinases. Neurons in compartmented cultures were treated or not with UO126. UO126 prevents *bcl-w*, *mef2d* and *c-fos* induction by target-derived neurotrophins. Neurons were infected with DN-MEK5 adenovirus for three days, then distal axons were stimulated with neurotrophins. DN-MEK5 expression inhibits *bcl-w* and *mef2d* mRNA induction but not *c-fos* induction by target-derived neurotrophins. (E) Lentivirus expressing mef2d RNAi (M2) inhibits induction of *bcl-w* and *mef2d*, but not induction of *c-fos*, in response to target-derived neurotrophins (C=control lentivirus). All data show means ± SEM, *P<0.05. (F) *Bcl-w* and *mef2d* mRNA expression is specifically induced by distal axon stimulation and not by global stimulation (cultures maintained for 5–7 days).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript



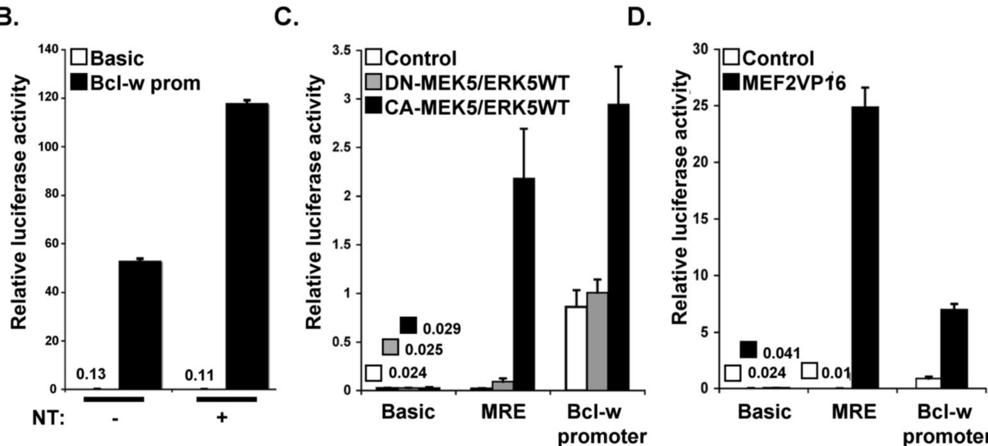


Figure 5. *Bcl-w* transcription is ERK5 and MEF2-dependent

(A) Schematic of *bcl-w* 5′ region. Sequence and locations of consensus MEF2 binding sites are indicated. The luciferase construct consisted of the 2.5kB promoter region, followed by ATG and luciferase reporter gene. (B) Luciferase reporter assay using *bcl-w* promoter-Luc co-transfected with pTK-Renilla-Luc for normalization. Two days after transfection by nucleofection, neurons were stimulated with neurotrophins for 2 hours. *Bcl-w*-luciferase expression is neurotrophin-dependent. (C, D) *Bcl-w* promoter is ERK5 and MEF2-dependent. Middle and right luciferase reporter assay using *bcl-w* promoter-Luc, 3xMRE-Luc or basic Luce cotransfected into COS cells with Renilla luciferase and the indicated expression plasmid (s). Three days after transfection with FuGENE 6, luciferase activity was measured. Data are from three independent experiments, each of which was conducted in triplicate, and show means ± SEM, *P<0.05.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

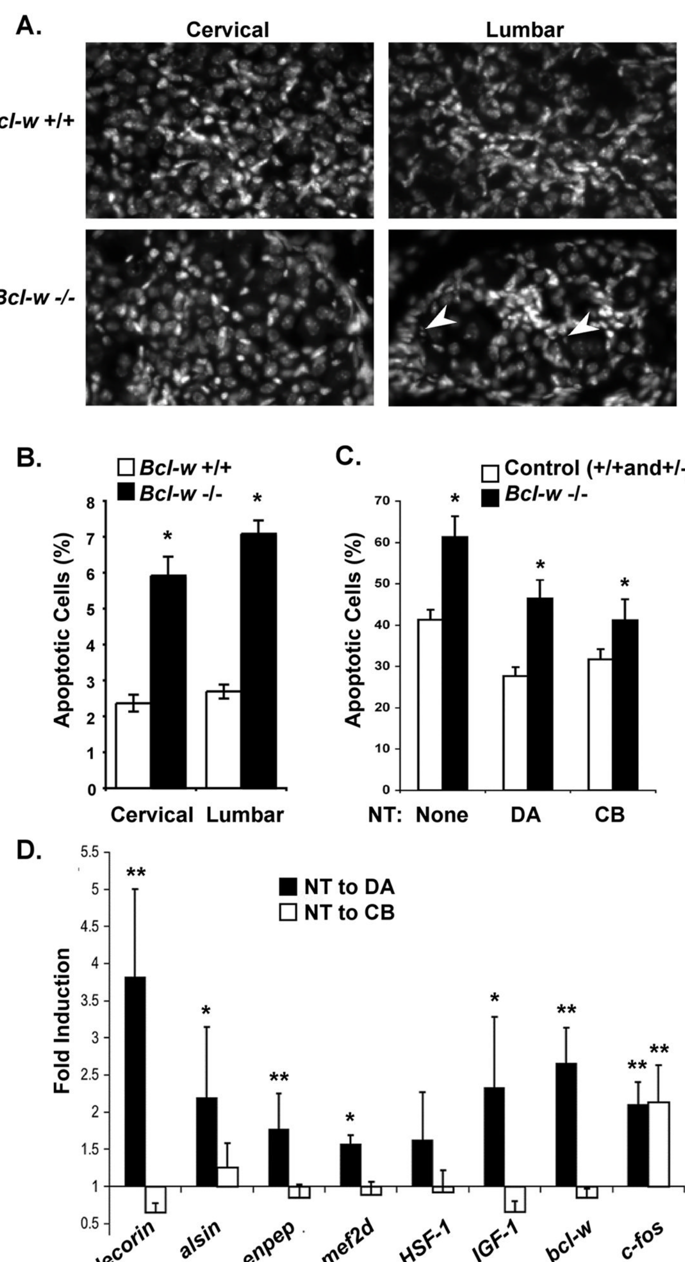


Figure 6. *Bcl-w* and other retrograde response genes are important for the survival of DRG sensory neurons

(A) Representative images of DAPI staining in dorsal root ganglia from cervical and lumbar regions of the spinal cord in WT (+/+) and *bcl-w* −/− mice. Arrows indicate apoptotic cells. Scale bar = 10 μm (B) *In vivo* analysis of apoptosis. *Bcl-w* −/− P0 mice show an increase in cell death compared to WT littermates, in both cervical and lumbar DRGs. The percentage of cells in the DRG with condensed nuclei when visualized by DAPI staining are shown. Three animals of each genotype were used, and 3–6 dorsal root ganglia from each lumbar and cervical region were counted. All data show means ± SEM, *P<0.0001. (C) Survival of *bclw*−/− sensory neurons in compartmented cultures stimulated with neurotrophin applied to the distal axons (DA) or the cell bodies and proximal axons (CB). DRG neurons were dissected at E15 and

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

seeded in compartmented cultures. *Bcl-w* −/− neurons are more prone to apoptosis in serum free-media compared to *bcl-w* +/+ and +/− under all conditions tested, *P < 0.05 (D) Retrograde response genes: DRG neurons in compartmented cultures were stimulated with NGF and BDNF or vehicle applied either to distal axons (DA), or the cell bodies and proximal axons (CB), for 2 hours. RNA was prepared and expression of *decorin, alsin, enpeptidase, mef2d, IGF-1, bcl-w* and *c-fos* were assessed by Q-RT-PCR and normalized to *gapdh* in the same sample. Fold induction was measured by comparing normalized level of expression in neurotrophin-treated/vehicle treated cells for 4 experiments each involving 5 cultures for each condition (DA shown first in black). Statistical analysis by Z-test, **P<0.05, *P<0.10 for a difference from 1.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

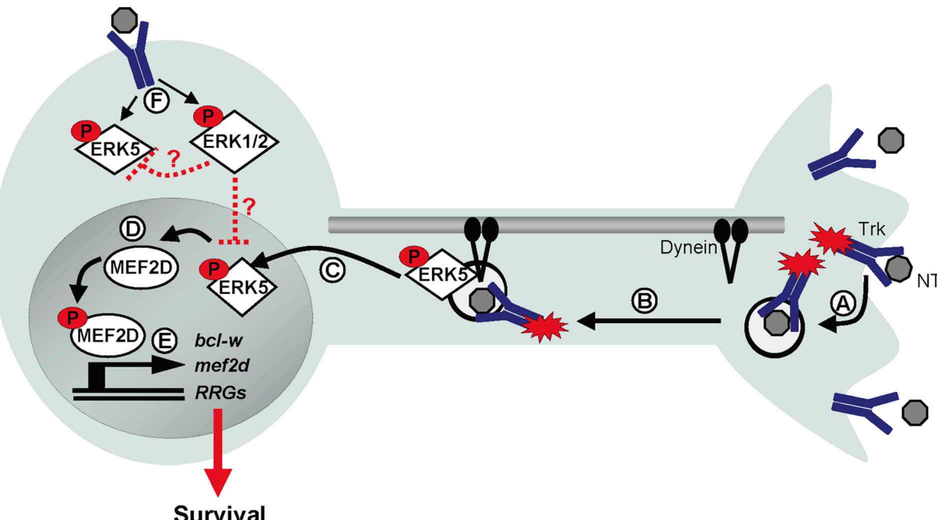


Figure 7. Target-derived neurotrophins activate an ERK5/MEF2 transcriptional program to promote survival

(A) Neurotrophin binding and Trk activation results in endocytosis of the activated receptor complex. (B) The signaling endosome, including downstream messengers, is retrogradely transported by microtubule dependent dynein motors. (C) Upon reaching the cell body, phosphorylated ERK5 translocates to the nucleus. (D) MEF2D is phosphorylated and activated in response to a retrograde ERK5 signal. (E) Activated MEF2D initiates transcription of *bcl-w*, *mef2d* and other retrograde response genes (RRGs) to promote survival mediated by target-derived neurotrophins. (F) In the case of cell body neurotrophin stimulation, both ERK5 and ERK1/2 are activated, however, expression of *bcl-w*, *mef2d* and other retrograde response genes are not induced. One possible mechanism is through suppression of ERK5 by the ERK1/2 pathway.