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Axonal transcription factors: Novel regulators of growth cone-to-nucleus signaling

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Abstract

Developing axons contain transcripts that are locally translated to influence the axonal proteome. Recent studies have shown that axonal transcripts include mRNAs encoding transcription factors. These mRNAs are translated to produce transcription factors that can be retrogradely trafficked back to the nucleus, where they regulate gene expression programs. These findings point to a novel mechanism of growth cone-to-nucleus signaling that occurs when growth cones encounter extracellular signaling molecules that stimulate local translation of these transcription factors, thereby influencing gene transcription. Here we summarize recent findings on local translation of transcription factors in axons and their roles in different neuronal processes such as neuronal specification, survival and axon regeneration. Comprehensive axonal transcriptome studies have revealed transcripts that encode many more transcription factors and cofactors, suggesting a potentially broad role for this type of signaling. We review the progress on the approaches and tools that have been developed to study local translation and retrograde trafficking of transcription factors. We also highlight the challenges in the field and discuss the potential routes to resolving them.

INTRODUCTION

During embryonic development, axons extend over long distances in order to navigate towards their targets. As axons grow, they encounter a variety of different extracellular signaling molecules that activate signaling pathways in the axon and thereby influence axon guidance. This process also involves communication between distal axons and the nucleus. Several studies show that signaling events in the growth cones at axonal tips results in changes in gene transcription in the cell body (For review, see (da Silva and Wang, 2011)). This communication between the growth cone and the nucleus allows neurons to adjust gene transcription as axons encounter different cues, and therefore potentially acquire expression of different sets of genes for the different stages of axon pathfinding or neuronal development.

The mechanisms that enable growth cone-to-nucleus signaling are poorly understood. Some of the earliest studies identified the existence of signaling endosomes, which are vesicles

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that are endocytosed in distal axons and retrogradely trafficked to the cell body (Riccio et al., 1997; Watson et al., 1999). These signaling endosomes contain receptor-ligand complexes (Curtis et al., 1995; Grimes et al., 1996; von Bartheld et al., 1996). The endocytosed ligand remains bound to the receptor throughout the process of retrograde trafficking, presumably due to the high concentration of the ligand within the lumen of the vesicle. Because the ligand is bound to the receptor, the receptor is in an active form, and may also contain bound signaling effector proteins (Atwal et al., 2000; Watson et al., 2001). These endosomes are thought to activate downstream signaling components when it arrives in the soma, thereby influencing gene transcription. The signaling pathway has been shown to occur for certain neurotrophins that are encountered by axons as they reach various target tissues (For review, see (Harrington and Ginty, 2013)). Additionally, recent work has shown that other molecules are capable of this type of signaling such as BMP4 (Ji and Jaffrey, 2012). Thus, signaling endosomes constitute an important mechanism by which diverse signals at the growth cones can influence gene transcription in the cell body.

Recent studies have suggested that other molecules may be retrogradely trafficked from axons. These include transcription factors that are localized to axons (Cox et al., 2008; Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012). Transcription factors have been shown to play key roles in different aspects of nervous system development (Thiel, 2006). In particular, these transcription factors control gene expression programs that regulate survival, neuronal differentiation, axon pathfinding, and a variety of other processes (Thiel, 2006). These effects occur when transcription factors bind to the promoters of various genes and activate their transcription, often by inducing the recruitment of coregulatory proteins that influence chromatin organization or the binding of transcription machinery (Kumar et al., 2004).

Transcription factors are typically in the nucleus adjacent to DNA, so the finding that some are detectable in axons was surprising. In many of these cases, the transcription factors are locally synthesized from axonally targeted mRNAs. The retrograde trafficking of these axonally synthesized transcription factors from growth cone to nucleus is mediated by importin-dependent nuclear transport (For review see (Otis et al., 2006)). In general, this pathway involves the tethering of cargo proteins to microtubule-dependent motors that traffic towards the cell body. Importin proteins bind nuclear-localization sequences (NLS) in their cargos and facilitate their attachment to microtubule motors via adapter proteins (Rollenhagen et al., 2003). A major motor for retrograde trafficking is dynein, which is a minus-directed motor (Goldstein and Yang, 2000), although the full complement of motor proteins and their adapters in axons is still not completely understood. Local translation of these transcription factors, followed by their retrograde trafficking to the nucleus, is a potential mechanism by which growth cone signaling pathways can affect gene transcription in the nucleus. In this review, we focus on the transcription factors that are synthesized locally, and we discuss the evidence both in support and against the potential role for this novel form of signaling.

How do transcription factors get to axons?

Like most axonal proteins, transcription factors could appear in axons as a result of anterograde transport from neuronal cell body. The challenge with anterograde transport is

that most transcription factors contain NLS sequences that promote transport to the nucleus. Proteins that bind the NLS, such as importins α and β, are known to be present in axons and mediate retrograde transport (Hanz et al., 2003; Perlson et al., 2005). Therefore, anterograde transport of NLS-containing proteins is expected to be inefficient unless the NLS can be inhibited during transport. Various transcription factors have an NLS that can be regulated in diverse ways. For example, the NLS in NFAT is phosphorylated, which inhibits its function (Loh et al., 1996). Thus, inhibition of the NLS could enable anterograde transport of transcription factors from the nucleus to the axon. After axonal transport is achieved, retrograde transport could be initiated upon removal of these inhibitory pathways in response to specific signals. For example, the NLSs of the p50 and p65 subunits of the transcription factor NF-κB are masked by inhibitors of NF-κB (IκBs) (Blank et al., 1991). These inhibitors retain NF-κB in the distant neuronal processes including axons (Mikenberg et al., 2007). Upon receptor activation, IκB is degraded and the NLSs are unmasked, which triggers the retrograde trafficking of NF-κB to the nucleus (Mikenberg et al., 2007). Thus, masking and unmasking of NLS sequences may be a mechanism to enable transcription factors to be anterogradely trafficked to axons and then retrogradely trafficked to the nucleus.

Transcription factors could also accumulate in axons from “lateral” transport mechanisms. Court and colleagues provided evidence that Schwann cells can transfer ribosomes and potentially other biomolecules into axons after injury (Court et al., 2008). This mechanism could potentially also result in the transfer of protein, or even mRNA. Thus, transcription factors mRNA or protein could be conveyed into the axoplasm, which could then be retrogradely trafficked in order to influence gene transcription.

Another mechanism that could result in the appearance of axonal transcription factors could be direct translocation through cellular membranes. Recent studies have shown that transcription factors such as Engrailed and Otx2 can be released from cells and translocate through membranes into target cells (Joliot et al., 1998; Prochiantz and Joliot, 2003). This has been shown specifically in the case of Engrailed-2 to induce growth cone collapse and repulsive turning in *Xenopus* retinal axons (Brunet et al., 2005). This effect involves a positively charged membrane translocation domain in Engrailed-2. This domain enables the protein to cross membranes, similar to the mechanisms that enable proteins such as Tat and Antennapedia to cross membranes (Frankel and Pabo, 1988; Joliot et al., 1991). Conceivably, these transcription factors could enter axons, and be retrogradely trafficked to influence gene transcription.

Transcription factors could also appear in axons as a result of local translation. As will be described below, four different transcription factors have been found to be locally translated in axons (Table 1). The initial description of axonally localized CREB transcripts followed several studies from Eberwine and colleagues that documented CREB mRNA and protein in dendrites (Crino et al., 1998; Lee et al., 2005). These previous studies also documented the trafficking of CREB from dendrites to the nucleus. The finding that these transcripts are also present in axons is perhaps not surprising since some of the mechanisms that mediate trafficking of mRNA into dendrites overlap with those mediating axonal transport (Job and Eberwine, 2001). However, subsequent studies described several axonal transcription factor

mRNAs that have not been previously reported in dendrites (Yan et al., 2009; Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012). Additionally, as will be described below, there is evidence from axonal transcriptome analysis that axons contain mRNAs encoding a variety of other transcription factors and cofactors. Although these mRNAs have not been shown to be translated in axons and a function for the encoded transcription factors has not been established, the presence of various transcription factor-encoding mRNAs in axons raises the possibility that other transcription factors are localized to axons by local synthesis.

Local translation of CREB in sensory axons

The first description of a transcription factor that is locally synthesized in axons was CREB (Cox et al., 2008). We used a cDNA library approach to identify transcripts that are present in the distal-most portion of axons (Cox et al., 2008). In this approach, we used a modified Boyden chamber in which embryonic dorsal root ganglia (DRG) sensory explants produced axons that grew at least 5 mm across a glass cover slip before they could grow through a microporous membrane into a region containing high levels of NGF. These distal axon segments were harvested and cDNA was prepared. One of the interesting cDNAs identified in the library encoded CREB. *CREB* transcripts were detected in the axons of cultured sensory neurons, and reporters containing the *CREB* 3′UTR were selectively induced by NGF and not Sema3A (Cox et al., 2008). These data suggest that CREB protein is synthesized in response to NGF. Endogenous levels of CREB protein are low in axons, but an increase in protein can be detected upon treatment with NGF. Experiments using a photoconvertible fluorescent protein (Dendra) tagged to CREB show that the tagged protein can be trafficked back to the nucleus. These experiments support a model in which locally synthesized CREB in axons could contribute to neuron survival-linked gene transcription in the nucleus after growth cones are exposed to NGF by target tissues.

One concern about this model is that CREB protein levels are very low in axons in culture. Indeed, we showed that the levels of CREB protein in axons are substantially lower than levels in the cell body (Cox et al., 2008). *CREB* mRNA was also not detected in sympathetic ganglia axonal serial analysis of gene expression (SAGE) libraries, but *CREB* was also not in the SAGE libraries prepared from cell body cDNA (Andreassi et al., 2010). Thus, axonal localization of CREB may be specific to certain neuronal types.

How could a small amount of CREB potentially impact gene transcription in the nucleus? If axonal CREB functions by being trafficked to the nucleus and changing the total amount of CREB in the nucleus, a substantial amount of axonal CREB synthesis would be needed. Evaluating this possibility is difficult since it is difficult to measure how much CREB could be produced over time by an axon, and how much of the NGF-exposed axon synthesizes CREB. Nevertheless, the assessment of the total synthetic capacity in the axon, and the fraction of the somatic CREB that is derived from the axon will help establish the biological significance of axonal CREB.

An alternative model does not require large amounts of axonal CREB synthesis. In this model, a small amount of axonal CREB could influence the cell body if it is in a phosphorylated form. Most of the CREB in the cell body is presumably not phosphorylated

because cell bodies are not exposed to NGF during embryonic development (Watson et al., 2001). Locally synthesized CREB could be preferentially regulated by signaling endosomes due to their colocalization in axons. Indeed, our work suggests that CREB can be phosphorylated in axons (Cox et al., 2008). Thus, although axonal CREB may not materially influence the total amount of CREB in the cell body, retrograde trafficking of axonal pCREB could substantially influence somatic pCREB levels, which is typically present at low levels.

For this model to explain functions of axonal CREB, the amount of cell body-derived CREB that is phosphorylated by retrogradely trafficked signaling endosomes needs to be at a level that is not substantially larger than the retrogradely trafficked axonal CREB. Otherwise, axonal pCREB would not be able to substantially alter cellular pCREB levels.

Lastly, axonal CREB could acquire unique modifications that could confer unique transcriptional effects. Different post-translational modifications induce binding of distinct regulatory proteins that can modify gene transcription pathways (Benayoun and Veitia, 2009). Indeed, selective phosphorylation of CREB in different sites such as Ser133, Ser142 or Ser143 induce specific gene expression programs (Kornhauser et al., 2002). It remains to be determined how the pattern of axonal CREB phosphorylation differs from the phosphorylation of pre-existing CREB in the nucleus.

An important future direction will be to determine if CREB is present in axons *in vivo*. CREB is not readily detectable in axons by immunostaining of tissue sections, indicating that any CREB that is present in axons does not readily accumulate. This may be because any newly synthesized CREB is retrogradely trafficked, so it cannot accumulate. Studies using axon ligature of peripheral axon bundles to allow proteins from the axon periphery to accumulate at the distal side of the ligature as they are retrogradely trafficked could be performed to test if CREB is synthesized in axons. Indeed, Delcroix et al. showed that a transcription factor related to CREB, ATF2, accumulates at the distal side of an axonal ligature in adult rat sciatic nerve (Delcroix et al., 1999). Thus, a protein that is otherwise undetectable can be detected once it accumulates to a detectable level. This approach may be valuable to detect diverse types of retrogradely trafficked proteins *in vivo*.

Control of neuronal patterning by axonally synthesized SMAD1/5/8

Local translation of axonal transcription factors may also have roles in regulating neuronal specification that occurs when axons reach target tissues. For example, the trigeminal ganglia comprise distinct neuronal subtypes, each of which have axonal projections that relay sensory information from the face to brainstem nuclei. As the peripheral axonal projections of the trigeminal ganglia reach specific target fields in the face, target-derived signals enable the central projections of the trigeminal ganglia to innervate the brainstem in a somatotopic distribution (Oury et al., 2006).

Initial studies identified one target-derived factor, BMP4 which is present in the ophthalmic and maxillary regions, and is relatively depleted in the mandibular portions of the face (Hodge et al., 2007). The trigeminal ganglia axons that enter into the ophthalmic and maxillary regions encounter BMP4, which initiates a retrograde signal that induces an

increase in the levels of phosphorylated SMAD1, 5, and 8 (pSMAD1/5/8) in the nuclei of these trigeminal ganglia neurons. The axons that enter into the mandibular region do not encounter BMP4, so their neurons lack pSMAD1/5/8. The increase in the levels the phosphorylated form of SMAD1/5/8 regulates the expression of the target genes encoding transcription factors such as *Tbx*3 and *OC2* (Hodge et al., 2007). The expression of these target genes is necessary for the central projections of these neurons to properly innervate the brainstem (Hodge et al., 2007). Thus, target-derived BMP4 induces the differentiation of trigeminal neurons into subtypes that express unique transcription factors and reflect the target fields that are innervated by the axons.

One of the interesting findings of this initial study was that pSMAD1/5/8 was not just in the nucleus, but also in the ophthalmic and maxillary axonal projections (Hodge et al., 2007). In contrast, the axons that comprise the mandibular projection lacked detectable staining of pSMAD1/5/8. Thus, pSMAD1/5/8 is a marker of the ophthalmic and maxillary axonal projections. This is in contrast to CREB, which exhibits low levels in axons and is not detectable in axons by routine staining in histologic sections. The axonal localization of these transcription factors was unexpected since these proteins are known to contain a NLS (Xiao et al., 2001). Because of the relatively high levels of SMAD in axons, we suspected that axonal SMAD could have roles in axon-to-nucleus signaling.

We found that SMAD1, 5, and 8 are locally synthesized within axons, and mediate the specification of trigeminal ganglia neurons into ophthalmic/maxillary subtypes (Ji and Jaffrey, 2012). Although SMAD1/5/8 protein is only detectable in the ophthalmic and maxillary axons, the *SMAD1/5/8* transcripts were detected in mandibular axons as well. These data suggested that specific target-derived factor(s) induce the translation of SMAD1/5/8, and that these target-derived factors are likely to be only in the ophthalmic and maxillary target fields. Although BMP4 is an ideal candidate to induce the translation of SMAD1/5/8 in axons because it has this specific distribution, we found that BMP4 failed to induce the synthesis of SMAD1/5/8 in isolated axons from trigeminal ganglia explants (Ji and Jaffrey, 2012). However, previous studies had shown that BDNF was also highly expressed in the face, and has a distribution that is similar to that of BMP4 (Arumae et al., 1993). Neurotrophins are a good candidate for regulating protein synthesis since neurotrophins have previously been shown to induce the translation of various transcripts including *PAR3* and *β-actin* (Yao et al., 2006; Hengst et al., 2009). We therefore considered the possibility that BDNF induced the translation of SMAD1/5/8. We found that BDNF, but not other neurotrophic factors, was highly effective in inducing SMAD1/5/8 levels in axons. This effect was also seen in axons from mandibular-trigeminal ganglia neurons (Ji and Jaffrey, 2012). This indicates that the mandibular axons have the capacity to induce SMAD1/5/8 synthesis, but because they do not encounter BDNF when they enter their target field, they lack the expression of these proteins.

What is the role of BMP4 in this signaling pathway? We showed that BMP4 induced the formation of signaling endosomes that are retrogradely trafficked to the nucleus (Ji and Jaffrey, 2012), which was very analogous to the types of signaling endosomes that had previously been seen with neurotrophins (Riccio et al., 1997; Watson et al., 1999). The signaling endosomes appear to maintain axonal SMADs in a phosphorylated form as they

are retrogradely trafficked, and as they enter into the cell body. This idea is supported by data from the original studies which showed that the SMAD1/5/8 that is in the phosphorylated form along the entire length of the ophthalmic and maxillary axons (Hodge et al., 2007). This distribution of pSMAD1/5/8 is counterintuitive since BMP4 is only present in the target fields. Thus, SMAD1/5/8 should have only been phosphorylated in this portion of the axon because when it is retrogradely trafficked, it would become dephosphorylated by the numerous phosphatases in the cytoplasm. However, SMAD1/5/8 is clearly maintained in a phosphorylated form along the length of the axons. Thus, signaling endosomes likely maintain SMAD1/5/8 in a phosphorylated form as they are retrogradely trafficked along with SMADs to the nucleus.

One of the interesting aspects of this study is that the local translation of *SMAD1/5/8* in axons was explored in a physiological context. Many examples of local protein synthesis in axons in culture have not been tested in a physiological setting due to the difficulty in inhibiting protein synthesis selectively in distal axons *in vivo*. Thus, alternate approaches are needed to assess the physiological relevance of proposed local translation pathways. In this case, we examined whether SMAD1/5/8 is present in axons in *BDNF* null mice. Because BDNF induces SMAD1/5/8 expression in transected trigeminal ganglia axons, we predicted that removal of BDNF from target fields would result in a loss of SMAD1/5/8 from the axons. BDNF expression is largely restricted to target fields during the embryonic stage while trigeminal ganglia axons innervate the face. Consistent with the idea that BDNF controls the local translation of SMAD1/5/8, SMAD1/5/8 was markedly reduced in the ophthalmic and maxillary axonal branches in *BDNF* null mice (Ji and Jaffrey, 2012). Thus, these data support the idea that target-derived BDNF controls the expression of SMAD1/5/8 in axons during embryonic development.

Regulation of regeneration gene programs by local translation of STAT3 and CEBP-1 after axon injury

Nerve injury in peripheral neurons triggers retrograde signaling events which turn on master regulators and induce gene transcription programs that is required for regeneration (Rossi et al., 2007). During this process, the transcription factor STAT3 is activated and translocated to nucleus to regulate gene expression programs (Schwaiger et al., 2000). Recent studies showed that mRNA encoding STAT3 is detected and can be locally translated in response to injury in adult rat sciatic and DRG axons (Ben-Yaakov et al., 2012). The active phospho-STAT3 (pSTAT3) interacts with dynein in axons during retrograde trafficking. The retrograde transport of STAT3 is mediated by importin α5 and β1 in an NLS-dependent manner. Interestingly, importin β1 is locally translated in response to injury as well (Hanz et al., 2003).

Local translation of another transcription factor, CEBP-1, was also shown to be required for axon regeneration in *C. elegans* (Yan et al., 2009). *cebp-1* mRNA is present in axons and its stability is regulated by the DLK-1 kinase via its 3′UTR. Local translation of CEBP-1 is induced by axotomy in a DLK-1-dependent manner. CEBP-1 was suggested to potentially have roles due to retrograde trafficking to the nucleus where it could influence gene transcription (Yan et al., 2009). Interestingly, CEBP-1 is a member of the basic-leucine-

zipper (bZip) domain superfamily of transcription factors. Thus, the finding that diverse bZip domain transcription factors, including CEBP-1 and CREB family members ATF-2 and CREB are each in axons suggests that this transcription factor family may have broad roles in axons. These results from both mammals and invertebrates suggest that local translation of transcription factors might be a common strategy used by different organisms to control axonal regeneration.

Methods used to study axon-to-nucleus signaling in cultured neurons

Although various mRNAs encoding transcription factors have been found in axons, it is important to note that the mere existence of an axonal mRNA encoding a transcription factor does not mean that the encoded protein is translated in sufficient quantities to exert biological effect, or that this transcript is indeed involved in any growth-cone-to-nucleus signaling pathway. One approach to address the idea that these mRNAs have a functional role is axon-specific knockdown (Hengst et al., 2006). This technique allows mRNAs to be selectively removed from axons, without interfering with their levels in cell bodies. Global knockdown approaches are problematic since they interfere with both cell body pools of an mRNA as well as the axonal pool of an mRNA. By selectively removing the axonal pool of an mRNA, and then evaluating axonal signaling, the role of the axonal pool can be established.

Another valuable tool for studying axon-to-nucleus signaling is compartmentalized culturing chambers. A requirement for recapitulating the signaling pathways *in vitro* is to be able to stimulate axons with a signaling molecule, without stimulating the cell bodies. Thus, after application of a signaling molecule to axons, any change seen in the nucleus must derive from a signal sent from the axon to the nucleus. Devices such as microfludiic culturing devices and Campenot chambers have been used (Taylor et al., 2005; Deglincerti and Jaffrey, 2012). Thus, compartmentalized culturing devices are critical for studying axon-to-nucleus signaling since they enable the axonal mRNA pool to be knocked down, and they allow selective application of signaling molecules to axons without affecting cell body.

Monitoring retrograde trafficking of transcription factors from axons to the nucleus

What types of experiments can be done to confirm that axonal transcription factors exert their functions by regulating gene transcription in nucleus? One possibility is to determine if they are indeed retrogradely trafficked. Several studies have directly monitored retrograde trafficking of these axonal transcription factors after they are tagged with various fluorescent proteins, or photo convertible proteins (Cox et al., 2008; Ji and Jaffrey, 2012).

However, it is important to assess if the endogenous, untagged protein is retrogradely trafficked to the nucleus. To look at the endogenous protein, we have used transected axons, and monitored how the levels of specific axonal transcription factors are depleted over time (Cox et al., 2008). In general, if the transcription factor is continuously being retrogradely trafficked, its levels will be rapidly depleted from axons after protein synthesis is inhibited, and new transcription factor cannot be synthesized. Indeed, we observed rapid depletion of

locally synthesized SMAD1/5/8 from axons only when protein synthesis is inhibited (Ji and Jaffrey, 2012). The depletion of SMAD1/5/8 can be blocked by inhibition of the motors involving retrograde transport (Cox et al., 2008; Ji and Jaffrey, 2012). These data demonstrate that SMAD1/5/8 is actively being depleted through a process involving motor-dependent retrograde transport. Thus, the presence of retrograde transport suggests that an axonal transcription factor may function in the nucleus.

Actively transported axonal transcription factors can also be identified by screening for proteins that interact with dynein, the retrograde motor (Ben-Yaakov et al., 2012). Fainzilber and colleagues developed an approach which combined co-immunoprecipitation with a dynein antibody and a protein-DNA array technique to identify retrogradely trafficked transcription factors (Ben-Yaakov et al., 2012). Briefly, the immunoprecipitates from axons were used to “fish” out biotinylated DNA probes, which were identified by screening against an array consisting of consensus transcription factor binding sites. Although this approach might be limited by starting axonal material, it is useful for identifying transcription factors that are retrogradely trafficked.

In addition to transcription factors, transcription cofactors might also be locally synthesized and then retrogradely trafficked. Recently described axonal transcriptome studies show the existence of many axonal mRNAs that encode transcription cofactors, including BRD, FHL, LMO and MORF4L (Gumy et al., 2011). Some of these proteins could potentially be locally translated, retrogradely trafficked, and then influence gene transcription.

Kinases or other signaling proteins could be retrogradely trafficked to activate gene transcription, thereby providing an alternate route to activate gene transcription. For example, retrograde transport of c-Jun N-terminal kinase (JNK) in injured axons regulates the activity of c-Jun and ATF3 in nucleus after peripheral nerve injury (Lindwall and Kanje, 2005). Recent axonal transcriptome analysis identified many mRNAs encoding different kinases and other signaling molecules such as Ca^{2+}–calmodulin-dependent protein kinases (CaM kinase), mitogen-activated protein kinases (MAPK), casein kinases (CSNK) (Zivraj et al., 2010; Gumy et al., 2011). Each of these proteins is known to influence gene transcription through diverse mechanisms. Retrograde transport of kinases and other signaling proteins could be highly efficient since it would potentially lead to an amplification of the initial translation event by activating multiple transcription factors in the cell body.

Unique features of local translation as a mechanism to localize transcription factors to axons

Local translation of a transcription factor confers certain advantages over anterograde trafficking of pre-existing transcription factors from the cell body. In cases where the transcription factor is synthesized locally, the levels of the transcription factor can be regulated rapidly in response to extrinsic cues. The transcription factor could be essentially in a latent form while it is localized in distal axons as an mRNA, and then only expressed when the growth cone encounters a signal that induces its translation. If there are low levels

of the transcription factor in the nucleus, a switch that induces translation and retrograde trafficking of a transcription factor could result in a large change in gene transcription.

The concept that locally translated transcription factors acquire unique post-translational modifications is supported by work on Elk1 in dendrites. Eberwine and colleagues directly introduced mRNA encoding Elk1 into dendrites and showed that the translated protein induced an Elk1-dependent pathway leading to cell death (Barrett et al., 2006). When Elk1 expression was targeted only to cell bodies, the Elk1 did not induce cell death. These data suggested that dendritic Elk1 acquired unique modifications such as SUMOylation and phosphorylation in dendrites and was transported to the nucleus, resulting in the activation of gene transcription pathways leading to cell death. Conceivably, similar types of modifications can occur on axonally synthesized transcription factors. For example, in addition to phosphorylation, SUMOylation appears to modify certain proteins in axons (van Niekerk et al., 2007), and this modification could potentially confer unique transcriptional effects on the axonally derived transcription factor once it arrives in the nucleus.

Local roles for axonal transcription factors

Although transcription factors are traditionally thought to exert their functions in nucleus by regulating gene transcription, it is important to consider the possibility that an axonal transcription factor might mediate its effects directly within the axon. Transcription factors that have roles locally would function through mechanisms that do not involve their DNA-binding ability and effect on gene expression. For example, Engrailed-2 is internalized in the growth cone and triggers local synthesis of new proteins that cause repulsive turning of *Xenopus* retinal axons (Brunet et al., 2005). Similarly, in Drosophila, nervy is localized to axons where it regulates axon guidance by linking cAMP-PKA signaling to semaphorin-plexin-mediated repulsion (Terman and Kolodkin, 2004). This protein was previously predicted to be a transcriptional factor (Feinstein et al., 1995), and its mammalian homologs, MTG8/ETO, act as transcriptional corepressors (Wang et al., 1998). Recent studies show that STAT3 can have a local effect by inhibiting stathmin, which stabilizes microtubules (Selvaraj et al., 2012). Thus, there are several examples where axonal transcription factors can have local roles. It is possible that the axonal transcriptional factors have distinct roles (local or nucleus) in different neurons. Indeed, as discussed above, axonal STAT3 functions locally to rescue axon degeneration in motor neurons, and signals retrogradely to regulate neuronal survival after injury in sensory neurons (Ben-Yaakov et al., 2012; Selvaraj et al., 2012).

Local translation of transcription factors might be a commonly used signaling mechanism in neurodevelopment

Although only a small number of mRNAs that encode transcription factors have been characterized in axons, the number of axonally synthesized transcription factors might be larger than currently appreciated. Indeed, several axonal mRNA libraries have revealed more transcription factor mRNAs (Taylor et al., 2009; Zivraj et al., 2010). Our analysis of these data showed that there are 49 and 59 transcription factor and co-factor mRNAs from embryonic cortical axon and retinal ganglion cell (RGC) axon, respectively (Table 2). A

recent transcriptome analysis of rat DRG axons revealed the most axonal transcription factor mRNAs so far (Gumy et al., 2011). Considering that there are about 350 transcription factors and cofactors which are expressed in the mouse nervous system (Gray et al., 2004), the presence of mRNAs encoding over 100 of them in axons reflects that there is potentially a large transcription factor mRNA repertoire localized in axons (Table 2). Although it is important to be cautious about concluding that these mRNAs have physiologic roles or are produced in sufficient quantities to have a biologic effect, the diversity of axonal mRNAs raises the possibility that local translation of transcription factors could have broader roles than those described above.

Interestingly, the capacity for local translation of transcription factors could be developmentally regulated. Characterization of the axonal transcriptome from embryonic (E16) and adult (3–5 months old) rats (Gumy et al., 2011) shows different sets of transcription factor mRNAs (Table 2). Although 71 transcription factor mRNAs are present in both embryonic and adult axons, there are 39 and 75 transcription factors which are expressed only in embryonic or adult axons, respectively (Table 2 and Figure 1). Conceivably, the temporal regulation of transcription factor mRNAs in axons could reflect the different signaling requirements of axons during different stages of development. Indeed, mRNAs encoding transcription factors which are known to regulate early neuronal development such as Hoxa5, SMAD1 and SMAD4 are found only in embryonic sensory axons, while those known to be involved in neuronal regeneration such as CEBPD, CREBBP, CREM, MAX and STAT are detected only in adult axons (Table 2).

Local translation of certain transcription factors might be utilized by multiple different neurons in the nervous system. Analysis of axonal transcription factor pools from different neuronal types and different developmental stages support this idea. For example, expression of transcriptional factors such as ATF4 (also known as CREB-2), HMGB1, HMGB2 and RUNX1 have been detected in DRG, RGC and cortical axons in both embryonic and adult stages (Table 2 and Figure 2), suggesting that these proteins might have a general role in neuronal function.

In addition to the transcription factors predicted by axonal mRNA libraries, it is interesting to speculate whether other axonal transcription factors could be locally synthesized. For example, ATF2 has been shown to be localized to axons, and it accumulates on the distal side of an axon ligature (Delcroix et al., 1999). The accumulation on the distal side of the ligature suggests that ATF2 is actively retrogradely trafficked. Other transcription factors, including Engrailed and Otx2 have also been identified in distal processes, although their localization in dendrites is more established (Di Nardo et al., 2007; Alvarez-Fischer et al., 2011). Conceivably, these transcription factors could also be locally synthesized from axonally localized mRNA.

Importantly, the published axonal libraries may not have revealed all the potential transcription factors that are localized in axons. First, because these libraries are limited to axons from just a few neuronal types, the transcription factors locally synthesized in other types of neurons may not be reflected by these libraries. Indeed, analysis of previous axonal transcriptome data have shown that different neuronal types have different pools of axonal

transcription factor transcripts (Table 2 and Figure 2). Second, some physiological axonal transcription factor mRNAs might have been missed from existing libraries because they were obtained from neurons growing in culture, rather than from axons growing *in vivo*. This is an important consideration because some of the axonal mRNAs that are seen in cultured neurons could reflect an adaptation to the culturing environment. Therefore, obtaining axonal mRNA libraries from axons growing in an animal will be very valuable to help focus attention on the mRNAs that are physiologically targeted to axons.

One way to identify these physiological axonal mRNAs is to use the EGFP-L10a mice (Heiman et al., 2008). We recently described the use of EGFP-L10a mice to study the localization of ribosomes in axons (Walker et al., 2012). The transgene expression of the EGFP-L10a ribosomal fusion protein is expressed in layer 5b cortical neurons under the control of the *Glt25d2* promoter in this mouse line (Heiman et al., 2008). In this experiment we took advantage of the spatial separation between the axons and the cell bodies of the long projection neurons: the cell bodies are located in the cortex, while the axons descend through the spinal cord. Thus, any EGFP-tagged ribosome derives exclusively from neurons in the cortex, excluding the possibility that it derives from dendrites or glial processes. This approach could be used to identify ribosome-bound mRNAs using an assay called translating ribosome affinity purification (TRAP) by doing immunoprecipitation with anti-EGFP antibodies (Doyle et al., 2008). By sequencing the ribosome-bound mRNA, the mRNAs that are being actively translated can be identified.

This approach provides an additional advantage over simply sequencing axonal mRNAs, since some axonal mRNAs may not be recruited to ribosomes. These mRNAs might not contain the *cis*-acting elements which are required for recruitment by axonal ribosomes. Thus, the ribosome-bound mRNA pool is likely to be a more accurate reflection of the axonal mRNAs that have physiological roles. Conceivably, promoters other than *Glt25d2* can be used to drive the expression of the transgene in other neuronal populations which have their axons distant from their cell bodies. For example, promoters which are specific to retinal ganglia neurons can be used to label the ribosomes that are found in axons that navigate towards and through the optic chiasm. These axons can be readily isolated, providing axonal libraries that could be used to characterize translating mRNA pools before, during, and after guidance through the chiasm. This technique will be valuable in identifying axonal mRNAs which are actively translated in a spatiotemporal-specific manner *in vivo*.

Establishing whether axonal transcription factors have roles *in vivo*

How can local translation in axons be established *in vivo* for any given transcription factor? *In vivo* experiments are important because experiments with cultured neurons have many potential artifacts. For example, the axonal localization of various mRNAs could reflect an adaptation to the culturing environment. Many mRNAs that are thought to encode locally translated proteins have not been shown to exhibit axonal localization in histologic sections. This is because axonal mRNAs exhibit punctate localizations in axons, which are often difficult to definitively identify in axons. mRNAs in axons appear to be present in granules, which may make them less accessible to probes used for in situ hybridization. The few examples in which the mRNAs are seen in tissue sections likely are due to their high

expression levels. For instance, *SMAD1/5/8 mRNAs* are detectable using *in situ* hybridization in tissue sections (Ji and Jaffrey, 2012). Newer *in situ* hybridization techniques (Sotelo-Silveira et al., 2011; Swanger et al., 2011) will be required to provide researchers with confidence that an mRNA encoding a transcription factor is indeed present in axons *in vivo*.

It is also important to detect local translation of transcription factors in axons in tissues. This is important since stimuli used to induce local translation in culture may not be an accurate reflection of the stimuli seen *in vivo. In vivo*, many signaling molecules are present in gradients, which cannot be recapitulated by a micropipet puffing system. Thus, studies of neurons *in vivo* can allay concerns related to neuronal adaptation and non-physiological stimuli used to induce translation. The challenge with testing the requirement for local translation in an animal is that protein synthesis inhibitors applied cannot be applied selectively to axons. Any local application system is likely to result in systemic distribution of the inhibitor. One way to potentially overcome this problem is to use cultured explant tissue. Although explants are in culture, neurons are still surrounded by the endogenous extracellular matrix, and extend axons that encounter physiological gradients of signaling molecules. Thus, explant tissue provides a way to monitor axonal behavior in a physiological context, without the artificial aspects of neuronal culture. Protein synthesis inhibitors could be applied to axons without systemic distribution through the blood, but experimental proof would be required to show that this inhibitor is not affecting translation in the cell body.

There are several examples where explant tissue could be used to address the physiological role of local protein synthesis. For example, sensory ganglia can be dissected out with embryonic limb bud attached and cultured to allow for the extension and guidance of sensory axons (Tucker et al., 2001). As another example, embryonic spinal cord explants can be used to study guidance of commissural axons to the spinal midline. In both cases, these axons can be transected, and then protein levels can be monitored in axons. By carefully comparing protein levels in severed axons treated with protein synthesis inhibitors and vehicle controls, the role of local protein synthesis in achieving the localization of an axonal transcription factor can be established. A potential problem with this approach is that the axons may begin to undergo degeneration, which could conceivably confound the results of this experiment. If axonal degeneration is a concern, these experiments can be performed in explant tissue derived from Wld^S mouse embryos. These animals express a transgene that encodes a fusion of NMNAT1, an NAD biosynthetic enzyme, with Ube4a. This transgene causes axons to exhibit markedly delayed degeneration after transfection (Conforti et al., 2000). As a result, these axons do not exhibit signs of degradation for weeks. By maintaining axonal health, any concerns related to altered expression levels due to axonal degeneration can be mitigated. Indeed, we use this approach to demonstrate the local translation of Robo3.2 in commissural axons by endogenous spinal floor plate-derived factors (Colak et al., 2013).

It should be noted that these experiments do not fully constitute "*in vivo*" experiments. However, by performing experiments in explants, which are thought to faithfully reflect the cell types and physiological gradients of extracellular signaling molecules, axons are

exposed to a complex environment which is likely to contain similar concentrations and gradients of the diverse factors found *in vivo*. Thus, explants represent a powerful model to establish whether local protein synthesis is likely to be important in mediating the axonal localization of any specific protein, including transcription factors.

Axonal vs dendritic local transcription factor synthesis

Although the examples above focus on local synthesis of axonal transcription factors, many of the concepts and principles also apply to locally synthesized transcription factors in dendrites. Recent dendritic transcriptome studies include transcripts encoding transcription factors (Zhong et al., 2006; Matsumoto et al., 2007). These transcription factors could mediate various types of dendrite-to-nucleus signaling analogous to the signaling pathways described here, and consistent with the precedent established by previous studies of dendritic transcription factors such as CREB and Elk1 (Crino et al., 1998; Barrett et al., 2006). Many of these transcription factors may be regulated by neurotransmitter signaling pathways (Wang et al., 2010), which have a more prominent role in regulating local translation in dendrites than they do in axons. Many of the issues described above, such as distinguishing between local and nuclear roles of these transcription factors need to be addressed in dendrites as well. Because dendrites have high levels of protein synthesis that persists beyond embryonic development, it is possible that local synthesis of transcription factors has roles in adult animals, and potentially during learning and memory, where local translation has important roles.

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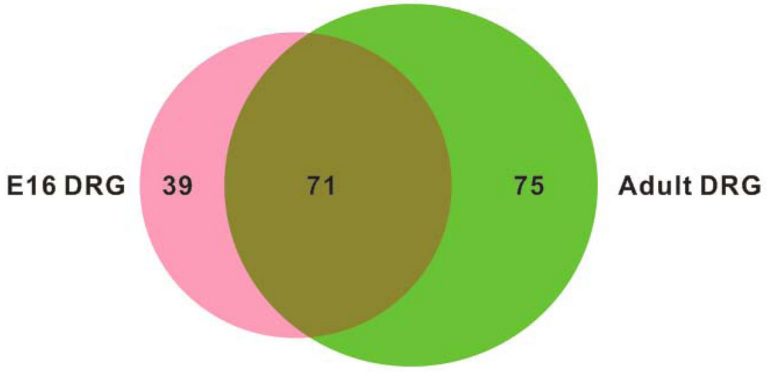

Figure 1.

Transcriptome comparison analysis of embryonic and adult DRG axons. Transcripts encoding transcription factors and cofactors were identified from the published axonal mRNA lists (Gumy et al., 2011) and by referring to available transcription factors lists and databases (Gray et al., 2004; Zhang et al., 2012; Wingender et al., 2013). Transcripts encoding 71 transcription factors were detected in both embryonic (E16) and adult DRG axons. 39 and 75 transcription factors were expressed only in E16 and adult DRG axons, respectively.

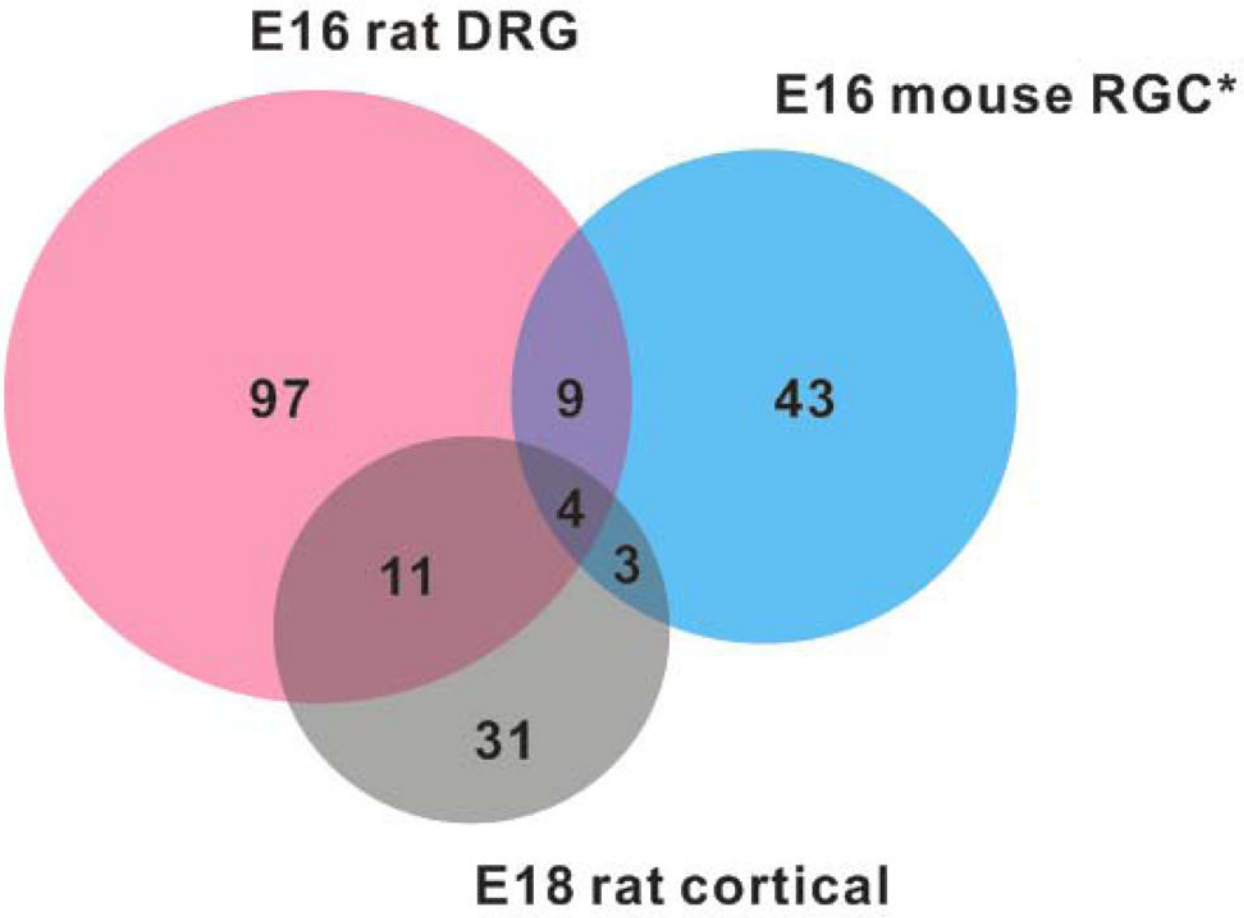

Figure 2.

Transcriptome comparison analysis of embryonic DRG, RGC and cortical axons. This study was carried out by analyzing the published data (Taylor et al., 2009; Zivraj et al., 2010; Gumy et al., 2011) using the method described as Figure 1. Four transcription factors were found to be expressed in axons of all three neuronal types. 97, 43 and 31 transcription factor mRNAs were unique to DRG, RGC and cortical axons, respectively. The asterisk indicates that the data from E16 mouse RGC was obtained from growth cones only.

Table 1

Known roles for local translation of transcription factors in axons

Signals that induce local translation	Organism	Stage	Neuronal type	Target mRNA	Function	Reference
NGF	rat, mice	embryo	DRG	*CREB*	Neuronal survival	Cox et al., 2008
Axotomy	*C. elegans*	adult	motor neuron	*C/EBP1*	Axon regeneration	Yan et al., 2009
BDNF	rat, mice	embryo	trigeminal ganglia	*SMAD1/5/8*	Neuronal specification	Ji and Jaffrey, 2012
Axotomy	rat, mice	adult	DRG, sciatic nerve	*STAT3*	Neuronal survival	Ben-Yaakov et al., 2012

Table 2

A summary of axonal mRNA encoding transcription factors, cofactors and chromatin remolding factors[†]

	Embryonic axonal mRNA	Adult axonal mRNA	
E16 rat DRG*	*Adnp, Arid1a, Ash1l, Atf4, Atf5, Atf7ip, Baz1b, Bhlhb3, Bnip2, Bnip3l, Brd3, Brd4, Cbfb, Cebpz, Chd4, Cic, Crip2, Dek, Dmtf1///RGD1562889, Dnajc2, Dpf2, Dr1, Egr1, Elf2, Etv1, Fbxl10, Fhl1, Fhl2, Fos, Foxk2, Gtf2a2, Gtf2f1, Gtf2h5, Gtf2i, Gtf2ird1, Hes6, Hif1a, Hmgb1, Hmgb2, Hmgn1, Hmgn2, Hmgn3, Hoxa5, Hsf2, Id4, Irf6, Irf3///Stk22s1, Jun, Jund, Klf4, Lasp1, Litaf, Lmo4, Maf1, Mbd1, Mbd3, Mll5, Mllt11, Morf4l1, Morf4l2, Nab1, Ncoa4, Ncor1, Ncor2, Nfatc3, Nono, Nr2f2, Nsd1, Pdlim1, Pdlim7, Phf3, Phf14, Pole4, Pxn, Runx1, Smad1, Smad4, Smarca5, Smarce1, Sox4, Ssrp1, Sub1, Taf9, Taf13, Tcf4, Tcf25, Tgfb1i1, Thrap3, Tle3, Trim28, Trip12, Tsc22d1, Tsc22d3, Ybx1, Yy1, Zcchc12, Zeb1, Zeb2, Zfhx3, Zfp36l1, Zfp91, Zfp207, Zfp219, Zfp260, Zfp278, Zfp422, Zfp553, Zfp580, Zfp771///Zfp553, Zfr*	*Arid1a, Ash1l, Atf1, Atf3, Atf4, Baz1a, Baz1b, Baz2b, Bcl6, Bcl10, Bhlhb2, Bnip2, Bnip3, Bnip3l, Brd1, Brd3, Brd4, Brd8, Cebpd, Cebpz, Chd2, Chd8, Cic, Cited2, Cnot2, Creb3, Crebbp, Crem, Crip2, Dek, Dpf2, Dr1, Egr1, Elf2, Elk3, Ets1, Ets2, Etv1, Etv3, Fbxl10, Fhl2, Fhl3, Fos, Foxj3, Foxn3, Foxp1, Fubp1, Gabpa, Gtf2a2, Gtf2b, Gtf2f1, Gtf2i, Hif1a, Hmgb1, Hmgb2, Hmgn1, Hmgn2, Hmgn3, Hopx, Hsf2, Id2, Irf3///Stk22s1, Jmjd1a, Jmjd1c, Jun, Jund, Klf4, Klf9, Lasp1, Litaf, Lmo2, Lmo4, Maf1, Max, Mbd2, Mll1, Mll3//LOC502710, Mll5, Mllt11, Mlx, Morf4l1, Morf4l2, Mtf1, Mxi1, Nab1, Ncoa3, Ncoa4, Ncor2, Nfat5, Nfatc3, Nfe2l2, Nfx1, Nfyc, Nono, Nr3c1, Nsd1, Pias2, Pms1, Pura, Pxn, Rfxap, Rreb1, Runx1, Sart1, Smarca5, Smarce1, Sox4, Sp1, Srf, Ssrp1, Stat1, Stat3, Sub1, Taf13, Tbl1xr1, Tcf4, Tcf25, Thrap3, Tle3, Tox4, Trip12, Tsc22d1, Tsc22d2, Tsc22d3, Ybx1, Yy1, Zbtb4, Zbtb11, Zdhhc3, Zdhhc6, Zeb1, Zeb2, Zfp36l1, Zfp36l2, Zfp91, Zfp148, Zfp180, Zfp207, Zfp292, Zfp386, Zfp513, Zfp644, Zfr, Zkscan3, Zmiz1, Zmiz2*	Adult rat DRG**
E16 mouse RGC[#]	*Atf4, Bach2, Baz2b, Brd8, Crebzf, Dnajc2, Eny2, Foxp1, Gatad2b, Hmg20a, Hmgb1, Hmgb2, Hmgn1, Hod, Homez, Hoxa5, Isl1, Jazf1, Lasp1, Lcor, Lmo6, Lrf, Mll5, Morf4l1, Morf4l2, Mxd3, Myc2, Nab1, Nono, Nr3c1, Nrf1, Pax3, Pdlim5, Prrx1, Ran, Rhox2, Runx1, Six4, Smad2, Sox9, Sp4, Stat6, Taf10, Tbl1xr1, Tbr1, Ubp1, Vezf1, WWc1, Zbed3, Zbtb1, Zbtb4, Zfp398, Zfp410, Zfp455, Zfp457, Zfp503, Zfp606, Zfx, Zfy1*		
E18 rat cortical[‡]	*Atf4, Bhlhb2, Bnip3l, Brd8, Bteb1, Cited2, Dnajc2, Fhl1, Foxa2, Foxe1, Foxo1A, Fshprh1, Gata3, Gbx2, Gtf2ird1, Hmgb1, Hmgb2, Hmgn2, Hoxa2, Hoxc4_mapped, Hoxd3_mapped, Id2, Id4, Jmjd1c, Lhx1, Lhx5, Litaf, Lmo4, Madh7, Nfia, Nfyc, Nr2f6, Nr6a1, Olig1, Pax8, Runx1, Smarca2, Sox11, Sp3, Stat1, Stat5A, Stat5B, Tcf4, Trim28, Yy1, Zbtb1, Zfp14, Zfp161, Zfp292*		

[†] These results are acquired by analyzing data from the previous studies (Taylor et al., 2009; Zivraj et al., 2010; Gumy et al., 2011).

* Found in embryonic axons only; found in both embryonic and adult axons.

** Found in adult axons only; found in both embryonic and adult axons.

[‡] Also found in: both embryonic and adult DRG axons; embryonic DRG axons only; adult DRG axons only.

[#] Growth cones only.