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Transcriptional Control of Axon Growth Ability

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UNIVERSITY OF MIAMI

TRANSCRIPTIONAL CONTROL OF AXON GROWTH ABILITY

By

Darcie L. Moore

A DISSERTATION

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TRANSCRIPTIONAL CONTROL OF AXON GROWTH ABILITY

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Mammalian central nervous system (CNS) neurons lose their ability to regenerate their axons after injury during development. For example, optic nerve injury studies in hamsters have shown that optic nerve axons injured around the time of birth retain the ability to regenerate to their target, but this ability is lost during development (So et al., 1981). The development of an inhibitory CNS environment has been implicated in the inability of the adult CNS to regenerate, however there is also support for this loss being a result of changes in developmental programs intrinsic to the neurons themselves (Goldberg et al., 2002a; Goldberg, 2004). While some molecules have been identified as being involved in intrinsic mechanisms controlling axon growth, there is still much to be discovered. Using genes shown to be regulated in retinal ganglion cells (RGCs) during development (Wang et al., 2007), I performed an overexpression screen in embryonic primary neurons measuring changes in neurite growth. Of these genes, the most significant effect in neurite growth was seen with overexpression of Krüppel-like factor 4 (KLF4), resulting in a greater than 50% decrease in growth. KLF4 is a member of the KLF family of transcription factors which all possess a DNA binding domain containing 3 zinc finger motifs. Outside of the nervous system, KLF4 has been implicated in cancer (Black et al., 2001; Rowland and Peeper, 2006), mitotic growth arrest (Shields et al.,
and most recently in the induction of pluripotency (Yamanaka, 2008; Zhao and Daley, 2008). In the CNS, KLF4 has recently been implicated in increasing the sensitivity of cortical neurons to NMDA insult (Zhu et al, 2009), though no effect of KLF4 on neurite growth or regeneration has yet been described. I found that KLF4 overexpression in RGCs results in decreased neurite growth and neurite initiation. KLF4 overexpression also leads to decreases in polarity acquisition in hippocampal neurons, though even when they acquire polarity, they still display decreased neurite growth. Additionally, KLF4 knockout targeted to RGCs leads to an increased neurite growth ability and increased neurite initiation in vitro. In vivo, KLF4 knockout increases RGC axon regeneration after optic nerve injury. Interestingly, KLF4 is one of 17 members of the KLF family, known for their ability to act redundantly and competitively amongst family members for their binding sites. Therefore, we looked to see if other KLFs could affect neurite growth ability. 15 of 17 KLF family members are expressed in RGCs, and their overexpression results in differential effects on neurite growth in both cortical neurons and RGCs. Additionally, many of the family members are developmentally regulated in a manner that typically correlates with their ability to affect neurite growth. For example, KLF6 and -7, whose expression decreases during development, when overexpressed, increase neurite growth, whereas KLF9, whose expression increases developmentally, when overexpressed, decreases neurite growth. Surprisingly, there are multiple KLFs expressed in RGCs that are neurite growth-suppressors, and further study has revealed that the combination of KLF growth enhancers with KLF growth suppressors results in a suppressive or neutral phenotype (Moore et al., 2009), suggesting that to further enhance regeneration after injury in vivo, we will need to additionally
remove the growth suppression from other KLF family members. Taken together, these
data suggest that KLFs may play an important role in the intrinsic loss of axon growth
and regeneration seen during development. Further characterization of downstream
targets of KLF4 and other KLF family members may reveal specific neuronal gene
targets that could mediate the phenotypic effects of these transcription factors. It is my
hope that by determining the developmental programs that underlie the loss of intrinsic
axon growth ability of CNS neurons, we may ultimately determine how to revert adult
CNS neurons to their embryonic axon growth ability.
This dissertation is dedicated to:

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PUBLICATION OF CHAPTERS AND ROLE OF AUTHOR

Chapter 1 is a review on optic nerve regeneration that will be published in the Journal of Neuro-ophthalmology in June 2010. I researched and wrote the article, with editing assistance by my mentor Jeffrey Goldberg. Figure 1.3 was contributed by Ying Hu.

Chapter 2 was published in Science October 9, 2009. I prepared and wrote the paper based upon my work during my training and in collaboration with Murray Blackmore in the Lembix lab. I was responsible for the majority of the figures in this chapter. Significant contributions came from Jeff Goldberg for figures 2.1A, 2.2A, and S10, and from Murray Blackmore for figures 2.4E (top portion), 2.4F, S11, S12, and S13. Editorial assistance came from co-authors Jeff Goldberg, Murray Blackmore, John Bixby, and Vance Lemmon.

Chapters 3, 4 and 5 are unpublished experiments that I have performed. I prepared and wrote these chapters, with editing assistance from my mentor, Jeff Goldberg. This work may become part of future paper submissions.

Chapter 6 is a review on KLF family members and their roles in axon growth and regeneration. I researched and wrote this review with editing assistance from my mentor Jeff Goldberg. This review will be submitted for publication into an appropriate journal in 2010.
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FOUR STEPS TO OPTIC NERVE REGENERATION

CHAPTER ONE

Overview

The failure of the optic nerve to regenerate after injury or in neurodegenerative disease remains a major clinical and scientific problem. Retinal ganglion cell (RGC) axons course through the optic nerve and carry all of the visual information to the brain, but they fail to regrow through the optic nerve after injury, and RGC cell bodies typically die, leading to permanent loss of vision. Why do RGCs fail to survive and regenerate their axons down the optic nerve and reconnect to the brain? In principle, there are at least four main hurdles for scientists and clinicians to overcome. First, we must increase RGC survival; second, we must overcome the inhibitory environment of the optic nerve; third, we must enhance RGCs’ intrinsic axon growth potential; and fourth, we may have to consider optimizing the mapping of RGC connections back into their targets in the brain. Here we discuss how research is targeting all four of these key steps, to hopefully give relief to patients with all types of retinal and optic nerve diseases.

Introductory remarks

Many disorders insult RGC axons in the optic nerve (Fig. 1.1), including traumatic optic neuropathy (Wu et al., 2008), ischemic optic neuropathy (Arnold and Levin, 2002; Hayreh, 2009), optic neuritis (Meyer et al., 2001; Guy, 2008), and glaucoma (Lebrun-Julien and Di Polo, 2008). The underlying causes of these diverse disorders vary: in some diseases like Leber’s hereditary optic neuropathy, the damage is thought to begin within the RGCs themselves; in others, such as in optic neuritis, damage to RGC
axons is secondary to dysfunction or loss of the surrounding optic nerve glial cells. While there are multiple well-characterized animal models for these conditions (Table 1.1), much is still unknown about their initial causes and progression. In none of these diseases, however, can RGC axon fibers regenerate back to their targets, and in most of these, RGCs die (Levin, 2007). Here we review critical advances in our understanding of why regenerating optic nerves is such a daunting task.

1. Survival of RGCs after optic nerve injury

One of the effects following optic nerve axon injury is RGC death, which can be seen in histopathological samples from human optic neuropathies (Quigley et al., 1989; Spencer, 1996; Wax et al., 1998), and can be studied in greater detail in animal models. For example, in adult rats, 85-90% of RGCs die by 2 weeks after crushing or cutting the optic nerve (Fig. 1.2; Berkelaar et al., 1994; Isenmann et al., 2003). The severity of initial RGC death is decreased the further the injury is from the eye itself (Villegas-Perez et al., 1993; Berkelaar et al., 1994; Hull and Bahr, 1994; Zeng et al., 1995), possibly due to either the support of optic nerve glial cells (see below, Fig. 1.1) or to the persistence of collateral axon branches to other supportive targets (Bernstein-Goral and Bregman, 1997). The majority of dying RGCs undergo apoptosis, or programmed cell death (Villegas-Perez et al., 1993; Berkelaar et al., 1994; Lingor et al., 2005). After RGC injury, there is an upregulation of pro-apoptotic proteins (Berkelaar et al., 1994; Isenmann et al., 1999; Levin, 1999; Qin et al., 2004; Homma et al., 2007); conversely, overexpression of anti-apoptotic proteins such as Bcl-2 results in an increased survival of
injured RGCs (Bonfanti et al., 1996; Cenni et al., 1996; Goldberg et al., 2002b; Inoue et al., 2002). Alternatively, some RGCs undergo necrotic death or secondary degeneration after optic nerve injury, although this number is minimal (Bien et al., 1999; Weise et al., 2001). The location of the injury, whether somatic or axonal, results in activation of distinct RGC death pathways (Whitmore et al., 2005).

What causes RGCs to die after optic nerve injury? Axon injury disrupts RGCs’ connection to their target, resulting in a loss of target-derived neurotrophic support. Target-derived signals are retrogradely transported to the cell body, and are hypothesized to be required for neuronal survival (Aguayo et al., 1996; Bhattacharyya et al., 1997; Grimes et al., 1997; Riccio et al., 1997; Senger and Campenot, 1997; Ure and Campenot, 1997; Howe and Mobley, 2005). Removal of this support leads to apoptosis, and addition of exogenous neurotrophic factors has been shown to increase both survival and regeneration (Yip and So, 2000). One of the neurotrophins shown to regulate RGC survival is brain-derived neurotrophic factor (BDNF), a factor expressed in the superior colliculus (Hofer et al., 1990; Wetmore et al., 1990), targeted by ~100% of RGC axons in the rodent and ~30% of RGC axons in humans. BDNF supports RGC survival both in vitro (Johnson et al., 1986; Meyer-Franke et al., 1995) and in vivo (Mey and Thanos, 1993; Cui and Harvey, 1995) by binding to its receptor, tropomyosin-receptor kinase B (trkB), resulting in activation of downstream effectors, including the Ras-MAPK and PI3K-Akt pathways. The increased survival after axotomy following BDNF application appears to be due to a combination of these two pathways (Nakazawa et al., 2002).
Are trophic factor injections alone likely to be enough to save RGCs? After injury, intraocular injections of either BDNF, or other neurotrophic factors such as neurotrophin 4/5 (NT-4/5), nerve growth factor (NGF), or insulin-like growth factor 1 (IGF-1), into the retina leads to a temporary, not sustained, increase in RGC survival (Mey and Thanos, 1993; Villegas-Perez et al., 1993; Berkelaar et al., 1994; Mansour-Robaey et al., 1994; Peinado-Ramon et al., 1996; Klocker et al., 1997; Di Polo et al., 1998; Cui et al., 1999; Yan et al., 1999; Yip and So, 2000; Koeberle and Ball, 2002; Cui et al., 2003a; Chidlow et al., 2005; Zhang et al., 2005; Zhi et al., 2005; Homma et al., 2007). For example, 25% of RGCs are alive at 3 weeks with application of BDNF or ciliary neurotrophic factor (CNTF), but by 7 weeks, 95% are dead, the same as in control conditions (Mey and Thanos, 1993). Sustained overexpression of trophic factors does not solve this problem. For example, transducing other retinal cells, such as Müller glial cells, to overexpress BDNF is neuroprotective for RGCs, but again these effects are not long-lasting (Mansour-Robaey et al., 1994; Di Polo et al., 1998). Neurotrophic factors such as glial-derived neurotrophic factor (GDNF; Klocker et al., 1997; Koeberle and Ball, 1998; Yan et al., 1999) and CNTF (Mey and Thanos, 1993; Weise et al., 2001; Watanabe and Fukuda, 2002; Cui et al., 2003a; Lingor et al., 2008) have also been found to similarly increase RGC survival after injury.

Why is the response to trophic factors so limited? Receptors for these factors are expressed in RGCs (Jelsma et al., 1993; Pachnis et al., 1993; Avantaggiato et al., 1994; Suzuki et al., 1998; Jomary et al., 1999; Ju et al., 2000; Yoles et al., 2001), however after injury, RGCs only transiently upregulate their neurotrophin receptors, followed by a
long-term decrease in their expression (Ju et al., 2000; Cui et al., 2002). Interestingly, recent studies demonstrate that exogenous application of BDNF can lead to a decrease in regeneration (Pernet and Di Polo, 2006), and application of neurotrophic factors in general can lead to downregulation of the receptors, creating a longer-term reduced responsiveness to these factors (Spalding et al., 2005), at least in animal models. It is not known whether this limited responsiveness would also be seen in humans, and neurotrophic factors for retinal neuroprotection are just starting to be tested in humans (Tao et al., 2002; Tao, 2006; Lambiase et al., 2009).

This reduced responsiveness may not be due solely to the exogenous treatment, but to a decreased ability of RGCs to respond to neurotrophins following optic nerve axon injury (Goldberg and Barres, 2000). After optic nerve injury in animal models, RGCs lose their trophic responsiveness, such that they are unable to respond to neurotrophic factors or activate their downstream intracellular signaling components, even in the presence of BDNF, for example (Meyer-Franke et al., 1995; Shen et al., 1999). This trophic responsiveness can be restored by increasing the number of trkB receptors present on the plasma membrane, either by overexpression of trkB receptors with BDNF treatment (Cheng et al., 2002) or by increasing RGCs’ intracellular cyclic-AMP (cAMP) levels either by pharmacologic treatment or by depolarization (Meyer-Franke et al., 1995; Shen et al., 1999). RGCs exhibit a decrease in cAMP levels after injury, possibly due to decreased electrical activity (Shen et al., 1999). These findings suggest a therapeutic approach, to increase RGCs’ cAMP levels post injury, although
cAMP injections alone do not increase survival (Monsul et al., 2004), and may need to be accompanied by neurotrophic factors.

Alternatively, trophic responsiveness and neuroprotection of RGCs after axon injury can also be enhanced by electrical stimulation (Morimoto et al., 2002). RGCs are less electrically active after optic nerve injury (Duan et al., 2009), and increasing activity through electrical stimulation increases cAMP levels in RGCs (Shen et al., 1999), and greatly potentiates the neuroprotective effects of neurotrophic factor treatment (Goldberg et al., 2002b), possibly by increasing the number of neurotrophin receptors on the neuronal surface (Meyer-Franke et al., 1995). Interestingly, in the peripheral nervous system (PNS), electrical stimulation after injury results in an accelerated expression of regeneration associated genes (Nix and Hopf, 1983; Al-Majed et al., 2004; Geremia et al., 2007), suggesting electrical activity may also positively influence axon growth. Indeed, recent studies have shown that transcorneal stimulation after optic nerve crush not only increased the number of surviving RGCs, but also the number of axons projecting past the lesion (Miyake et al., 2007; Tagami et al., 2009). This suggests that electrical stimulation may be used as a therapeutic strategy to increase RGC survival and growth in future studies.

In addition to the loss of positive signals such as trophic support and electrical activity, there is an increase in negative, pro-death signals after optic nerve injury. For example, there is an increase in superoxide levels in RGCs from the mitochondrial electron transport chain; blocking this increase by treatment with reactive oxygen species (ROS) scavengers or through inhibition of complex III in the mitochondria leads to a
reduction in RGC death (Lieven et al., 2006). Interestingly, providing extra 
neurotrophins cannot block this increase in superoxides (Lieven et al., 2006), suggesting 
that the cell death pathway for trophic factor withdrawal is separate from that of free 
radical-induced cell death. Treating RGCs with novel reducing agents to block this rise 
in superoxides is neuroprotective in RGCs at low doses (Schlieve et al., 2006).

All of these studies suggest that there are multiple strategies for increasing RGC 
survival after injury, including elevating cAMP, providing multiple exogenous trophic 
factors, electrically stimulating RGCs to increase their activity, and reducing superoxide 
levels. It is likely that multiple combinations of these will be required to optimally 
increase RGC survival and regeneration of RGCs. Interestingly, some of the same 
signals which increase survival also increase RGC’s ability to regenerate (reviewed in 
Goldberg and Barres, 2000).

2. The inhibitory environment of the optic nerve

Why do RGC axons fail to regenerate in the injured or diseased optic nerve (Fig. 
1.3)? Indeed after injury anywhere in the adult central nervous system (CNS), the ability 
of axons to regenerate is actively inhibited by the mature CNS environment and the 
cellular response to injury. The response by meningeal cells, microglia, 
oligodendrocytes, and astrocytes can include migration to the site of injury, proliferation, 
and changes in cellular morphology and protein expression. The expression and 
secretion of inhibitory molecules and proteins, and the presence of myelin debris, creates 
an unfavorable environment for axon regeneration in the CNS. This is in contrast to the
PNS, where axons do regenerate after peripheral nerve injury. One difference between the CNS and PNS is the makeup of the glial cells: whereas peripheral nerve Schwann cells are supportive for axon growth due to their secretion of neurotrophins and lack of associated inhibitory factors, optic nerve oligodendrocytes and reactive astrocytes are inhibitory to axon growth, expressing many inhibitory proteins, as has been demonstrated throughout the brain and spinal cord as well (Yiu and He, 2006; Abe and Cavalli, 2008). Damaged oligodendrocytes degenerate after injury, leaving myelin debris containing inhibitory proteins such as Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (Omgp) at the site of injury. Astrocytes respond to injury by becoming hypertrophic and proliferating, forming a “glial scar” at the optic nerve injury site. Astrocytes release inhibitory extracellular matrix molecules such as chondroitin sulfate proteoglycans (CSPGs), creating a molecular barrier to regeneration (Selles-Navarro et al., 2001; Yiu and He, 2006). In the spinal cord, treatment with a bacterial enzyme chondroitinase ABC to degrade the sulphated glycosaminoglycan sidechains can somewhat reverse this inhibition (Rhodes and Fawcett, 2004). In addition to actively inhibiting axon growth, CSPGs may also mask growth-promoting proteins such as laminin (Bovolenta and Fernaud-Espinosa, 2000), or may change normally growth-attractive proteins like Semaphorin 5A into repulsive cues (Kantor et al., 2004). Other semaphorins secreted from infiltrating meningeal cells and expressed by oligodendrocytes and neuroepithelial cells also inhibit axon regeneration (Goldberg et al., 2004; Fawcett, 2006; Kaneko et al., 2006). Interestingly, some of these inhibitory molecules are used as guidance cues during development and may be re-expressed after
injury. For example, semaphorin 3A acts as a repulsive guidance cue during development (Giger et al., 1998), but is also upregulated after injury, inhibiting axon regeneration (Pasterkamp et al., 2000; Pasterkamp and Verhaagen, 2001). The expression of netrin-1 at the optic nerve head, normally attracting RGC axons to exit the retina and grow into the optic nerve in early development, becomes an inhibitory signal in later life due to the low levels of cAMP in adult RGCs (Shewan et al., 2002). Therefore, the complexity with which these inhibitory proteins interact with RGCs still needs to be further defined.

Can we simply turn off RGCs’ response to such negative cues? Semaphorin receptors such as neuropilin-1 are upregulated following injury (Nitzan et al., 2006; Pasterkamp and Verhaagen, 2006); Nogo-A, MAG, and Omgp activate neuronal Nogo receptor (NgR) protein complexes that may be constitutively expressed; and downstream signaling molecules such as Rho and Rho kinase (ROCK; see below) are normally found in RGC axons. Research involving these proteins has led to varied results, limiting the ability to draw complete conclusions as to their exact mechanisms. For example, inactivation of MAG through chromophore assisted laser inactivation (CALI) after optic nerve crush in embryonic day 15 (E15) chick explants, led to axon regeneration to the edge of the explant (~2mm; Wong et al., 2003). In MAG knockout mice, however, optic nerve crush in ~7 week-old animals was not followed by RGC regeneration 2 weeks after injury (Bartsch et al., 1995). The differences between these experiments (in situ vs. in vivo, chick vs. mouse, 36 hours vs. 2 weeks, E15 vs, 7 weeks old) make them difficult to
compare, but they suggest that MAG inactivation alone is not sufficient to increase axon regeneration in mammals.

CNS injuries performed on the multiple different Nogo and NgR knockouts have revealed varied results in regeneration, from none to modest regeneration and sprouting, although more positive results have been seen using dominant-negative and pharmacoinhibition strategies against these same molecules (Chaudhry and Filbin, 2006). Treatment with the IN-1 antibody, for example, which neutralizes Nogo-A, resulted in greater regeneration than Nogo-A gene knockouts (Buchli and Schwab, 2005; Teng and Tang, 2005b). Treatment of spinal cord injury with anti-Nogo antibodies has entered clinical trials in Europe and, if successful, is almost certain to be followed by optic nerve regeneration clinical trials (http://www.axregen.eu/team/university-of-zurich/).

Interestingly, pre-treating neurons with neurotrophins prior to exposure to MAG and myelin significantly decreases their ability to inhibit axon growth. This neurotrophin pre-treatment increases levels of intracellular cAMP (Cai et al., 1999). In agreement, a developmental decrease in cAMP correlates with a developmental increase in the negative response to MAG/myelin (Cai et al., 2001). This again identifies cAMP as an important modulator of axon growth after injury.

Many of these inhibitory environmental signals converge on a downstream target, the small GTPase, Rho (Gross et al., 2007). Targeting a convergent downstream signal relieves the need to block each glial-associated inhibitor separately. Rho signaling leads to actin cytoskeleton remodeling (Maekawa et al., 1999) and growth cone collapse (Fournier et al., 2000; Benarroch, 2007). Experiments inactivating Rho with C3
transferase, which ADP-ribosylates Rho proteins, showed increased regeneration of CNS fibers (Lehmann et al., 1999; Dergham et al., 2002; Winton et al., 2002; Bertrand et al., 2005), specifically when Rho was inactivated soon after injury (Bertrand et al., 2007). Multiple injections of this inhibitor after injury additionally resulted in an increase in RGC survival (Bertrand et al., 2007). Additionally, the combination of Rho inactivation, overexpression of CNTF, cAMP treatment and peripheral nerve grafting after axotomy enhanced viability as well as increased regeneration of those surviving fibers (Hu et al., 2007). Recently, collapsing-response mediator protein 4b (CRMP4) was identified as interacting with Rho to carry out its inhibitory functions. Knocking down CRMP4 or blocking CRMP4 and Rho interaction resulted in attenuation of inhibition from myelin substrates, identifying an even more specific therapeutic target than Rho itself (Alabed et al., 2007). Inhibition of ROCK, a downstream effector of Rho, has also shown promising results in overcoming environmental inhibition and promoting neurite outgrowth both in vitro and in vivo (Borisoff et al., 2003; Fournier et al., 2003; Chan et al., 2005; Lingor et al., 2007; Sagawa et al., 2007; Ichikawa et al., 2008; Lingor et al., 2008).

Besides activation of RhoA by these inhibitory environmental signals, there is an increase in intracellular calcium which may be involved in the downstream activation of the epidermal growth factor receptor (EGFR) and PKC, though whether these two are interrelated has yet to be determined. Inhibition of PKC activity from CSPG and myelin-based activation increases regeneration in dorsal column axons (Sivasankaran et al., 2004). Inhibiting EGFR pharmacologically after optic nerve crush blocks myelin and CSPG inhibition on neurite growth, and promotes regeneration of RGCs (Koprivica et al.,
2005), though recently it has been suggested that this EGFR inhibitor may be acting through other mechanisms (Ahmed et al., 2009; Douglas et al., 2009). A number of drugs that block RGCs’ and other CNS neurons’ responses to inhibition are now in clinical trials for spinal cord injury, and identification of further downstream targets of inhibitory signaling will create more specific therapeutic targeting strategies for future studies (Thuret et al., 2006).

Not all of the cellular responses to injury or disease are negative or inhibitory. For example, macrophages associated with inflammation can potentially be neuroprotective and induce axon outgrowth (Filbin, 2006). Macrophages are recruited with lens injury and elicit an 8-fold increase in RGC survival and a 100-fold increase in regeneration of RGC axons past the site of an optic nerve crush (Fischer et al., 2000; Leon et al., 2000; Fischer et al., 2001). Similar macrophage activation can be elicited by injection of zymosan, a yeast cell wall preparation which activates macrophages (Leon et al., 2000; Yin et al., 2003; Lorber et al., 2005). How does lens injury create these effects? One possibility is that macrophages migrating into the retina express oncomodulin, which causes extensive outgrowth of RGCs (with concurrent elevation of cAMP) after optic nerve crush in the adult optic nerve (Yin et al., 2006). In contrast, recent studies suggest that the effect of lens injury is not due to macrophage activation or oncomodulin, but primarily occurs through an upregulation of CNTF expression in retinal astrocytes (Leibinger et al., 2009). Finally, combinatorial approaches may further enhance regenerative response, for example using macrophage-derived factors to “sensitize”
neurons prior to dominant-negative suppression of the activity of NgR (Fischer et al., 2004a), or in combination with Rho inactivation (Fischer et al., 2004b).

The immune system has recently been targeted for clinical trials in optic nerve neuropathies and spinal cord injuries. Immunization with a peptide derived from Nogo-A, an inhibitory protein present on myelin, can increase recovery after spinal cord injury (Hauben et al., 2001). In addition, vaccination with copolymer 1 (Cop-1), a synthetic chain of amino acids which cross-reacts with myelin basic protein (MBP), could activate the immune system and decrease secondary degeneration of surviving fibers after optic nerve injury. Cop-1 is a drug presently used to treat multiple sclerosis and is not known to create any additional immunogenic effects, therefore making it a good candidate for clinical testing (Kipnis et al., 2000). Clinical trials were started for Cop-1 treatment in progressive optic nerve degeneration, and initiated but suspended for transplantation of autologous activated macrophages into the injured spinal cord (Knoller et al., 2005).

Further studies to find additional proteins released following lens injury or macrophage activation may reveal potential candidates involved in increasing both CNS survival and regeneration.

Finally, bypassing the inhibitory optic nerve environment entirely is a reasonable solution—and is the oldest approach as well. As early as 1911, Tello used peripheral nerve grafts attached to cut optic nerve to demonstrate that RGCs could regenerate a short distance if given a permissive substrate (Tello, 1911). Such experiments were rejuvenated by Aguayo and colleagues in the 1980s, using sciatic nerve transplants to connect the retina to the superior colliculus (Aguayo et al., 1987). While the majority of RGCs died
as a result of the optic nerve injury, replacement of a portion of the injured optic nerve with a piece of peripheral nerve enabled about 20% of the surviving RGCs (~5%) to regrow long axons back to their targets, though this took approximately 2 months (Bray et al., 1987; Vidal-Sanz et al., 1987; Aguayo et al., 1990). These results suggest that there is a small percentage of RGC axons that are able to regenerate in a permissive environment; however, whether this small percentage is enough to mediate a functional outcome in humans is unclear.

Why are peripheral nerve grafts able to support CNS regeneration? The less inhibitory environment of the peripheral nerve, as well as the positive trophic environment secreted by Schwann cells creates a very permissive substrate for growth. In addition, the peripheral nerve graft may act to change the role of cells already present in the injured optic nerve (Dezawa et al., 1999). While astrocytes typically respond to CNS injury by hypertrophy, proliferation, expression of inhibitory proteins such as CSPGs, and creation of a glial scar, in peripheral nerve grafts, astrocytes were found to encircle axonal bundles and act in conjunction with the Schwann cells of the peripheral nerve to guide those axons which regenerated through the peripheral graft, ultimately changing the environmental response at the injury site (Dezawa et al., 1999).

In addition to peripheral nerve grafts, other substrates have been grafted in experiments to increase RGC outgrowth and regeneration. Transplanted perinatal optic nerves (Sievers et al., 1995), RGC target tissue from fetal brain (Harvey et al., 1987; Hausmann et al., 1989; Sievers et al., 1989; Harvey and Tan, 1992), cell transplants (Lu et al., 2003; Girard et al., 2005; Lu et al., 2005), various bridge matrices containing
Schwann cells (Berry et al., 1988; Xu et al., 1995a; Dezawa et al., 1997; Plant and Harvey, 2000; Negishi et al., 2001), the addition of neurotrophic factors (Hagg et al., 1991; Xu et al., 1995b; Yick et al., 1999; Terris et al., 2001; Iannotti et al., 2003; Hu et al., 2005), olfactory ensheathing cells (Smale et al., 1996; Cui et al., 2003b; Ruitenberg et al., 2006), and peripheral nerve transplants into the vitreous (Berry et al., 1996; Berry et al., 1999) have all shown varied results. The graft and transplantation studies have shown that, at a minimum, some CNS neurons can regenerate to their targets, however, it is important to now determine if these techniques can be translated into therapeutic treatments for patients.

3. The developmental loss of RGCs’ intrinsic axon growth ability

In essentially all of the above experiments, even when some of the inhibitory signals are neutralized, only a small percentage of RGCs regenerate, and at a very slow rate. Could there be a problem with the adult neurons themselves losing their capacity to re-grow their axons? In spinal cord injury studies in cats, the neonatal nervous system retains its ability to regenerate, but this ability is lost as the animals develop (Bregman and Goldberger, 1982). This finding was confirmed in rats whose spinal cords were injured at birth or as adults, demonstrating again that the neonatal CNS retains the ability to regenerate while the adult CNS does not (Kunkel-Bagden et al., 1992). Is this simply because the adult glia have turned on their expression of inhibitory molecules? One way to address this question has used “heterochronic cultures.” For example, embryonic retinal explants can extend axons into embryonic or adult brain explants, but adult retinal
explants cannot extend axons into either, suggesting that the problem is with the retina or RGCs (Chen et al., 1995). Similarly, co-culturing mature postnatal explants with young, environmentally permissive explants have demonstrated a differential effect of tissue age on regenerative ability in hippocampal (Li et al., 1995), cerebellar (Dusart et al., 1997; Bouslama-Oueghlani et al., 2003), and hindbrain tissues (Blackmore and Letourneau, 2006). Such studies suggested that intrinsic changes within the neurons themselves limited their regenerative ability, although in heterochronic co-culture experiments the glial environment surrounding the injured neurons remains, leaving the possibility that local (e.g. retinal) inhibitory signals are still responsible for the lack of growth from adult tissues.

Pure RGC cultures were finally used to definitively demonstrate that RGCs do indeed turn off their intrinsic capacity for rapid axon growth during early development. To remove all influence of any potential extrinsic, inhibitory environment, RGCs from embryonic or postnatal ages were purified away from all other cell types and cultured in a strongly trophic environment (Fig. 1.4) or even transplanted back in vivo, where in all cases, embryonic RGCs extended their axons up to 10-fold faster than postnatal or adult RGCs (Goldberg et al., 2002a). Altering the extrinsic environment did not change this fundamental observation, pointing to intrinsic limitations in RGC axon growth ability. Additionally, it was found that this developmental decrease can be initiated by a membrane-associated signal on presynaptic amacrine cells (Fig. 1.1; Goldberg et al., 2002a). These findings confirm the in vivo and in situ experiments above, suggesting that a developmental program in RGCs is involved in their inability to regenerate.
Can RGCs’ intrinsic capacity to regenerate be increased in the adult back to embryonic levels? One method of increasing their intrinsic axon growth ability may be to alter the expression of specific genes that are up- or downregulated during development or after injury or in disease. For example, overexpression of Bcl-2, an anti-apoptotic gene whose expression is decreased developmentally, increases RGC survival and slightly increases the regenerative capacity of RGCs in tissue explants (Chen et al., 1997). In vivo, Bcl-2 overexpression leads to increased regenerative ability in early postnatal but not later postnatal rodents, even after the negative influence of astrocytes is also minimized (Cho et al., 2005). Using pharmacological approaches to induce Bcl-2 expression using lithium and removing astrocytes at the injury site using an astrocyte toxin resulted in an increase in optic nerve regeneration, without increasing RGC survival (Cho and Chen, 2008), though how much of this is related to other effects of lithium is unclear. Bcl-2 may yet be a promising target to increase RGC survival and possibly regeneration in vivo despite these mixed results.

The levels of cAMP within neurons also appear to be developmentally regulated such that embryonic neurons have high cAMP levels which drop sharply postnataally and remain low throughout adulthood (Cai et al., 2001). The response of these neurons to MAG/myelin is also dependent on the neuron’s stage of development and the neuron’s intrinsic level of cAMP, as described previously. Embryonic axons, possessing endogenously high cAMP levels, are promoted by MAG/myelin, and this effect requires the activation of the transcription factor, cAMP response element-binding protein (CREB; Gao et al., 2004). CREB’s upregulation of Arginase I (Arg I), an enzyme which
synthesizes polyamines, has been suggested to be part of the molecular pathway involved in overcoming myelin inhibitors (Cai et al., 2002; Gao et al., 2004; Deng et al., 2009). The expression profile of Arg I parallels that of cAMP levels during development, and its overexpression has been shown to be sufficient to block the switch from promotion to inhibition by MAG/myelin (Cai et al., 2002; Gao et al., 2004). Therefore, the change in cAMP levels and its downstream effectors during development is a type of switch which allows neurons to respond differently to extrinsic inhibitory environments (Cai et al., 2001; Cai et al., 2002).

Other developmentally regulated signaling pathways could be important in axon regeneration. For example, the activation of the MAP kinase pathway was recently found to be important in the regeneration of motor neurons in c. elegans. The Dlk-1/MKK-4/PMK-3 MAP kinase pathway was not only required for axon regeneration but also for normal growth cone formation and morphology. Interestingly, this pathway was not necessary during development, suggesting that there are specific signaling pathways activated during regeneration that are separate from those activated during development (Hammarlund et al., 2009). Dlk has also been shown to be important for the degeneration of severed axons, a necessary component in successful regenerative systems, clearing the way for regeneration following injury (Miller et al., 2009). Thus, the effect of Dlk seen in regeneration could be due, at least in part, to its effect on degeneration as well.

Another signaling pathway that is developmentally regulated, at least in part, centers on the mammalian target of rapamycin (mTOR) protein. Deleting either the phosphatase and tensin homolog (PTEN) protein, or tuberous sclerosis complex 1
(TSC1), both of which normally inhibit mTOR, leads to a dramatic increase in the number of regenerating axons after optic nerve injury, as well as to an increase in RGC survival (Park et al., 2008). mTOR has been shown to be important in cell growth and in protein synthesis, and suggests that an active growth state may require this pathway to remain active.

Could the activation or inactivation of transcriptional programs be important in the loss of intrinsic axon growth ability? In cerebellar granule neurons (another type of CNS neuron), the ubiquitin ligase Cdh1-anaphase promoting complex (Cdh1-APC) and its downstream targets have been identified as important players in the intrinsic regenerative ability of CNS neurons. Cdh1-APC was first identified to be a cell cycle ubiquitin ligase, however, a new role for this complex has been discovered in regulating axonal growth, in particular for axon growth inhibition (Konishi et al., 2004; Stegmuller and Bonni, 2005; Teng and Tang, 2005a; Lasorella et al., 2006; Stegmuller et al., 2006; Stegmuller et al., 2008; Huynh et al., 2009). Cdh1-APC targets inhibitor of differentiation 2 (Id2) for degradation, releasing a basic helix-loop-helix transcription factor (E47) to upregulate genes involved in axon growth inhibition, including NgR (Lasorella et al., 2006). Cdh1 also targets the transcription factor SnoN, whose degradation results in reduced axonal growth (Stegmuller et al., 2006). These results suggest that the loss of intrinsic axon growth ability could be induced by critical transcriptional changes. It is not known, however, if these exact pathways function similarly in RGCs.
Interestingly, our lab has recently found that a family of transcription factors called Krüppel-like factors (KLFs) may affect axon growth ability during development and regeneration. There are 17 members of the KLF family; 15 KLFs are expressed in RGCs, and many of these are developmentally regulated (Moore et al., 2009). For example, the expression of both KLF4 and KLF9 increases during postnatal development. When overexpressed in RGCs, they decrease neurite growth significantly. Using a gene knockout of KLF4 in RGCs, we have found that removal of KLF4 increases neurite growth of RGCs in vitro, and more importantly, increases axon regeneration after optic nerve injury. Taken together, these results suggest that the KLF family of transcription factors may be involved in the loss of intrinsic loss of axon growth ability seen during development in RGCs.

This large number of signaling pathways, proteins, and transcription factors suggests that not one single target will be adequate to increase optic nerve regeneration. Coaxing RGC axons through an injured or degenerating optic nerve may take a multifactorial approach, addressing RGC survival, glial inhibitors, and RGCs’ intrinsic capacity for rapid axon regeneration.

4. Target reinnervation after optic nerve injury

Once we are able to enhance RGC survival after injury, overcome the inhibitory molecules present at the lesion, and re-establish an embryonic growth phenotype, will the axons be guided back to their developmental targets and create functional maps of visual space? Work on regeneration is too early to comment on what will happen “post-
regeneration;” however, much is known about developmental mapping from which we can draw assumptions. For example, during embryonic development, RGC axons are attracted to the optic nerve head by glial cells expressing netrin-1 (Kennedy et al., 1994; Serafini et al., 1996; Deiner et al., 1997), half are guided by ephrin B2 ligands to remain ipsilateral at the optic chiasm (Williams et al., 2003), and are funneled within the optic nerve by Semaphorin-5A, expressed by neuroepithelial cells (Adams et al., 1996; Raper, 2000; Oster et al., 2004), and Slit (Erskine et al., 2000; Niclou et al., 2000; Plump et al., 2002). To keep spatial orientation intact, RGC axons must topographically map onto the superior colliculus, their target in the midbrain. This occurs through gradients of ephrin ligands which create the specificity of the visual map, allowing RGCs to communicate their positional relevance (Inatani, 2005).

Are any of these molecules available for regenerating axons finding their way to their target brain regions in the adult? Experiments have shown that after deafferentation of the superior colliculus (again, the major target for RGCs in rodents), there is re-expression of some of the same guidance cues, creating a crude topographic map (Bahr and Eschweiler, 1991, 1993; Wizenmann et al., 1993; Sauve et al., 2001). After optic nerve injury, ephrin A2 expression in the superior colliculus is upregulated (Knoll et al., 2001; Rodger et al., 2001; Rodger et al., 2005), and if RGC survival is enhanced, RGCs express the appropriate Eph A5 receptors in a gradient that mimics development (Symonds et al., 2007). Other developmental guidance molecules such as ephrin B1, however, are only minimally expressed in the adult and deafferented superior colliculus (Knoll et al., 2001). Interestingly, in experiments in which a small percentage
of regenerating axons were able to reach their target through peripheral nerve grafts, functional synapses were identified at the target tissue (Carter et al., 1989; Keirstead et al., 1989; Vidal-Sanz et al., 1991; Carter et al., 1994; Sauve et al., 1995; Thanos, 1997; Thanos et al., 1997; Vidal-Sanz et al., 2002), though the axonal arbors made by these neurons were much smaller than normal (Carter et al., 1998). Those fibers that did reinnervate the superior colliculus created a rough topographic map, suggesting developmental cues may be re-expressed in the target tissue, allowing for appropriate mapping (Sauve et al., 2001). Additional grafting studies connecting a severed optic nerve with the pretectum (important for pupillary reflexes) revealed a restoration of a functional pupillary reflex after 2-4 months (Thanos, 1992; Whiteley et al., 1998; Vidal-Sanz et al., 2002), again supporting the notion that regenerating axons can functionally reinnervate their target tissues. Taken together, these findings suggest that if RGCs can be promoted to regenerate, there are some developmental cues available to guide axons back to their targets and possibly re-create topographic maps of visual space.

Conclusions

Promoting optic nerve regeneration may seem an insurmountable task, and indeed we often communicate to patients with various optic neuropathies that the vision lost is lost for good. Research in the field of CNS regeneration to date suggests that we must simultaneously address RGC death after optic nerve injury, the inhibitory glial environment, changes in RGCs’ intrinsic potential for axon regeneration, and ultimately think about how regenerating axons may innervate their targets in the brain. With a
number of these approaches entering into human clinical trials in the optic nerve or the spinal cord, and with many new technologies and strategies, we are getting closer to offering real hope to those with optic nerve disease.

Acknowledgements

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## CHAPTER ONE TABLES

Table 1.1

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<tr>
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<td>Decreasing aqueous outflow</td>
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<td><strong>Ischemic optic neuropathy</strong></td>
<td>Photochemically induced ischemic optic neuropathy</td>
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<td><strong>Optic neuritis</strong></td>
<td>2D2 myelin oligodendrocyte glycoprotein (MOG)-specific T cell receptor (TCR) mice</td>
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<td>Optic nerve crush</td>
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<td><strong>Leber’s Hereditary Optic Neuropathy</strong></td>
<td>Mutated human ND4 gene targeted to mitochondria</td>
<td>(Qi et al., 2007)</td>
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For an additional review of animal models of optic nerve diseases, see (Levkovitch-Verbin, 2004)
Figure 1.1. The retina contains at least 6 major classes of neurons plus Müller glial cells and other astrocytes, but retinal ganglion cells (RGCs) are the only output neurons of the retina, sending their axons out of the optic disc and into the optic nerve. Most of the processing of light begins with rod and cone photoreceptors in the outer nuclear layer (ONL), which transduce photons into a chemical code passed onto neurons of the inner...
nuclear layer (INL). The signal is modified and enhanced by these interneurons before providing input onto RGCs, which transmit both visual and non-image-forming information to the brain.

In the optic nerve, RGC axons are myelinated by oligodendrocytes, increasing the speed and probably the fidelity of electrical signaling to the brain. Astrocytes also play critical roles throughout the optic nerve, communicating with both blood vessels and with RGC axons in the spaces between the ensheathing myelin. The RGC axons in turn propagate an electrical signal down their length, arriving at their target structures in the brain.

Figure 1.2. RGC survival is dramatically decreased following optic nerve crush in adult mice. RGCs can be labeled by injections of a fluorescent dye into the superior colliculus, the primary target for RGC axons in the rodent. The dye (in this example, Fluorogold, shown in red) is retrogradely transported along the length of the axons back to the cell bodies in the retina, allowing for specific labeling of RGCs. The dissected retina can be viewed en face in flatmount as above, and can also be immunostained for RGC markers like beta-III tubulin (shown in green), which label RGC cell bodies as well as their bundled axons in the nerve fiber layer (NFL). When one optic nerve is crushed after such retrograde labeling, by two weeks a severe loss of retrogradely labeled or tubulin-positive RGCs is noted, along with atrophy of the axon bundles in the NFL. Scale bar, 50 µm.
Figure 1.3. Optic nerve crush experiments allow researchers to test approaches to increase optic nerve regeneration. Rodent optic nerves can be crushed a short distance behind the eyeball using fine forceps and left for 2 or more weeks to assess for any regeneration to occur. One day before collecting the nerves for processing, a fluorescent anterograde tracer is injected into the vitreous and is taken up by retinal cells including RGCs, which transport the dye down the length of their axons. In animals with no therapeutic treatment (control, top), the axons typically stop at the crush site (asterisk) and very few will regenerate any further into the distal optic nerve. In contrast, animals treated with multiple injections of ciliary neurotrophic factor (CNTF) plus cyclic-AMP (cAMP) demonstrate increased RGC axon regeneration past the crush site (bottom). Scale bar, 200 µm. (Y. Hu and J. Goldberg, unpublished data)
Figure 1.4. RGCs can be purified away from all other cells of the retina by immunopanning, and cultured in vitro to measure their survival and axon and dendrite growth. Here, RGCs cultured for 3 days are fixed and immunostained for Tau to label axons and dendrites. Scale bar, 50 µm.
CHAPTER TWO

KLF FAMILY MEMBERS REGULATE INTRINSIC AXON REGENERATION ABILITY


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CHAPTER TWO

Overview

Neurons in the central nervous system (CNS) lose their ability to regenerate early in development, but the underlying mechanisms are unknown. By screening genes developmentally regulated in retinal ganglion cells (RGCs), we identify Krüppel-like factor-4 (KLF4) as a transcriptional repressor of axon growth in RGCs and other CNS neurons. RGCs lacking KLF4 show increased axon growth both in vitro and after optic nerve injury in vivo. Related KLF family members suppress or enhance axon growth to differing extents, and several growth-suppressive KLFs are upregulated postnatally, whereas growth-enhancing KLFs are downregulated. Thus, coordinated activities of different KLFs regulate the regenerative capacity of CNS neurons.

Introductory remarks

Adult mammalian central nervous system (CNS) axons are unable to regenerate after injury, but immature CNS neurons regenerate axons robustly (Bregman et al., 1989; Chen et al., 1995; Nicholls and Saunders, 1996). In addition to the development of an inhibitory CNS environment (Case and Tessier-Lavigne, 2005; Yiu and He, 2006), a developmental loss in neurons’ intrinsic capacity for axon growth is thought to contribute to regeneration failure. For example, after birth, axonal outgrowth from rat retinal ganglion cells (RGCs, a type of CNS neuron) slows substantially (Goldberg et al., 2002a). Similar developmental declines in axon growth ability have been observed in mammalian tissue explants of brainstem (Blackmore and Letourneau, 2006), cerebellum
(Dusart et al., 1997; Bouslama-Oueghlani et al., 2003), entorhinal cortex (Li et al., 1995), and retina (Chen et al., 1995). Various cell-autonomous factors such as cAMP and CREB (Cai et al., 2001; Gao et al., 2004), Bcl-2 (Chen et al., 1997; Cho et al., 2005), Rho/ROCK (Lehmann et al., 1999), Cdh1-APC (Konishi et al., 2004; Lasorella et al., 2006), and PTEN (Park et al., 2008) have been suggested to play roles in this process. However, manipulating these regulators of axon growth, even when simultaneously overcoming environmental inhibition, only partially restores regeneration, suggesting that additional intrinsic axon growth regulators remain to be identified.

**Results**

To investigate the molecular basis for the developmental loss of axon growth ability in RGCs, we took advantage of the fact that co-culture with amacrine cell membranes is sufficient to signal embryonic RGCs to decrease their rapid axon growth (Goldberg et al., 2002a). Addition of the transcriptional inhibitor actinomycin D blocked this effect of amacrine membranes, and embryonic RGCs retained their capacity for axon growth (Fig. 2.1A). These data suggest that gene transcription is required for the developmental loss of intrinsic axon growth ability in RGCs.

To identify candidate genes, we profiled gene expression from embryonic day 17 (E17) through postnatal day 21 (P21) RGCs (Wang et al., 2007), spanning the period when axon growth ability declines in vivo (Chen et al., 1997; Goldberg et al., 2002a). We screened 111 candidates whose expression changed greater than 3-fold by overexpression in embryonic hippocampal neurons, and used automated image
acquisition and neurite tracing (KSR instrument, Cellomics) for rapid, unbiased quantification of neurite length (Buchser et al., 2006); the investigator (DLM) was blinded to gene identity until the screen was complete. The zinc-finger transcription factor, Krüppel-like factor-4 (KLF4), was the most effective suppressor of neurite outgrowth, decreasing average length by 50% (Fig. 2.1B). In a separate, blinded screen examining growth cone morphologies, KLF4 again emerged as the most interesting candidate gene as growth cones in KLF4-overexpressing hippocampal neurons were consistently enlarged (e.g. Fig. 2.1C).

Although KLF4 regulates cell survival in other systems (Ghaleb et al., 2007; Hagos et al., 2009; Zhu et al., 2009), we detected no differences in survival between KLF4- and control-transfected hippocampal neurons (Suppl. Fig. 1A). To determine if the growth-suppressive effect was specific either to axons or dendrites, we manually traced Tau+ and MAP2+ neurites (Suppl. Fig. 1B). Overexpression of KLF4 in embryonic hippocampal neurons significantly decreased the lengths of both axons (Tau+/MAP2-) and dendrites (Tau+/MAP2+; Suppl. Fig. 1-2). We also observed a reduction in branching (Suppl. Fig 3) and in the percentage of neurons that extended neurites (Suppl. Fig. 1C). Taken together, these findings suggest that KLF4 acts independently of cell survival to suppress axon and dendrite initiation and elongation by hippocampal neurons in vitro.

We next asked whether KLF4 regulates axon growth of RGCs. KLF4 expression increased postnatally both by microarray analysis (Wang et al., 2007; Fig. 2.2A) and by quantitative reverse transcriptase PCR (qRT-PCR; Fig. 2.2B) of acutely purified RGCs.
We purified RGCs from E20 rats and transfected them with FLAG-tagged KLF4 (Zhang et al., 2006) or a FLAG-only control. Overexpression of KLF4 in embryonic RGCs reduced the percentage of neurons extending neurites (Fig. 2.2E), reduced neurite branching (Suppl. Fig. 4), and reduced axon and, less so, dendrite lengths (Fig. 2.2C-D). The average axon length of KLF4-transfected RGCs continued to increase over three days, but at a slower rate than control transfected neurons (Suppl. Fig. 5), suggesting that KLF4 overexpression decreases elongation rate. Furthermore, truncated KLF4 that lacked a C-terminal DNA-binding domain (Suppl. Fig. 2A; Zhang et al., 2006) had no effect on axon growth (Fig. 2.2C-D). Thus, KLF4 suppresses axon growth in embryonic RGCs, and KLF4’s DNA-binding domain is required for its growth-suppressive activity.

We next tested whether knocking out KLF4 in developing RGCs enhances axon growth ability. Because KLF4-null mice die perinatally (Segre et al., 1999), we used a Cre/lox strategy to target KLF4 knockout to RGCs. Floxed-KLF4 mice (Katz et al., 2002) were crossed to ROSA-EYFP reporter mice and Thy-1-promoter Cre recombinase mice. Approximately 50% of RGCs purified from Thy-1-cre/ROSA-EYFP mice were EYFP+ (Suppl. Fig. 6). There was no effect of transgenic Cre expression on RGC neurite growth, neurite initiation or survival in vitro (Suppl. Fig. 7, 8). To examine axon growth from KLF4-deficient RGCs in vitro, RGCs were purified from P12 Thy1-cre+/−/KLF4fl/fl/ROSA-EYFP+ ("KO") or Thy1-cre−/−/KLF4fl/fl/ROSA-EYFP+ ("WT") littermate mice and cultured for 3 days (Fig. 2.3A). No effect of KLF4-KO was seen on survival (Suppl. Fig. 8). P12 KLF4-KO RGCs showed a statistically significant increase in neurite initiation compared to controls (Fig. 2.3B), mirroring our previous finding that
overexpression of KLF4 decreases neurite initiation (Fig. 2.2E). We also observed a significant increase in neurite lengths in KLF4-KO RGCs (Fig. 2.3C). These data demonstrate that knocking out KLF4 enhances axon growth ability in P12 RGCs in vitro.

We next asked if knocking out KLF4 during development enhances regeneration from adult RGCs in vivo. Thy1-cre+/KLF4fl/fl (KO), Thy1-cre+/KLF4fl/+ (Het), or Thy1-cre+/KLF4+/+ (WT) littermate mice were subjected to optic nerve crush, and after two weeks we assessed regeneration of RGC axons in the optic nerve. By adulthood, there were no differences in RGC number between KO, Het and WT animals (Suppl. Fig. 9A). Compared to controls, however, KLF4 KO mice showed an increase in the number of regenerating axons at multiple distances from the injury site (Fig. 2.3D-E). KLF4 KO did not affect RGC survival after injury (Suppl. Fig. 9B), showing that this increase in regenerating axons was not secondary to an increased RGC number. Thus, knocking out KLF4 expression during development increases the regenerative potential of adult RGCs.

Although knocking out KLF4 enhanced axon growth and regeneration, the size of the effect led us to speculate that other KLF family members might compensate for the loss of KLF4. The KLF family is comprised of 17 related transcription factors with homologous DNA-binding domains and divergent activation and repression domains (Kaczynski et al., 2003). KLFs often regulate gene expression interactively, with both cooperative and competitive relationships among family members (Dang et al., 2002; Eaton et al., 2008; Jiang et al., 2008). Our microarray data suggested that many KLFs are expressed by RGCs (Wang et al., 2007), and that some are developmentally regulated (Suppl. Fig. 10). We re-profiled the expression of all 17 KLF family members in
developing RGCs by RT-PCR, and detected transcripts for 15 (Fig. 2.4E). Furthermore, qRT-PCR revealed that KLF6 and KLF7 transcripts decrease more than 10-fold, while KLF9 increases more than 250-fold (Fig. 2.4A-C). Thus expression of multiple KLFs is regulated in developing RGCs.

Do other KLF family members also regulate neurite growth? Other KLFs can affect neurite branching in response to thyroid hormone (KLF9; Cayrou et al., 2002) or neurite outgrowth in zebrafish retinal explants (KLF6 and -7; Veldman et al., 2007). In RGCs, overexpression of KLF9 significantly decreased growth, similar to KLF4, and KLF6 and -7 increased neurite growth 13% and 23%, respectively (Fig. 2.4D). We comprehensively surveyed all 17 KLF family members’ effects on neurite growth in cortical neurons in vitro, and found that although no KLFs affected cell survival (Suppl. Fig. 11), eight KLFs including KLF4- and -9 suppressed neurite growth, and KLF6 and -7 again significantly increased neurite growth, 35% and 60%, respectively (Fig. 2.4E). As with KLF4, effects on neurite growth depended on the DNA-binding domain (Suppl. Fig. 12-13). Interestingly, clustering KLFs by sequence similarity revealed an association between functional domains (Kaczynski et al., 2003) and effects on neurite outgrowth (Fig. 2.4E). For instance, overexpression of the BTEB cluster and the cluster containing KLF4 (orange and pink bars, respectively, Fig. 2.4E) decreased neurite growth. The TIEG and PVALS/T-containing clusters (blue and green bars, Fig. 2.4E) had no effect on neurite length. KLF6 and KLF7, with 85% homologous activation domains, both increased neurite length (yellow bars, Fig. 2.4E). To explore coordinate regulation of neurite growth by KLFs, we co-expressed all two-way combinations of
KLFs -4, -6, -7 and -9 in cortical neurons. The negative effects of KLF4 on neurite growth were dominant over the otherwise positive effects of KLF6 or -7; the negative effects of KLF9 summed with KLF6 or -7 to no net effect (Fig. 2.4F), suggesting a complexity to KLF-KLF interactions in regulating neurite growth. Thus, during development, RGCs downregulate at least two growth-enhancing KLFs (KLF6 and -7), and upregulate at least two growth-suppressive KLFs (KLF4 and -9), which may be dominant in their effect over KLF6 and -7.

Conclusions

These findings that the KLF family of transcription factors regulates axon growth in a number of CNS neurons have important implications. First, although KLF4 has been implicated in a wide variety of cellular events including differentiation (Dai and Segre, 2004; Ghaleb et al., 2005), cancer progression (Black et al., 2001; Safe and Abdelrahim, 2005; Rowland and Peeper, 2006), and stem cell reprogramming (Zhao and Daley, 2008), this function for KLF4 in postmitotic neurons advances our knowledge of the transcriptional regulation of axon regeneration. KLF4 targets relevant for regeneration may include genes selectively expressed in neurons, or important in growth cone function. Second, the clustering of KLF gene function according to domain homology may provide a key for understanding how KLFs cooperate and compete to determine cellular phenotype, whether for axon regeneration or for other systems (Kaczynski et al., 2003). Third, the decrease in RGCs’ intrinsic axon growth ability (Goldberg et al., 2002a) parallels changes in expression within the KLF family; postnatal RGCs express
higher levels of axon growth-suppressing KLFs and lower levels of axon growth-enhancing KLFs; similar changes can be found in published corticospinal motor neuron data (Arlotta et al., 2005). Thus manipulating multiple KLF genes may be a useful strategy to add to existing approaches to increase the intrinsic regenerative capacity of mature CNS neurons damaged by injury or disease.

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Figure 2.1. A screen of developmentally regulated genes identifies KLF4 as an inhibitor of neurite growth. A) Purified embryonic RGCs were cultured in the presence (white bars) or absence (black bars) of amacrine cell membranes for 3 days, and replated away from amacrine cell membranes, after which RGC axon growth was measured. Actinomycin D blocked RGCs’ decrease in axon growth caused by amacrine cell membranes (Mean ± SEM). B-C) E18 hippocampal neurons were co-transfected with 111 candidate genes and EGFP, cultured for 3 days on laminin, and immunostained for Tau to visualize neurites. B) Neurite length of co-transfected (EGFP+) neurons. Bars represent average neurite length normalized to EGFP control (far left). KLF4 (arrow) decreased neurite growth by 50%. C) EGFP+ growth cones of EGFP+/KLF4 transfected neurons (right) were enlarged compared to control-transfected neurons (left). (Scale bar, 10µm)
Figure 2.2. KLF4 is developmentally regulated in RGCs, and its overexpression decreases axon growth in a zinc-finger-dependent fashion. A-B) KLF4 expression in RGCs increases at birth, as measured in acutely purified rat RGCs by microarray (3 probe sets, A; (Wang et al., 2007) or in acutely purified mouse RGCs by qRT-PCR (B; fold change from E18). Two biological replicates are plotted with their average in B. C-E) FLAG-KLF4-WT, FLAG-KLF4-^C lacking the C-terminal zinc finger DNA binding domain, or FLAG or mCHERRY controls were transfected into E20 RGCs. C) RGCs after 2 days were immunostained for FLAG or GFP (green, transfected cells), and Tau or MAP2 (red) as marked (nuclear DAPI is blue). (Scale bar=50µm, 10µm inset) D) Hand-tracing revealed that FLAG-KLF4-WT overexpression decreased axon growth; overexpression of FLAG-KLF4-^C was similar to controls (** p<0.001, * p<0.02, unpaired t-test, post-Bonferroni correction; mean ± SEM). E) E20 RGCs were transfected with either mCherry-pIRES2-eGFP (control) or KLF4-pIRES2-EGFP and plated for 1, 2 or 3 DIV. At 1-3DIV, more control-transfected RGCs extended at least 1 neurite >10 µm than KLF4-transfected RGCs (*p<0.001, paired t-test; mean ± SEM).
Figure 2.3. KLF4 knockout increases RGC neurite growth in vitro and regeneration of adult RGCs in vivo. A-C) Purified P12 RGCs were cultured from Thy1-cre⁺/-/KLF4fl/fl/Rosa⁺ (Cre- WT) and Thy1-cre⁺/-/KLF4fl/fl/Rosa⁺ (Cre+ KO) mice, and plated for 3 DIV prior to Tau immunostaining and automated tracing.  A) Immunostaining for Tau (red) demonstrated low levels of growth of Cre- WT RGCs (left panel) but increased levels of axon growth of Cre+ KLF4-KO RGCs (right panel; Rosa⁺ yellow cells, arrowheads).  B) KLF4-KO RGCs have a higher percentage of cells with neurites, compared to controls (N=3; * p<0.02, t-test; mean ± SEM).  C) When all YFP+ RGCs were measured, KLF4 KO RGCs extended longer neurites than WT RGCs (representative experiment shown; * p<0.001; mean ± SEM).  D-E) Two weeks after optic nerve crush of Thy1-cre⁺/-/KLF4fl/fl (WT), Thy1-cre⁺/-/KLF4fl/fl (Het), and Thy1-cre⁺/-/KLF4fl/fl (KO) mice, regenerating fibers were anterogradely labeled by intravitreal injection of Alexa 594-labeled cholera toxin B. Regenerating fibers were counted at specified distances from the lesion site.  D) More fibers regenerate in KO mice compared to WT or Het (n=10 WT, 4 Het, 7 KO; p<0.001 for KO vs WT or Het; no difference between WT and Het by mixed model analysis of covariance; mean ± SEM).  E) Partial projections of sectioned optic nerve from WT and KO mice show regenerating axons more than 1mm distal to the lesion site in KO nerve. (Scale bar, 200µm).

From this point forward, “N” represents the replicates of that experiment, while “n” represents the number of components (neurons) tested within that “N.”
Figure 2.4. Multiple KLF family members are developmentally regulated in RGCs and differentially affect CNS neurite growth. A-C) RGCs from multiple ages were purified by immunopanning and analyzed by qRT-PCR. Transcript abundance is normalized to E19. KLF6 (A) and KLF7 (B) decrease more than 5-fold postnatally, while KLF9 (C) increases-250 fold. Each marker type is a separate experiment, line is average; N=2-3. D) P4 RGCs were co-transfected with KLFs and EGFP reporter and plated for 2 days on laminin. Bars represent average total neurite length of transfected (EGFP+) neurons. (n>700; * p<0.05, ** p<0.01; ANOVA with post hoc Dunnett’s test; mean ± SEM; pooled data from two replicate experiments.) E) P5 cortical neurons were co-transfected with individual KLFs and mCherry, plated for 3 days on laminin, and immunostained for beta-III tubulin. (Top) KLF family members are grouped according to defined structural domains (Kaczynski et al., 2003), and clustered by amino acid similarity (Clustal analysis, Vector NTI). (Middle) Bars represent average total neurite length of transfected (mCherry+) neurons, and are colored by the presence of known motifs (above). Nine KLFs significantly decreased neurite length, and two increased neurite length (N>3, n>100; * p<0.05, ** p<0.01, ANOVA with post hoc Dunnett’s test; mean ± SEM). (Bottom) Purified RGCs from different ages were analyzed by RT-PCR with KLF-specific primers, ordered according to the overlying bar graph. Transcripts for all KLFs except -1 and -17 were detected in developing RGCs. (F) P5 cortical neurons were co-transfected with combinations of KLFs with IRES-mCherry (red) or IRES-EGFP (green) reporters and cultured as above (DNA loading controls, Suppl. Fig. 13). Bars represent average neurite length of dually transfected neurons (mCherry+, EGFP+). Co-expression of KLF4 or -9 blocked the growth-promoting effects of KLF6 or -7. (N=3, n>25; * p<0.05, ** p<0.01, ANOVA with post hoc Dunnett’s test; mean ± SEM).
Figure S1. KLF4 overexpression in hippocampal neurons decreases neurite growth and neurite initiation. A-D) E18 hippocampal neurons were co-transfected with KLF4 or control plus EGFP, cultured on laminin-coated plates, and immunostained for Tau (neurites) and MAP2 (dendrites). A) There was no difference in survival by nuclear morphology and DAPI intensity between control- and KLF4-transfected neurons (Mean ± SD). B) Transfected EGFP+ cells (arrows) were imaged to detect DAPI, EGFP, and either Tau (top) or MAP2 (bottom). KLF4-transfected neurons had shorter axons and dendrites. (Scale bar, 50µm) C) KLF4 overexpression decreased the percentage of transfected neurons that were able to extend at least 1 neurite >10 µm (N=5; * p<0.01, paired t-test; mean ± SEM). D) KLF4 overexpression decreased both axon (Tau+/MAP2-) and dendrite (MAP2+) length (* p<0.01, t-test; mean ± SEM).
Figure S2. KLF4-mediated suppression of neurite growth requires the C-terminal zinc finger domain. E18 hippocampal neurons were transfected with either FLAG-KLF4-WT, FLAG-KLF4-^C lacking the C-terminal zinc finger DNA binding domain (A), or mCherry-pIRES2-eGFP as control. B) After 3DIV, neurons were stained for Tau (neurites) and MAP2 (dendrites) prior to imaging and analysis (Cellomics KSR). Transfected neurons are indicated by arrows. C) Neurite growth was normalized to control transfected neurons (not graphed, equal to 100%). WT KLF4 overexpression significantly decreased neurite growth in both Tau stained and MAP2 stained neurites, while deletion of KLF4’s C-terminus led to growth indistinguishable from that of controls (* p<0.01, one representative experiment of 2 shown; mean ± SEM).
Figure S3. KLF4 overexpression decreases numbers of both neurites and branches in embryonic hippocampal neurons. E18 hippocampal neurons were electroporated with EGFP and either KLF4 or a pcDNA3 vector control and cultured for 3 days on PDL- and laminin-coated plates in growth media. Following immunostaining, transfected neurons were imaged and hand-traced. There was a decrease in the number of neurites originating from the cell body (A), the number of branches from all neurites (B), and the number of branches normalized to the total neurite length for each transfected neuron (C) after KLF4 overexpression (*p<0.001 for each graph, unpaired t-test; n>50 per condition; mean ± SEM).
Figure S4. KLF4 overexpression in embryonic RGCs decreases the numbers of both neurites and branches. E20 RGCs were purified and transfected using Lipofectamine 2000 with a FLAG control plasmid, FLAG-KLF4, or FLAG-KLF4-^C deletion mutant lacking the C-terminal zinc finger DNA-binding domain. Neurons were plated for 3 days on PDL- and laminin-coated plates in growth media. Following immunostaining, transfected neurons were imaged and hand-traced. KLF4 overexpression decreased the average number of neurites (A), branches (B), and branches normalized to total neurite length of each neuron (C), whereas RGCs overexpressing the truncated Flag-KLF4-^C behaved similarly to controls (p<0.01, unpaired t-test post-Bonferroni; n>25 for each; mean ± SEM).
Figure S5. RGCs overexpressing KLF4 continue to extend neurites, but at a slower rate. E20 RGCs were purified and transfected with either KLF4-pIRES2-eGFP or mCherry-pIRES2-eGFP and cultured for 1, 2, or 3 days (DIV) prior to immunostaining for Tau and MAP2. Hand tracing revealed that while KLF4 transfected cells have decreased growth ability, they are still able to grow over a period of days whether looking at axon length (Tau+, MAP2-, A) or dendrite length (Tau+,MAP2+, B) (*p<0.003, unpaired t-tests comparing 1 to 3 DIV for each condition; N=1, n>70; mean ± SEM).
Figure S6. Half of RGCs activate Cre in the Thy1-cre+/-/Rosa+/+ mice. Alexa Fluor 594-labeled cholera toxin B was injected into the superior colliculus of P7 Thy1-cre+/-/Rosa+/+ mice to retrogradely label RGCs (red). Eyes were fixed, sectioned and immunostained to amplify the EYFP signal (green). A) Retinal cross sections reveal that YFP was expressed in RGCs, as well as in other retinal cells. B) RGCs from P10 Thy1-cre+/+/Rosa+/+ and Thy1-cre-/+/Rosa+/+ mice were purified by immunopanning, cultured on PDL- and laminin for 3 days, and immunostained for Tau (neurites) and GFP (to amplify YFP). Images were taken both with a Zeiss microscope and by the Cellomics Kineticscan software to determine intensity of YFP fluorescence. C) Two times the standard deviation of background intensity in Thy1-cre-/+/Rosa+/+ RGCs yielded a baseline threshold for “YFP +”. 46% of RGCs were YFP +, suggesting that this Thy1-cre line is targeting approximately half of immunopanned RGCs.
Figure S7. Transgenic Cre expression does not affect RGC neurite growth. RGCs from P10 Thy1-cre\textsuperscript{+/−}/Rosa\textsuperscript{+/−} and Thy1-cre\textsuperscript{−/−}/Rosa\textsuperscript{+/−} mice were purified by immunopanning, cultured on PDL and laminin for 3 days, and immunostained for Tau (neurites) and GFP (to amplify YFP). Cellomics Kineticscan software imaged and traced neurites, and measured YFP intensity. The baseline threshold of YFP intensity indicating cre targeting was determined as in Supplemental Figure 6, above. RGCs were grouped either as all RGCs from Thy1-cre\textsuperscript{−} animals (no Cre expression, black bars), YFP\textsuperscript{−} cells from Thy1-cre\textsuperscript{+} animals (also no Cre, hatched bars), or YFP\textsuperscript{+} cells from Thy1-cre\textsuperscript{+} animals (Cre-expressing RGCs, white bars). Neurons with growth <10 µm were not included in the length analysis. Quantification of total neurite length (A) or of percent of RGCs with at least one neurite >10 µm (B) revealed no differences between genotype (A: ANOVA revealed no significant differences between genotype; 1 representative experiment shown, n>2000 for each condition; mean ± SEM).
Figure S8. KLF4 knockout does not affect survival of RGCs in vitro. Purified P12 RGCs were cultured from Thy1-cre\(^{-/-}\)/KLF4\(^{+/+}\)/Rosa\(^{+}\) (Cre- WT) and Thy1-cre\(^{+/-}\)/KLF4\(^{+/+}\)/Rosa\(^{+}\) (Cre+ KO) mice. MTT survival assays at 1-3 DIV showed no significant differences in survival between KLF4 KO and WT RGCs (N=3; mean ± SEM).
Figure S9. KLF4 knockout during development does not affect adult RGC number or survival after injury. Two weeks after optic nerve crush of Thy1-cre$^+/\text{KLF4}^{+/+}$ (WT), Thy1-cre$^+/\text{KLF4}^{+/+}$ (Het), and Thy1-cre$^+/\text{KLF4}^{+/+}$ (KO) mice, retinas from both the control eye (uninjured nerve) and injured eye (crushed nerve) were flatmounted and immunostained for βIII tubulin (Tuj1) to label RGCs. Confocal imaging of retinas from knockout animals, normalized to WT, showed no differences in basal RGC number in the contralateral uninjured retinas (A; mean ± SEM; n=8 WT, 4 Het, 9 KO) or in RGC survival two weeks after optic nerve crush (B; mean ± SEM; n= 6 WT, 4 Het, 9 KO).
Figure S10. Multiple KLFs are expressed in RGCs and are developmentally regulated. RNA was isolated from acutely purified RGCs from multiple ages and analyzed by microarray analysis on Affymetrix chips (Wang et al., 2007). 9 of 17 KLFs were probed on these arrays using between 1-3 probes; probes not present in at least 2 samples within one age by the Affymetrix algorithm are marked as “absent” with an asterisk at the end of the line. Occasionally one probe would not detect message while the other probe would, as often happens in microarray datasets. All of these KLFs except for KLF1 were detected in RGCs by RT-PCR (Fig. 2.4).
Figure S11. Overexpression of KLF transcription factors does not affect cell survival. P5 cortical neurons were dissociated, transfected with EGFP or KLFs, and cultured on PDL- and laminin-coated plates in growth media. After 72 hours, the percent of cells that excluded SYTOX orange dye was quantified (Cellomics Kineticscan). Transfection with KLFs did not significantly change neuronal survival (p>0.50, ANOVA with Dunnett’s post-test; N=3, n>500; mean ± SEM).
KLF-mediated regulation of neurite length in cortical neurons requires the C-terminal zinc finger DNA-binding domain. mCherry control, full length KLF-IRES-mCherry, or zinc finger deletion KLF-^C –IRES-mCherry constructs encoding KLF4, -9, -6, or -7 were transfected into P5 cortical neurons. Neurons were plated for 3 days on laminin and immunostained for beta-III tubulin. Bars represent average total neurite length (Cellomics KSR) of transfected (mCherry+) neurons. Compared to mCherry control-transfected neurons, full length but not truncated KLFs significantly affected neurite lengths. (N=3, n>100; * p<0.05, ** p<0.01, ANOVA with post hoc Dunnett’s test; mean ± SEM).
Figure S13. Effect of KLFs in combinatorial experiments is independent of µg of plasmid transfected. 4µg of control mCherry, 4µg of full length KLF–IRES-mCherry, or 2µg of full length KLF–IRES-mCherry plus 2µg of truncated (non-functional, see Fig. S12) KLF-^C –IRES-mCherry were transfected into P5 cortical neurons. Neurons were plated for 3 days on laminin and immunostained for beta-III tubulin. Bars represent average total neurite length (Cellomics KSR) of transfected (mCherry+) neurons. Neurons transfected with 4µg and 2µg of functional KLFs had similar neurite lengths (N=3, n>100; p>0.05, ANOVA with post hoc Dunnett’s test; mean ± SEM).
CHAPTER THREE

THE EFFECT OF KLF4 OVEREXPRESSION ON NEURONAL POLARITY
CHAPTER THREE

Introductory Remarks

For neurons to functionally integrate into the circuitry of the brain, they typically polarize their neurites asymmetrically through segregation of proteins and organelles to specific cellular compartments, namely axons and dendrites. Dendrites, typically shorter and thicker processes, receive much of the synaptic input to the neuron, whereas axons are long, thin processes that transmit the output of the neuron. These functionally different domains of the neuron are specified early in development and contribute to the neuron’s ability to integrate and interact in the nervous system (reviewed in Arimura and Kaibuchi, 2007). We have previously shown that KLF4 overexpression leads to a reduction in neurite growth as well as neurite initiation in multiple neuronal types. Its developmental deletion in RGCs leads to an increase in neurite length and neurite initiation in vitro, and an increase in regeneration in vivo following injury (Moore et al., 2009). Here we investigated the role of KLF4 in neurite polarization first in embryonic hippocampal neurons, where the acquisition of neuronal polarity has been well-characterized (Dotti et al., 1988), and then also in RGCs.

Results

We first asked if KLF4 overexpression affects the acquisition of polarity in embryonic hippocampal neurons. Using the stages of polarity previously characterized in hippocampal neurons (Figure 3.1; Dotti et al., 1988), a blinded observer classified all transfected neurons after 3 days in culture. Whereas the largest percentage of transfected
control neurons were at stage 4 of maturation, the KLF4-overexpressing neurons were more likely to be at earlier stages of polarization, and were equally distributed among all four stages of neuronal polarity, even after 3 days in culture (Fig. 3.2A). This suggests that KLF4-overexpressing neurons have a defect or delay in their acquisition of polarity.

We next asked if the decrease we see in neuronal growth in embryonic hippocampal neurons following KLF4 overexpression (Moore et al., 2009) could be due to this decrease in the acquisition of polarity. Using the immunostaining markers Tau (all neurites) and MAP2 (dendrites), we hand-traced the length of axons and dendrites of only those transfected neurons that were in stage 3 or greater. When these polarized neurons were compared, we found that KLF4 overexpression still led to a decrease in length in both axons and dendrites (Fig. 3.2B). This suggests that the decrease in neurite growth seen with KLF4 overexpression is not solely due to a disproportionate percentage of neurons that are still in the early stages of maturation. Such a conclusion is limited, however, as the shorter length could also reflect a later acquisition of stage 3 or 4 polarity, and thus the appearance of shorter axons. We did not perform a time lapse experiment to more closely characterize the rate of elongation once hippocampal neurons enter stage 3 or 4 of neurite polarization.

While the stages of polarity are well-characterized in hippocampal neurons, they have not yet been well-characterized in RGCs. In cultured RGCs in vitro on laminin, there are often multiple axons (Tau+/MAP2-; unpublished observations, DLM). Therefore, we sought to measure the number of axons (Tau+/MAP2-) of transfected neurons in vitro. First, we performed this analysis on transfected hippocampal neurons to
observe the effect of KLF4 overexpression on the number of axons from these cells. While the majority of transfected control hippocampal neurons had 1 axon, as expected, the highest percentage of KLF4 overexpressing neurons had no axons, as identified by Tau+/MAP2- staining (Fig. 3.4A). While many of these neurons were growing neurites, in many cases a light MAP2 immunoreactivity was seen in all of the neurites, resulting in the lack of “axons” for that neuron, and all neurites coming from the cell were characterized as dendrites (Fig. 2S1). In embryonic RGCs, we found similar results (Fig. 3.3). Control RGCs were more likely to have multiple axons than hippocampal neurons (Fig. 3.4), although the majority of transfected control RGCs again had primarily one axon (Fig. 3.4B). In addition, the majority of KLF4-overexpressing RGCs again had no axons as defined by the absence of MAP2 staining (Fig. 3.4B). In the future, it will be interesting to also perform immunostaining against a dephospho Ser-202 form of Tau, using the monoclonal antibody Tau-1, which labels the distal axon (Rebhan et al., 1995). This will allow us to determine if this axon-specific marker is similarly segregated in both control and KLF4-overexpressing neurons. These data suggest that in RGCs and hippocampal neurons, there is a reduced polarization of axons after KLF4 overexpression.

We also determined the effect of KLF4 on dendrite number, again defined as Tau+/MAP2+ neurites. Interestingly, the results were fairly similar between both hippocampal neurons (Fig. 3.5A) and RGCs (Fig. 3.5B). KLF4 overexpression led to increased numbers of neurons with 0-1 dendrites, while the majority of transfected
control neurons had 4 or greater dendrites. This further supports a role for a lack of polarity acquisition and maturation in KLF4-overexpressing neurons.

These data, together with the studies demonstrating that KLF4 overexpression leads to decreased neurite initiation (Fig. 2.2E; Fig. S1), suggest that KLF4 overexpression results in a delay in neuronal maturation based on the lack of neurite initiation, the lack of polarity acquisition, and the loss of segregation of dendrite-specific proteins to restricted neuronal compartments (Fig. 3.3).

**Discussion and Future Directions**

Here we show that KLF4 overexpression in embryonic hippocampal neurons and RGCs leads to a decrease in the acquisition of polarity. These data are limited in a number of ways. First, the study using hippocampal neurons has not yet been repeated, though interestingly, it is highly similar to the studies with RGCs which have a number of replicates. Second, while we have reported differences in “axons” on the basis of the lack of segregation of MAP2, we are not using a specific axonal marker such as Tau-1, which would more specifically delineate the effects of KLF4 overexpression on axons. Third, these studies should be further augmented with timelapse studies to determine if KLF4 overexpression leads to a delay in neurite initiation and elongation, or arrested neuronal polarity. In addition, these timelapse studies could lead to a better understanding of mechanisms of neurite growth in these transfected neurons. Therefore, additional experiments need to be done to support these initial findings, and to better define the effect of KLF4 overexpression on polarity.
These preliminary data do suggest, however, that KLF4 can affect neuronal polarization, though the mechanism by which this occurs is still unclear. Technically, it may be important to consider the timing of the expression from the transfected plasmid after transfection, as there may be variability in the timing and amount of expression. Depending on the stage of growth and polarity the neurons are in at the time that KLF4 protein is being produced, there may be different effects on neurite initiation, neurite polarization or neurite elongation. Additionally, these data do not address whether KLF4-overexpressing neurons would eventually reach the later stages of polarity given enough time in culture. Both of these questions could be addressed using time lapse microscopy to image the same neurons over an extended period of time.

While there is very limited information on KLF4 in the nervous system, research in other systems may suggest potential mechanisms. KLF4-overexpressing hippocampal neurons and RGCs show a reduction in neurite initiation, neuronal polarity, and a decrease in neurite growth. One possible explanation may be KLF4’s ability to globally decrease macromolecular synthesis, as has been reported from microarray analyses in cancer cell lines (Whitney et al., 2006). The need for new protein synthesis in the development and specification of axons and dendrites may be inhibited following KLF4 overexpression.

A second hypothesis could involve KLF4-mediated suppression of centrosome amplification, as has been observed in colon cancer cell lines (Yoon et al., 2005). Centrosomes are not only involved in cell division: neuronal polarity has been associated with the location, numbers, and presence of the centrosome in the neuron (Zmuda and
Rivas, 1998; de Anda et al., 2005; Zolessi et al., 2006). In the post-mitotic neuron, the centrosome acts as a microtubule organizing center (MTOC), shown to be important in neurogenesis, neuronal migration, neuronal differentiation, and establishment of polarity (reviewed in (Higginbotham and Gleeson, 2007). In cerebellar granule neurons (CGNs) in vitro, the centrosome is colocalized with the Golgi at the base of the first process that becomes the first axon of the typically bipolar neuron. It relocates after to the base of what will become the second axon, and after maturation, no longer localizes with either of the axons (Zmuda and Rivas, 1998). In cultured hippocampal neurons, there have been mixed reports on the importance of the localization of the centrosome for polarity. At the time of the development of a minor neurite into an axon, there is no co-localization of the centrosome, however, this was not studied prior to the initial extension of these processes (Dotti and Banker, 1991). More recent studies in hippocampal neurons demonstrated that the Golgi-centrosome complex congregates in the area of the cell where the first neurite forms, which consistently becomes the axon. Multiple centrosomes lead to multiple axons, while destruction of the centrosome leads to the loss of an axon (de Anda et al., 2005). Thus, in vitro, the centrosome appears to play an important role in establishing neuronal polarity.

In vivo studies in zebrafish RGCs, axons arise out of the basal portion of the cell prior to the retraction of its apical process. The centrosome remains in the apical process or elsewhere in the cell, however there was not a basal localization (Zolessi et al., 2006). RGCs have not been studied for their distinct polarity stages in vitro, where there
is no apical process remaining from migration. Therefore, it is difficult to determine if this centrosomal effect is just neuronal type specific or an in vitro phenomenon.

Thus, the importance of the centrosome in neuronal polarity in some cells, and the finding that KLF4 can affect the centrosome (Yoon et al., 2005) suggests a potentially interesting connection between KLF4 and the centrosome in non-neuronal cells. Therefore, it may be interesting to determine the location and state of the centrosome in KLF4-overexpressing neurons.

A third hypothesis for the effect of KLF4 overexpression on polarity in neurons is that KLF4 affects the stabilization of microtubules. Neurite initiation and growth, both of which are decreased in KLF4 overexpressing neurons, can be affected by changes in the stabilization of microtubules, as has been seen with Taxol treatment (Letourneau and Ressler, 1984). Additionally, recent papers have suggested that the stability of the microtubules may be very important to the process of neuronal polarization and axon regeneration (Gomis-Ruth et al., 2008; Witte et al., 2008; Kollins et al., 2009). Thus, it may be important to test the state of the microtubules in KLF4 transfected neurons through immunostaining against tyrosinated tubulin (dynamic microtubules) and acetylated α-tubulin (stabilized microtubules).

Interestingly, manipulation of another transcription factor in RGCs has also been shown to affect neuronal polarity. Knockout (KO) of Brn3b in RGCs leads to increased numbers of MAP2+ processes with dendritic features. Neurites growing from retinal explants from these mutants are disorganized, shorter and less bundled. While wildtype RGCs in vitro had a more traditional bipolar structure, KO RGCs had multiple short,
thick MAP2+ neurites with expanded growth cones (Gan et al., 1999; Wang et al., 2000). In Brn3b KO dorsal root ganglion (DRG), however, the opposite effect was seen, such that, neurites lacked MAP2 staining and were Tau-1 positive. This suggests that transcription factors can play a role in the acquisition of neuronal polarity, and that the effect of these transcription factors can be different in distinct neuron types (Wang et al., 2000). Interestingly, a similar phenotype was seen with KLF4-overexpressing cells, with thick, short processes and a dysregulation of polarized markers (Fig. 3.3). It may be important to determine if there is an interrelation between these two transcription factors.

Because of the small amount of information available about KLF4 in neurons, determining the mechanism that could mediate its polarity phenotype will have to be performed through a candidate approach. KLF4’s ability to decrease general protein synthesis (Whitney et al., 2006) and to affect the centrosome in non-neuronal cells (Yoon et al., 2005) suggests these functions as potential mechanisms behind this decrease in neurite polarization. Additionally, experiments revealing phenotypes similar to those seen with KLF4 overexpression also suggest potential targets and interactors. Therefore, these preliminary data not only demonstrate a role for KLF4 on neuronal polarity, but also lay the foundation for additional experiments that may further define the role of KLF4 in neurons.

Acknowledgements

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**Fig. 3.1** E18 hippocampal neurons acquire polarity through defined stages. Images were taken of hippocampal neurons transfected with either pMAX/pcDNA3 or pMAX/KLF4 and quantified according to their stage of polarity. Example images of control transfected neurons in each of the previously defined stages (Dotti et al., 1988) are seen above. Stage 1 - formation of lamellipodia; Stage 2 - multiple fairly symmetrical processes extend from the neuron; Stage 3 - one neurite begins to take on axonal characteristics, elongating more than the other processes; Stage 4 - dendrites begin to further mature and elongate; Stage 5 (not pictured here) - the full maturation of the hippocampal neuron, when functional synaptic contacts are made (Dotti et al., 1988).
Fig. 3.2 KLF4-overexpressing hippocampal neurons demonstrate decreased neurite polarization; polarized neurons have decreased neurite length compared to controls. A-B) E18 hippocampal neurons were transfected with either pMAX/pcDNA3 or pMAX/KLF4 and plated on PDL- and laminin-coated plates for 3 days in vitro. A) EGFP+ cells were analyzed by a blinded experimenter for their stage of polarity (defined in Fig. 3.1). KLF4-overexpressing neurons have less well differentiated neurites. N=1; n>115 neurons for each transfected condition. B) Stage 3 and 4 EGFP+ hippocampal neurons immunostained for Tau (neurites) and MAP2 (dendrites) were hand traced and analyzed for their neurite growth. KLF4 overexpression in polarized hippocampal neurons led to decreased axon (Tau+/MAP2-) and dendrite (Tau+/MAP2+) lengths. N=1, n=26 (KLF4), n=45(ctrl); *p<0.05, ** p<0.001; Mean ± SEM.
Fig. 3.3 KLF4 overexpression in E20 RGCs leads to a decreased segregation of dendrite-specific markers within neurites. E20 RGCs were purified and transfected (Lipofectamine 2000) with FLAG-KLF4 or a control vector, cultured on laminin for a period of 2 days, and subsequently immunostained for DAPI (A; nuclei), Tau (B; all neurites), and MAP2 (C; dendrites). KLF4-overexpressing neurons (right panels) displayed a light MAP2 immunoreactivity throughout all of their neurites in contrast to the specific MAP2 compartmentalization seen in controls (left panels). Scale bar, 20 µm.
Fig. 3.4  KLF4 overexpression results in an increased percentage of neurons without defined axons in both embryonic hippocampal neurons and RGCs.  A) E18 hippocampal neurons were electroporated (Amaza) with pMAX/pcDNA3 or pMAX/KLF4 and plated on PDL- and laminin-coated dishes for 3 DIV prior to immunostaining for Tau (neurites) and MAP2 (dendrites). The number of axons (Tau+/MAP2-) was counted for each cell that had at least 1 neurite greater than 10µm. N=1, n>50 for each transfected condition.   B) E20 RGCs were purified and transfected (Lipofectamine 2000) with a control plasmid (mCherry-pIRES2-eGFP or a Flag control) or KLF4 (KLF4-pIRES2-eGFP or Flag-KLF4) and plated for 3 DIV on PDL-and laminin-coated dishes prior to immunostaining and analysis as described above.  N=3-4; *p<0.05, **p<0.01, t-tests of equal variance; Mean ± SEM.
Fig. 3.5  KLF4 overexpression results in a larger percentage of neurons without defined dendrites in both embryonic hippocampal neurons and RGCs.  A) E18 hippocampal neurons were electroporated (Amaxa) with pMAX/pcDNA3 or pMAX/KLF4 and plated on PDL- and laminin-coated dishes for 3 DIV prior to immunostaining for Tau (neurites) and MAP2 (dendrites). The number of dendrites (Tau+/MAP2+) were counted for each cell that had at least 1 neurite greater than 10µm. N=1, n>50 for each transfected condition.  B) E20 RGCs were purified and transfected (Lipofectamine 2000) with a control plasmid (mCherry-pIRES2-eGFP or a Flag control) or KLF4 (KLF4-pIRES2-eGFP or Flag-KLF4) and plated for 3 DIV on PDL-and laminin-coated dishes prior to immunostaining and analysis as described above. N=3-4; *p<0.03, **p<0.01, t-tests of equal variance; Mean ± SEM.
CHAPTER FOUR

GENE TARGETS OF KLF4
CHAPTER FOUR

Introductory Remarks

The transcription factor KLF4 has been shown to negatively regulate both neurite growth in vitro and regeneration in vivo (Moore et al., 2009), yet how it does so is unclear. KLF4 can act as both an activator and repressor of gene transcription (reviewed in Kaczynski et al., 2003) and has been shown to regulate the expression of many genes in non-neuronal cells. No downstream targets of KLF4 signaling have been identified in neurons, however. Therefore, to begin to address this question, we screened a sampling of previously published downstream targets of KLF4 - cyclin-dependent kinase inhibitor 1A (p21; (Zhang et al., 2000), urokinase plasminogen activator receptor (u-PAR; Wang et al., 2004), ornithine decarboxylase (ODC; Chen et al., 2002), and cytochrome p450, family 1, subfamily A, polypeptide 1 (CYP1A1; Zhang et al., 1998) - to determine if modulation of their expression could phenocopy KLF4’s effects on neurite growth. Additionally, we also measured changes in expression levels of known KLF4 targets p21 and cyclin D1, as well as the growth-suppressing family member, KLF9 following KLF4 overexpression. By determining how specific target genes are regulated by KLF4 overexpression in neurons, or by observing effects of overexpression or knockdown of KLF4 targets on neurite growth in hippocampal neurons, we should further our understanding of the mechanisms behind KLF4’s suppression of neurite growth.
Results

We first asked if a series of known targets of KLF4 identified in other cell types could be mediating KLF4’s effect on neurite outgrowth. We electroporated E18 hippocampal neurons with pMAX (EGFP, Amaxa) as a marker of transfection and singly, each of the following plasmids: u-PAR, p21, ODC, 5 shRNAs against ODC, CYP1A1, and as a positive control, KLF4. After 3 days in culture, we immunostained for Tau to visualize neurites, and used Cellomics Kineticscan to image and measure the effect of overexpression or potential knockdown on the total neurite length and the length of the longest neurite. While KLF4 overexpression again led to a decrease of approximately 50% in both measurements, no other single treatment was able to phenocopy this effect (Fig. 4.1), though we did not verify if overexpression or knockdown actually occurred in the transfected cells. These results suggest that there may be neuronal-specific targets of KLF4, or that published downstream targets in non-neuronal cells may not be functioning similarly in neurons.

We next sought to determine whether a series of selected targets were regulated in RGCs by overexpression of KLF4 using qRT-PCR. We found that expression of p21, cyclin D1, and another KLF growth suppressor, KLF9, was increased by KLF4 overexpression (Fig. 4.2). While the levels of expression changes were highly variable between collection times, in all cases, expression levels were still increased compared to control transfected neurons. This inconsistency may have been due to the variability of transfection efficiency, which ranged from 30-50%. These data demonstrate that at least
some known downstream targets of KLF4 in non-neuronal cells can be similarly regulated by KLF4 in neurons, such as p21, and differentially regulated modulated in neurons, such as cyclin D1.

**Discussion and Future Directions**

Because overexpression of flag-tagged constructs of KLF4 demonstrated flag localization only in the nucleus (Fig. S2B), and deletion of the DNA-binding domain eliminates KLF4’s effect on neurite outgrowth (Fig. 2.2C-D; Fig. S2), we anticipated that KLF4 is affecting neurite growth through transcriptional activity and gene regulation. Due to the limited study of KLF4 in neurons, it is unclear what downstream targets of KLF4 could be mediating this effect. Thus, we screened a short list of these genes to look for similar neurite length phenotypes to those seen with KLF4 overexpression. While this list was by no means extensive, the majority of these genes have shown some function in neurite growth and regeneration, suggesting they were good candidates for KLF4’s function.

In non-neuronal cells, KLF4 positively regulates p21 and u-PAR, and negatively regulates ODC and CYP1A1. By overexpressing these positively regulated genes, and knocking down these negatively regulated genes, we hoped to see a decreased neurite length phenotype similar to that seen with KLF4 overexpression. Following analysis, we found that none of the genes tested in the screen were able to phenocopy the effect of KLF4. There could be many reasons for this, including the possibility that these genes may be regulated in a cell-type specific manner. An interesting case is p21, whose
expression is activated by KLF4 in non-neuronal cells (Zhang et al., 2000; Rowland and Peeper, 2006), while in cortical neurons KLF4 overexpression does not affect p21 mRNA levels (Zhu et al., 2009). In our experiments, KLF4 overexpression in RGCs led to an increase in p21 expression at multiple timepoints, more similar to what has been shown in non-neuronal cells. When we increased p21 expression in hippocampal neurons through overexpression, however, we saw no effect on neurite growth, despite previous studies demonstrating that p21 promotes neurite growth in multiple neuronal types (Borasio et al., 1989; Altin et al., 1991; Tanaka et al., 2002; Tomita et al., 2006).

Why is there so much variability in our studies with p21 compared to published findings? In our experiments detecting expression changes in p21 following KLF4 overexpression, we collected the samples at 1 or 2 DIV after electroporation, while in the previously published study in cortical neurons where they saw no effect on p21 expression with KLF4 overexpression, they collected samples at 11 DIV after lentiviral transfection (Zhu et al., 2009). Modulation of KLF4 in other tissues has also resulted in variable levels of p21. For example, knocking out KLF4 specifically in cornea results in little if any difference in p21 expression (Swamynathan et al., 2008). All of this variability in the effect of KLF4 on p21 expression, suggests that other genes or KLF family members may be consistently regulating this target. For example, the growth-enhancing KLF family members, KLF6 and -7 (Moore et al., 2009), are also able to positively regulate p21 (Laub et al., 2005; Narla et al., 2007), suggesting it may not be the downstream target that is responsible for the growth-suppressing effects in KLF4 overexpressing cultures. Importantly, while we have shown that p21 expression levels
are increased in RGCs after KLF4 overexpression, our overexpression experiments demonstrate that an increase in p21 expression does not lead to a decreased neurite growth phenotype, suggesting that this p21 expression alone is not mediating the effect of KLF4, though we cannot rule out a function for this gene in combination with other targets.

Another gene that has been shown to be differentially regulated by KLF4 in neurons and non-neuronal cells is cyclin D1. In colon cancer cells, KLF4 overexpression leads to repression of cyclin D1 (Shie et al., 2000), while in cortical neurons, KLF4 overexpression leads to increased expression of cyclin D1 (Zhu et al., 2009). Therefore, our results demonstrating upregulation of cyclin D1 with KLF4 overexpression in RGCs correspond with what has been reported previously in neurons. Interestingly, KLF7 has been shown to repress cyclin D1 (Laub et al., 2001b) and KLF6 can physically interact with cyclin D1, inhibiting its activity (Benzeno et al., 2004). Besides KLF4, other KLFs can activate expression of cyclin D1, such as KLF5 (Nandan et al., 2004), KLF6 (Sirach et al., 2007), KLF8 (Zhao et al., 2003; Wang and Zhao, 2007; Mehta et al., 2009), and KLF9 (Zhang et al., 2001b). These studies suggest that there may be a pattern where neurite growth suppressors increase cyclin D1 expression, while growth enhancers inhibit cyclin D1 expression, though the variability in the cell types tested in these experiments makes it difficult to interpret. Therefore, while cyclin D1 is a common target for many of the KLFs, its positive or negative regulation appears to be KLF specific as well as possibly cell type specific.
Because KLF4 normally suppresses ODC in colon cancer cells (Chen et al., 2002), we hypothesized that ODC knockdown may decrease neurite growth similar to KLF4 overexpression. ODC is a key enzyme in the biosynthesis of polyamines by catalyzing the decarboxylation of ornithine to putrescine. Recently, polyamines (specifically spermidine, spermine, and putrescine) have been found to be very important in increasing axon growth over inhibitory substrates as the downstream mediators of increased cAMP and CREB activation (Cai et al., 2002; Gao et al., 2004; Deng et al., 2009), and in increasing regeneration of injured hippocampal neurons in vitro and after optic nerve injury in vitro and in vivo (Chu et al., 1995; Deng et al., 2009). Additionally, ODC activity was shown to be increased after facial nerve axotomy (Tetzlaff and Kreutzberg, 1985). When we overexpressed ODC or shRNAs against ODC in hippocampal neurons, we did not see any effect on neurite growth, though it should be noted that we did not validate the 5 shRNAs for their ability to knockdown ODC in neurons. Additionally, more time in culture could be allowed for the actual knockdown prior to looking for an effect. Therefore, future steps include testing ODC shRNAs for their efficacy in knockdown, and optimizing culturing conditions for extremely low density to allow for accurate tracing of transfected neurons after 5-6 days in culture.

Another positively regulated target of KLF4 is urokinase plasminogen activator receptor (u-PAR), which is the receptor for urokinase-type plasminogen activator (u-PA; Wang et al., 2004). u-PA is a protease that cleaves plasminogen into an active form of plasmin, resulting in cleavage of extracellular matrix molecules. u-PAR tethers u-PA to the membrane, limiting its function to a localized area. u-PAR mRNAs are increased
after injury in the PNS (Siconolfi and Seeds, 2001b) and loss of any portion of the plasminogen activator system results in reduced regeneration after sciatic nerve injury in mice (Siconolfi and Seeds, 2001a). Additionally, u-PAR can also bind to integrins and participate in the signaling of beta 1 integrins (Wei et al., 1999). When we overexpressed u-PAR in embryonic hippocampal neurons, we did not see any effect on neurite growth. Thus, while u-PAR may be important for growth and regeneration, KLF4’s modulation of this gene may not be responsible for its effect on neurite growth.

CYP1A1 is a drug metabolizing enzyme whose expression is negatively regulated by KLF4 (Zhang et al., 1998). CYP1A1 is expressed in neurons and glia of the brain (Chinta et al., 2005b; Chinta et al., 2005a; Kapoor et al., 2006), though no specific function has yet been identified there. In our study, overexpression of CYP1A1 in hippocampal neurons did not affect neurite growth, suggesting this KLF4 target may not be responsible for KLF4’s effect on growth. Interestingly, the growth-suppressors KLF9, -13, and -16 have also been shown to repress expression of this gene through the same BTE binding site, similarly to KLF4 (Imataka et al., 1992; Kaczynski et al., 2002).

Another potential reason that we did not see a neurite length effect after modulating the expression of any of these genes may be that affecting one gene target is not enough for the full effect, but that multiple genes must be targeted at the same time. Therefore, another future experiment is to test combinations of these genes or knockdown constructs for changes in neurite growth phenotypes.

Additionally, we tested only a few of the published targets of KLF4, as we were limited to what was available at the time. More recent experiments have also shown
interesting potential downstream candidates of KLF4’s effect on neurite growth. For example, when KLF4 is knocked out in cornea, transcription factors such as Sox4, NFκB2 (p52) and ATF3 were found to have increased expression in knockout tissues (Swamynathan et al., 2008). This is very interesting, as Sox4 is a closely related family member of Sox11, important in axon regeneration (Jankowski et al., 2009), p52 is one possible subunit of the NFκB dimer which has also been shown to be important in regeneration and can interact with other regeneration-promising transcription factors p53 and STAT3 (Schumm et al., 2006; Perkins, 2007), and ATF3 is a transcription factor highly expressed during axon regeneration (Tsujino et al., 2000; Mason et al., 2003; Campbell et al., 2005; Seijffers et al., 2007; Jankowski et al., 2009). These findings in cornea would suggest that knockout of the growth suppressor KLF4 increases levels of regeneration-promoting transcription factors. Additionally, it has been shown that when KLF4 is knocked out in cornea, there is increased expression of matrix metalloproteases (MMPs) 2, 3, 9, and 13 (Young et al., 2009). MMPs have been shown in multiple studies to be upregulated in regenerating axons allowing for degradation of scarring and extracellular matrix materials to facilitate regeneration (Shubayev and Myers, 2000, 2004; Ahmed et al., 2005; Berry et al., 2008), thus again suggesting that in the absence of the growth-suppressor KLF4, proteins important in enhancing growth and regeneration are upregulated. Other gene targets determined from KLF4 knockout mice are the small proline rich (SPRR) family of proteins (Patel et al., 2003), including SPRR1a, upregulated in knockout mice, which has been identified to be important during peripheral nerve regeneration (Bonilla et al., 2002; Seijffers et al., 2007). Therefore, all of
these additional targets will be important to test to better understand the functional
downstream targets of KLF4 in neurons. Initial steps have been taken to answer this
question by overexpressing different KLF family members in RGCs and performing
microarray analysis on these samples to allow for more identification of more neuronal-
specific and as yet unidentified targets, though these data have not yet been obtained.

An important additional finding in these experiments is that for the first time we
have shown that KLF4 can positively regulate the expression of KLF9, another growth-
suppressor. During development in RGCs, KLF4 expression increases around birth (Fig.
2.2A-B), while KLF9 begins to slowly increase in expression at that time, increasing
dramatically through adulthood (Fig. 2.4C). This could suggest that the increase in KLF4
expression jumpstarts the expression of KLF9 which continues through adulthood. This
could be tested in KLF4 knockout RGCs to see if KLF9 expression is affected by KLF4
deletion.

Taken together, we have shown that KLF4 can regulate some known target genes
in neurons, though in some cases differently to what has been demonstrated in non-
neuronal cells. In particular, we have established a potential crossregulation of KLF9 by
KLF4, whereby KLF4 overexpression can lead to an increased expression of the strong
growth-suppressor KLF9. All of these experiments strongly suggest that a more global
search for targets and cross-regulation between family members is required.
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Fig. 4.1 Published KLF4 targets in non-neuronal cells do not decrease neurite growth after overexpression or knockdown similarly to KLF4. A-B) E18 hippocampal neurons were electroporated (Amaxa) with pMAX/pcDNA3 or pMAX/gene of interest and plated on PDL- and laminin-coated plates for 3 days in vitro prior to being immunostained for Tau (neurites) and imaged and measured by Cellomics Kineticscan software. EGFP+ cells extending at least 1 neurite greater than 10μm were analyzed to determine the effect on total neurite length (A) and the longest neurite including its branches (B). Neither overexpression nor knockdown of suggested targets were able to similarly replicate the overexpression of KLF4. N=1, n≥100 for each transfected condition; *p<0.001, ANOVA, t-tests with Bonferroni correction; Mean ± SEM.
Fig. 4.2  KLF4 overexpression leads to increased levels of published gene targets as well as KLF9. P4 RGCs were electroporated (Amaxa) with mCherry or Flag-KLF4 and plated for either 1 or 2 DIV prior to collection for RNA. RNA was purified (RNEasy; Qiagen) and reverse transcribed into cDNA (Bio-rad). q-PCR was performed for KLF9, p21, cyclin D1, and 18S for normalization. Following determination of primer efficiency (Pfaffl, 2001), fold change was determined using the transfected control samples as 100%. In most cases, multiple experiments were performed for each timepoint (2nd experiments for each timepoint display hatched marks). Despite the high variability (possibly due to the variability in efficiencies between 30-60%), in each case overexpression of KLF4 led to increases in expression of KLF9, p21, and cyclin D1. In data not shown, this same batch of cDNA was also probed for the presence of mRNA which was decreased compared to control, suggesting that KLF4 overexpression does not increase the expression of all mRNAs tested.
CHAPTER FIVE

KLF FAMILY MEMBERS AND THEIR REGULATION AND FUNCTION IN RGCS
RGCs lose their intrinsic axon growth ability during development (Goldberg et al., 2002a), and recently, the Krüppel-like factor (KLF) family of transcription factors has been implicated in this loss (Moore et al., 2009). In RGCs in vitro, overexpression of KLF4 leads to a suppression of neurite growth, while KLF4 knockout results in increased neurite growth. In vivo, KLF4 knockout in RGCs leads to increased axon regeneration following optic nerve crush. The effect of KLF4 knockout on regeneration and growth, however, is modest, suggesting that additional factors may be involved. Overexpression testing of all KLFs in cortical neurons revealed differential effects on neurite growth that could be separated through their structural subgroups (Moore et al., 2009). While initial testing of a few of these KLFs in overexpression studies in RGCs suggested that that their effect on neurite growth may be similar between CNS neuronal type (Moore et al., 2009), it is still unclear if all KLF family members will have similar effects on neurite growth in RGCs as was seen in cortical neurons. Additionally, while some KLFs were studied for their developmental regulation in rat (-6, -7, and -9; Moore et al., 2009), it is presently unknown if the expression of other KLF family members is also developmentally regulated in RGCs. Therefore, we sought to the reveal the developmental pattern of expression of KLF family members, as well as their effect on neurite growth in RGCs.
Results

We first asked if the expression of other KLF family members was developmentally regulated in RGCs. Using purified mouse RGCs from ages ranging from E18 through P18, we performed qRT-PCR to determine the expression profile for various KLFs. These preliminary experiments reveal that the expression of KLFs -2, -5, -9, and -15 increase during development, while the expression of KLFs -3, -6, -7, -8, -13, and -16 decrease. KLF10 did not show a specific developmentally regulated pattern, and KLFs -11, -12, -14, -1, and -17 have not been tested (Fig. 5.1A-B). Not shown here is KLF4, which was previously shown to increase around the time of birth through both qRT-PCR (Fig. 2.2B) and microarray (Fig. 2.2A). These results demonstrate that the expression of multiple KLF family members is developmentally regulated in mouse RGCs, further supporting the potential involvement of KLFs in loss of intrinsic axon growth ability.

What are the effects of these KLF family members on neurite growth? As overexpression of the KLFs in cortical neurons resulted in differential effects on neurite growth, we were interested to see if they would behave similarly in RGCs. We began by performing lentiviral overexpression of KLF9, a strong growth suppressor in cortical neurons. Lentiviral overexpression of KLF9 led to a decrease in neurite growth in embryonic day 20 (E20) RGCs similar to what was seen in young postnatal cortical neurons (Fig. 5.2). Do all KLFs behave similarly in RGCs? To address this question, we optimized a different technique whereby we electroporated small numbers of P4 RGCs with individual KLF family members and a pMAX (EGFP) reporter. After 2 days in
vitro, we immunostained cultures and used Cellomics Arrayscan VTI to image and trace transfected (EGFP+) neurons (Fig. 5.3). The majority of the KLFs behave similarly in RGCs as in cortical neurons when overexpressed, with the exception of KLFs -2, -3, and -12. In cortical neurons, KLF2 is an inhibitor of neurite growth, while in RGCs, it had no effect. Both KLF3 and -12 suppress neurite growth in RGCs, but have no effect in cortical neurons (Fig. 5.3). These results suggest that the effect of KLFs may be neuronal type-specific, and could involve the endogenous levels of the population of KLFs present in that neuron at that specific time when the overexpression occurred.

**Discussion and Future Directions**

We have previously shown that overexpression of KLF family members in cortical neurons has differential effects on neurite outgrowth (Fig 2.4E). We now sought to determine if comparable experiments in RGCs would result in similar phenotypes. We have further asked if the expression of all KLF family members is developmentally regulated in RGCs, and importantly, if the correlation of this expression pattern and the overexpression growth phenotype of KLFs in RGCs is maintained.

We found that overexpression of the majority of KLF family members in RGCs resulted in similar phenotypes to those seen in cortical neurons, with 3 exceptions (Fig. 5.3). How can overexpression of 3 KLFs result in different phenotypes in different CNS neurons? One possibility is that specific co-factors, repressors, or modifiers may not be present in one population of neurons versus another. To address this question, we could perform immunoprecipitation experiments to determine if KLFs are interacting with
similar co-factors between different CNS neuronal types. Separately, we could probe western blots of lysates from different CNS neuronal types with antibodies against modified forms of these proteins to determine if the KLFs were being similarly modified in multiple cell types.

Another possibility for the 3 different overexpression phenotypes seen in multiple CNS neurons may be that the endogenous expression levels of KLFs at the time of overexpression are different between distinct neuronal types. This could be tested by measuring the actual mRNA counts of KLF family members in both cortical neurons and RGCs at the specific ages tested in the experiments. Previous microarrays of corticospinal neurons during development (E18, P3, P6, P14; Arlotta et al., 2005) contained information on the expression of 12 members of the KLF family. Interestingly, 4 of the KLFs (-3, -6, -7, and -9) had similar developmental expression patterns to those seen in RGCs, while the rest were somewhat different. This further suggests that there may be differences in the expression levels and patterns of KLF family members in multiple neuronal types.

Finally, these qRT-PCR experiments were performed in mouse RGCs, while the overexpression of the KLF family members was tested in rat neurons. It is possible that there may be some variability in the expression patterns of the different KLFs between species. For example, our microarray data of KLF expression in rat RGCs during development (Fig. S10) display some variation from these mouse qRT-PCR experiments, such that the majority are similar in the direction of their expression changes, but KLFs -10 and -15 behave quite differently. In contrast, qRT-PCRs of a subset of these KLFs in
rat RGCs (KLFs -6, -7, and -9; Fig. 2.4A-C) revealed similar patterns of expression to those performed on mouse RGCs (Fig. 5.1A). Thus, it is unclear whether there are species-specific variations in KLF expression within a single cell type. Further understanding of species variability would require additional studies of KLF family member expression in rat RGCs to compare to the studies shown here.

Our results also suggest that while multiple KLFs are developmentally regulated in RGCs, their developmental pattern of expression may not always directly correlate with their effect on neurite growth in a manner consistent with RGCs’ development decrease in intrinsic axon growth ability. Previously we have shown that KLF6 and -7, whose expression decreases during development, when overexpressed lead to enhanced neurite growth, while KLF4 and -9, whose expression increases during development, when overexpressed lead to decreased neurite growth (Moore et al., 2009). This suggested that the expression of growth enhancers is reduced in older neurons, while the expression of growth suppressors is increased in older neurons. Our additional analysis of developmental expression patterns of more members of the KLF family suggests that perhaps this is not a generalizable concept. For example, KLF13 and 16, members of the basic transcription element binding protein (BTEB) subgroup of KLFs, both suppress neurite growth in cortical neurons and RGCs, however, in RGCs their expression decreases during development. Similarly, KLF3, a suppressor of neurite growth in RGCs, displays a decrease in expression developmentally. All three of these examples do not follow the pattern demonstrated by KLFs -4, -6, -7, and -9, where growth suppressors increase during development and growth enhancers decrease during
development. This suggests that our initial correlation based upon expression profile may not apply to all KLFs. We may need to further consider that the pattern of developmental expression may be less important than the relative expression between KLFs. As discussed above, total mRNA counts of each KLF at a particular developmental age would better illuminate the relationships of expression between the family members. Additionally, we are measuring mRNA levels over development, and protein levels may not be directly correlated. KLF proteins can undergo post-translational modifications, leading to activation, inactivation, or degradation of the protein, for example (Black et al., 2001; Dong and Chen, 2009). Thus, the expression data may not accurately represent the presence or activity of the protein. To further address this concern, levels of KLF proteins, both unmodified and modified, can be measured from western blots of different ages of RGCs when working antibodies become available.

In conclusion, these studies demonstrate that the majority of the KLFs affect neurite growth similarly in different types of neurons when overexpressed, with few exceptions. In addition, many of the KLF family members are developmentally regulated in RGCs, though the direct correlation between their developmental expression profile and their effect on neurite growth is not consistent throughout the family. It may be that by exploiting the differences between various CNS neurons in KLF expression profiles and overexpression phenotypes, we can begin to unravel those mechanisms which underlie their ability to affect neurite outgrowth.
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Figure 5.1 Multiple KLF family members are developmentally regulated in mouse RGCs. A-B) Floxed KLF4 wildtype mouse (CD1 background) RGCs were purified through immunopanning at different timepoints during development and snap frozen. In most cases, multiple preps were pooled for different ages. RNA was purified (RNEasy, Qiagen) and subjected to qRT-PCR (Bio-Rad) for levels of the specific KLF and 18S as a loading control. Efficiencies were determined for each set of primers, and fold change was measured using E18 as 100% (Pfaffl, 2001). Approximately 3 technical replicates were performed for each condition. A) N=1, B) N=2, each type of mark is a replicate, with the line representing the average.
Fig. 5.2 KLF9 lentiviral overexpression in RGCs decreases neurite growth. A-C) E20 RGCs were infected with lentivirus encoding flag-mCherry control or flag-human KLF9 and cultured on PDL- and laminin-coated plates in growth medium for 4 days. Neurons were immunostained for Tau (neurites, red) and FLAG (green, appears yellow in overlay). Transfected cells (flag+, localized to nucleus in FLAG-KLF9 treatment) were hand traced. Compared to controls, RGCs expressing flag-KLF9 extended shorter neurites. Bars represent the average neurite length of transfected neurons (>50 cells per treatment; *** p<0.001, paired t-test; Mean ± SEM).
Fig. 5.3 Overexpression of KLF family members results in differential effects on neurite growth in RGCs. P4 RGCs were electroporated (Amaxa, Small Cell Number Kit) with pMAX and the KLF of interest or mCherry. After 2 days in culture, neurons were stained for Tau (neurites) and imaged and traced using Cellomics Kineticscan. Average total neurite length for each transfected condition was normalized to control and these data were averaged over multiple days of experiments. KLF1 and KLF17 were not tested as they were previously found not to be expressed in RGCs (Fig. 2.4E). N=3-8; Mean ± SEM.
CHAPTER SIX

KLF FAMILY MEMBERS AND THEIR ROLES IN AXON GROWTH AND REGENERATION
CHAPTER SIX

Introductory Remarks

Why do neurons in the central nervous system (CNS) fail to regenerate their axons after injury? This has remained a fundamental question in neuroscience, with obvious implications for human disease. In the CNS, embryonic or neonatal neurons can regenerate their axons after injury, whereas postnatal or adult neurons cannot (Bregman and Goldberger, 1982; Kunkel-Bagden et al., 1992). This could be due to the development of an inhibitory CNS environment; however, overcoming this inhibition is still not sufficient on its own to lead to functional regeneration (Caroni and Schwab, 1988; Bartsch et al., 1995; Moon et al., 2001; Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2005). These findings suggest that intrinsic changes within the neurons themselves may also contribute to regenerative failure in the CNS.

Development of the extrinsic inhibitory CNS environment

Both mature astrocytes and mature oligodendrocytes contribute to an inhibitory environment for axon regeneration in the injured adult mammalian CNS. Between the first and second postnatal week of development in most CNS tissues, oligodendrocytes begin to form myelin sheaths around axons to allow for increased conduction of electrical impulses (Waxman, 1980; Foran and Peterson, 1992). After injury, damaged axons are exposed to the myelin-associated lipids and proteins that are inhibitory to axon growth and regeneration (reviewed in Yiu and He, 2006). Astrocytes respond to injury by secreting chondroitin sulphate proteoglycans (CSPGs) which are also inhibitory to
growth (Snow et al., 1990; McKeon et al., 1999; Becker and Becker, 2002; Jones et al., 2002; Jones et al., 2003; Tang et al., 2003). Many strategies have been attempted to overcome glial-associated inhibitory cues and thus increase CNS regeneration. For example, inhibitory proteins have been knocked out at the genetic level (Bartsch et al., 1995; Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003; Su et al., 2008), neutralized through antibody treatments (Caroni and Schwab, 1988; Bregman et al., 1995; Tang et al., 2001), or enzymatically digested (reviewed in Crespo et al., 2007). These studies have resulted in modest regeneration, leading to alternative strategies targeting the downstream signaling of these inhibitory pathways (reviewed in Schmandke and Strittmatter, 2007). The incomplete regeneration in all of these studies suggests that there may be additional inhibitory proteins that have yet to be discovered still acting to inhibit growth, and that there may be intrinsic changes within the neurons themselves that limit their regenerative ability.

Evidence for intrinsic control of axon regenerative ability

Significant data supporting neuron-intrinsic mechanisms began with spinal cord injury experiments in whole CNS preparations from neonatal opossums and embryonic rat, which demonstrated that the injured neonatal CNS can regenerate, and that this ability is lost postnatally (Saunders et al., 1992; Treherne et al., 1992; MacLaren and Taylor, 1995; Saunders et al., 1995). Similar results were reported in co-culture experiments using heterochronic tissue explants in multiple systems. In these studies, axons from older neurons did not have the ability to regenerate even on the permissive
substrate of embryonic explants, again suggesting that there may be a developmentally regulated intrinsic loss of axon growth ability (Chen et al., 1995; Li et al., 1995; Dusart et al., 1997; Blackmore and Letourneau, 2006). In both of these types of experiments, however, the local inhibitory environment around the neurons is still retained, with connections between adjacent neurons and glia possibly contributing to the neuronal response. Removing this inhibitory influence by purifying retinal ganglion cells (RGCs), a type of CNS neuron, away from all other retinal cells finally addressed the question of the intrinsic growth potential of different ages of CNS neurons. These studies revealed that embryonic RGCs grow their axons ten-fold faster than postnatal RGCs, and that this growth ability is specifically lost around the time of birth (Goldberg et al., 2002a). These data suggest that RGCs lose their intrinsic capacity for rapid axon growth during development, and that this may play a role in the regenerative failure of CNS axons after injury.

What is the molecular basis for this loss? Prior work has pointed to possible roles for cAMP (cyclic adenosine 3’,5’-monophosphate; Cai et al., 2001), CREB (cAMP response element-binding protein; Gao et al., 2004), Bcl-2 (B-cell lymphoma/leukemia; Chen et al., 1997; Cho et al., 2005), Cdh1-APC (anaphase promoting complex; Konishi et al., 2004; Lasorella et al., 2006) and PTEN (phosphatase and tensin homology; Park et al., 2008) in this loss. To identify new candidate genes that could contribute, Dr. Goldberg performed microarrays on RGCs of different ages to reveal developmentally regulated genes. These genes were screened in primary neurons for their effect on neurite outgrowth. Overexpression of the transcription factor Krüppel-like factor 4
(KLF4) resulted in a significant decrease in neurite outgrowth in hippocampal and cortical neurons, and RGCs. KLF4 knockout during early development resulted in increased neurite growth from RGCs in vitro, and increased axon regeneration in vivo after optic nerve injury. Interestingly, in RGCs, KLF4 expression increases postnatally, specifically during the period around birth, which is when RGCs decrease their intrinsic axon growth ability. These data support a model whereby the increase in KLF4 expression around birth leads to a loss of regenerative ability of RGCs.

This is the first description of KLF4’s ability to regulate axon growth and regeneration. In addition, KLF4’s function as a growth suppressive transcription factor is unique in the field of transcription factor modulation of neurite outgrowth, where most act as growth enhancers. How KLF4 limits regeneration and growth in neurons is not yet known. As KLF4 has the ability to both activate or repress gene transcription, its function may be to turn off genes that promote neurite growth, and/or turn on genes that suppress neurite growth. Thus, during development, KLF4 may play an integral role in slowing down axon growth as the axon nears or reaches its target, perhaps freezing the neuron in a morphologically stable state once the gross circuitry is established.

What is known about KLF4?

KLF4 has been most widely studied recently in stem cell reprogramming (Zhao and Daley, 2008), though it has also been shown to play roles in differentiation (Dai and Segre, 2004; Ghaleb et al., 2005), growth arrest (Shields et al., 1996; Chen et al., 2001; Chen et al., 2003; Yoon et al., 2003), and cancer progression (Black et al., 2001; Safe and
KLF4 has both activator and repressor capabilities, with known protein-protein interactions with p300/CBP (CREB-binding protein; Geiman et al., 2000; Evans et al., 2007) and CtBP1 (C-terminal-binding protein 1; Liu et al., 2009), respectively. Other co-activators or co-repressors may yet be discovered.

KLF4 function in neurons has only been reported once previously, when it was shown to be upregulated by NMDA or AMPA treatment in cortical neuron cultures. Overexpression of KLF4 in these neurons concurrent with NMDA treatment led to an increased activation of caspase-3, which was dependent on extracellular and intracellular calcium levels. Importantly, overexpression of KLF4 alone did not increase caspase-3 levels in these neurons. Therefore, KLF4 overexpression in cortical slices leads to increased caspase-3 activation after NMDA insult. We have found that KLF4 overexpression or knockout had no effect on survival in any of the neuronal types tested (Figs. 2S1, 2S8, 2S11). In addition, after optic nerve injury in vivo, KLF4 knockout had no effect on RGC survival (Fig. 2S9). Thus, it is possible that KLF4 may affect survival in cultures in combination with other stressors, however, it does not appear to affect survival in primary neurons in vitro or in RGCs in vivo.

What are the downstream mediators of KLF4’s effects on neurite growth and regeneration? Although little is known about KLF4’s targets in neurons, a number of KLF4 targets identified outside of the nervous system may be good candidates for mediating KLF4’s effect on axon growth, such as p21^{WAF1/Cip1} (p21), p53, urokinase plasminogen activator receptor (u-PAR), ornithine decarboxylase (ODC), three different
laminin chains (Rowland and Peeper, 2006), activating transcription factor 3 (ATF3), small proline rich protein 1a (SPRR1a), and matrix metalloproteinases (MMPs). For example, p21 inhibits Rho kinase (ROCK), promoting neurite outgrowth and branching in rat embryonic hippocampal neurons (Tanaka et al., 2002). p53 expression is required for neurite growth and regeneration (Di Giovanni et al., 2006; Tedeschi et al., 2009). u-PAR is the receptor for the plasminogen activator system which cleaves extracellular matrix molecules. Mice lacking any portion of this system show delayed regeneration in the peripheral nervous system following sciatic nerve crush (Siconolfi and Seeds, 2001a).

Upregulation of Arginase I (Arg I), which catalyzes arginine to ornithine, has been suggested to be the downstream modulator of elevated cAMP and CREB activation which allows for a decreased inhibition to myelin (Cai et al., 2002; Gao et al., 2004). ODC converts ornithine (the product of Arg I) to putrescine, which is subsequently catalyzed into the polyamines spermidine and spermine, found to be important in promoting regeneration of injured hippocampal neurons in vitro and after optic nerve injury in vitro and in vivo (Chu et al., 1995; Deng et al., 2009). The laminin gamma-1 chain is one of the laminin chains regulated by KLF4 (Higaki et al., 2002). Removal of the gamma-1 chain in a mossy fiber CNS injury model resulted in decreased axon growth as well as an inability of regenerating fibers to cross the lesion site (Grimpe et al., 2002), whereas application of a portion of the laminin gamma-1 chain to a rat model of spinal cord injury resulted in enhanced regeneration of CNS axons (Wiksten et al., 2004).

Finally, in studies in which KLF4 was knocked out in cornea, multiple genes relevant to regeneration were found to be upregulated, such as ATF3 (Swamynathan et al., 2008), a
growth-enhancing transcription factor expressed in regenerating neurons (Mason et al., 2003; Pearson et al., 2003; Campbell et al., 2005; Seijffers et al., 2007). SPRR1a is upregulated after KLF4 knockout (Swamynathan et al., 2008), and also during peripheral nerve regeneration (Bonilla et al., 2002). In addition, multiple MMPs have been shown to be upregulated after KLF4 knockout in the cornea (Young et al., 2009), and these enzymes can enhance regeneration in multiple systems through degradation of inhibitory proteins (Ahmed et al., 2005).

It is presently unknown whether KLF4 regulates the expression of these genes in neurons, or whether these or other target genes mediate the effect of KLF4 on CNS axon growth. To more fully understand the mechanism by which KLF4 exerts its influence on neurite growth, additional experiments to identify KLF4 gene targets in neurons, such as microarrays, are necessary.

The KLF family of transcription factors and their effects on neurite growth

KLF4 is one of 17 members of the KLF family of transcription factors. Each family member contains 3 highly homologous Cys2/His2-type zinc fingers in their C termini with highly conserved regions between them. KLFs bind DNA at CACCC/GC/GT boxes, which are highly represented throughout regulatory regions in the genome. They are often grouped with the Sp (specificity protein) family, though the KLF family is distinguished by the absence of the Sp family’s Buttonhead (BTD) box (Suske et al., 2005). Many KLFs have been studied in cancer, and several can play roles
in cell cycle, proliferation, and cell death (Black et al., 2001). Little is known, however, about the expression or function of the 17 KLFs in the mammalian nervous system.

We found that 15 of 17 KLF family members are expressed in RGCs (Moore et al., 2009), and 14 of 17 KLFs are expressed in cortical neurons (M. Blackmore, unpublished observations). Do all KLF family members affect neurite outgrowth? We found that overexpression of KLF family members resulted in differential effects on neurite growth which, in cortical neurons, correlated with their structural subfamily groupings (Moore et al., 2009). In RGCs, effects of KLF family member overexpression were only slightly different from the results seen in cortical neurons (Figs. 2.4E and 5.3), suggesting that the correlation between growth effect and subfamily is not completely conserved between neuron types. This difference could be attributed to the endogenous expression of different KLFs or co-factors in the specific types or ages of neurons studied. Thus, multiple KLFs are expressed in neurons, and can differentially regulate neurite growth.

As neurons express multiple KLFs, how do they interact to affect neurite growth? 8 growth suppressors are expressed in RGCs during development, while only 2 growth enhancers are expressed (Moore et al., 2009). Knocking out a single growth suppressor, KLF4, increases axon growth and regeneration, suggesting that the endogenous background expression of KLFs in postnatal neurons is primarily suppressive. It is not known whether certain KLFs dominate in function over others, however. In overexpression experiments using combinations of growth suppressive (KLF4 and -9) and growth enhancing (KLF6 and -7) KLFs, we found that the suppressors dominated:
KLF6 and -7 could never enhance growth in the presence of KLF4 or -9, and KLF4 could suppress neurite growth even when KLF6 or -7 were co-overexpressed (Moore et al., 2009). It is not yet known whether these factors are competing for similar binding sites or physically interacting with each other to inhibit function. These data imply that in the adult nervous system, the presence of many growth-suppressive KLFs is dominant, and suggest that to increase growth or regeneration, multiple suppressive KLFs may need to be removed.

Is the expression of all KLF family members developmentally regulated, and can this be correlated with their effect on neurite growth? Interestingly, the expression of many KLF family members was developmentally regulated. Some had a direct correlation with their effect on neurite outgrowth: KLF6 and -7 decreased in expression postnatally, and enhanced growth when overexpressed; others including KLF4 and -9 increased in expression postnatally, and suppressed neurite growth when overexpressed (Moore et al., 2009). This was not consistent with all other KLFs, however. Thus, the developmental regulation of at least several KLFs renders them additional candidates in the developmental loss of intrinsic axon growth ability. These studies also raise the hypothesis that KLF expression or function may be regulated at levels other than mRNA transcription. Further studies must be done to better understand their regulation at both the mRNA and protein levels.
**KLF family members in the nervous system**

While the majority of research on KLF family members has been studied in other systems, some characterization has been performed in neurons and the nervous system as a whole. The growth enhancers KLF6 and -7 were both found to be highly expressed in the developing nervous system, consistent with our findings in RGCs. In particular, KLF7 is highly expressed in both the PNS and CNS throughout development (Laub et al., 2001b; Lei et al., 2001). KLF6 is expressed in the developing nervous system (Laub et al., 2001a), and in the adult is present in neurons, endothelial cells and neuronal progenitors in the forebrain (Jeong et al., 2009). In addition, these two KLFs have been studied previously for their effects on axon growth in zebrafish retinal explants, where KLF7 was found, together with KLF6, to be necessary for axonal outgrowth. Interestingly, KLF6 and -7 were able to compensate for each other in their ability to affect axon growth (Veldman et al., 2007), suggesting a possible redundancy in the genes that they regulate. Similarly, we have found that concurrent overexpression of KLF6 and -7 in cortical neurons was not synergistic, further supporting a redundant role in their function (Moore et al., 2009). A more comprehensive study on KLF7’s functions in the nervous system revealed that KLF7 knockout results in deficits in axon growth and pathfinding in the olfactory system, retina, and brain (Laub et al., 2005; Laub et al., 2006), again supporting a role for KLF7 in enhancing axon growth. Interestingly, KLF7 has been shown to positively regulate p21, p27, L1, GAP-43, TrkA, TrkB, and genes important for synaptogenesis and cytoskeleton dynamics in neurons (Laub et al., 2001b; Laub et al., 2005; Lei et al., 2006; Kajimura et al., 2007; Kingsbury and Krueger, 2007).
KLF7’s ability to upregulate the Trk neurotrophin receptors may also play an important role in RGC survival after optic nerve injury. RGCs experience a loss of trophic responsiveness after injury, which may be due to the decreased expression of Trk receptors (Meyer-Franke et al., 1998; Shen et al., 1999; Goldberg et al., 2002b; Duan et al., 2009). Thus, therapeutically, overexpression of KLF7 may not only serve to enhance neurite growth, but also may increase trophic responsiveness in neurons, leading to increased neuroprotection after injury. In summary, these studies support our hypotheses that KLF6 and -7 act as growth-enhancers in neurons, and identify possibly downstream mechanisms for their effect.

KLF9 has also been studied previously in the nervous system and for its effect on neurite growth. It is a thyroid hormone (T3)-regulated transcription factor, with its expression in the brain developmentally regulated such that it is barely detectable from embryonic ages through P0, increases dramatically through one month, and then maintains this higher expression into adulthood (Denver et al., 1999; Martel et al., 2002; Morita et al., 2003), reflecting the pattern of plasma thyroid hormone levels. This pattern of expression is also consistent with KLF9’s developmentally increased mRNA levels in RGCs (Moore et al., 2009). KLF9’s expression is not only positively regulated by binding of T3 receptor-retinoid X receptor heterodimers to the T3 response element (T3RE) in the 5’ flanking region of the KLF9 gene (Denver and Williamson, 2009), but also by corticosterone (Bonett et al., 2009), and activity (Lin et al., 2008; Scobie et al., 2009). KLF9 itself can repress transcription through recruitment of the co-repressor Sin3A (Imataka et al., 1992; Zhang et al., 2001a). These findings make KLF9 an
interesting candidate for a role in the loss of axon growth ability, due not only to its
dramatic developmental regulation, but also to its ability to be modulated by electrical
activity. As activity enhances trophic responsiveness and axon growth (Goldberg et al.,
2002b), it will be interesting to explore the role of KLF9 in these processes.

Previous studies on the role of KLF9 in neurite outgrowth are contrary to our results. For example, in cell lines, overexpression of KLF9 led to an increase in the
number of cells extending neurites as well as increasing the number and length of the
neurites (Denver et al., 1999). In agreement, additional studies in embryonic cortical
neurons reported that knockdown of KLF9 decreased neurite branching to non-T3 treated
levels, without affecting elongation. A similar effect was seen in the small subpopulation
of small acetylcholinesterase (AChE) expressing cells, which are a target population for
T3 (Cayrou et al., 2002). Thus, previously reported data suggest that KLF9 functions to
increase neurite growth, branching, and elongation. In contrast, we have found in
embryonic (Fig. 5.2) and postnatal RGCs (Fig. 5.3), and young postnatal cortical neurons
(Fig. 2.4E) supplemented with T3, KLF9 overexpression dramatically decreased neurite
growth, suggesting possible differences for this transcription factor in different cell and
neuronal types (Moore et al., 2009).

Despite multiple reports of effects of KLF9 on neurite growth, KLF9 knockout
mice do not have defects in axon targeting and dendrite length (Scobie et al., 2009),
though there has been an unquantified observation of slightly less developed Purkinje cell
dendrites in the cerebellum (Morita et al., 2003). KLF9 knockout mice do display
deficits in dentate granule (DG) neuron maturation, such that there is an increase in
immature DG neurons in the hippocampus of developing knockout animals as defined by early and late maturation markers, spine maturation and electrophysiological properties (Scobie et al., 2009). This increase in the numbers of immature DG neurons is unrelated to proliferation and cell fate specification, suggesting that the early phase processes in neurogenesis in the KLF9 knockout hippocampus are normal. As newly born neurons are less able to functionally integrate into the adult hippocampus, there is reduced neurogenesis-dependent synaptic plasticity (Scobie et al., 2009). Behaviorally, KLF9 knockout mice have deficits in rotorod and contextual fear-conditioning tests (Morita et al., 2003; Scobie et al., 2009). Thus KLF9 is required for newly born DG neurons late-phase integration into the hippocampal circuitry (Scobie et al., 2009), but may have redundant factors that can compensate for its role in neurite growth.

Taken together, it appears that KLF9 may function differently between various neuronal types, and it is possible that it may act together with other KLFs in a redundant fashion to maintain its specific regulation of downstream targets.

Of the other KLFs we have found to suppress neurite growth, only three, KLF5, -15, and -16, have been studied to any degree in the nervous system. KLF16, a member of the basic transcription element binding protein (BTEB) subgroup of KLFs together with KLF9, -13, and -14, is expressed in the brain in embryos and adult animals (Hwang et al., 2001; D'Souza et al., 2002). It can bind to Sp1 sites in the promoters of at least 3 dopamine receptors to regulate their transcription, competing with Sp1 and Sp3 for binding site occupancy. KLF16 can either activate or repress transcription depending on
the cell type studied (Hwang et al., 2001), suggesting that the endogenous population of other proteins available directly affects its function.

KLF15, a KLF which we could not place into a specific subfamily, is expressed in various parts of the brain and retina. In the retina, KLF15 is present in neurons of the inner nuclear layer and some neurons of the ganglion cell layer (Otteson et al., 2004). Our studies suggest that its expression is developmentally upregulated in purified RGCs (Fig. 5.1), further supporting a role for KLF15 as a growth suppressor in neurons. Interestingly, KLF15 can repress the rhodopsin promoter, although deletion of its N terminus results in a switch to a transcriptional activator (Otteson et al., 2004). Its function in neurons has not been described previously.

KLF5, which also does not belong to a specific KLF subfamily, has been studied in the human prefrontal cortex where it is expressed, as well as in the granular and pyramidal cells of the hippocampus. Interestingly, KLF5 is downregulated in the prefrontal cortex in schizophrenia patients, and a polymorphism of KLF5 is associated with schizophrenia (Yanagi et al., 2008). Some targets of KLF5 identified in non-neuronal systems may prove to be good candidates for its effects on neurite growth in neurons. For example, KLF5 activates transcription of ILK (integrin-linked kinase), as well as the ILK targets Cdc42 and myosin light chain in keratinocytes (Yang et al., 2008). The signaling pathways and post-translational modifications of KLF5 have been well-characterized in other systems, and its function is highly regulated by these post-translational modifications (reviewed by Dong and Chen, 2009), which may prove to be important to its function in neurite growth. Its expression pattern in RGCs is similar to
that of KLF4, with a peak around the time of birth. Whether it functions similarly or redundantly to KLF4 is unknown, but in embryonic stem cells, KLF5 has been shown to bind to the same sites as KLF4 when KLF4 is removed, compensating for its absence (Jiang et al., 2008).

Interestingly, while each of these three KLFs was shown to suppress neurite growth when overexpressed, their developmental expression patterns in RGCs are varied, such that KLF16 expression decreases and KLF15 expression increases during development, whereas KLF5 exhibits a peak in expression similar to that of KLF4.

Furthermore, KLF12, a member of the PVALS/T subgroup of KLFs, did not affect neurite outgrowth at all when overexpressed in cortical neurons (Moore et al., 2009), even though it is expressed at its highest levels in the brain shortly after birth (Imhof et al., 1999).

Taken together, these data suggest that KLF roles in neuronal development are not limited to effects on neurite growth. Less than half of the KLFs have had some previous characterization in the nervous system, leaving much to be revealed about their function, regulation, interactions, and downstream targets in neurons, and specifically in neurite growth.

*KLF family members function as a “network”*

Our discovery that multiple KLFs are expressed in RGCs suggests that this family acts as a “network”, as has been shown in other systems (Turner and Crossley, 1999; Black et al., 2001; Eaton et al., 2008). KLFs can function and interact differently in
different cell types due to the expression of other KLFs or other co-factors, or to differences in post-translational modifications of the transcription factors themselves. These features may explain how the KLF family of transcription factors creates a cell type-specific and perhaps developmental period-specific “code” resulting in the neurite growth phenotypes we are studying.

**Cell-Type Specific Functions** - The effects of KLFs differ depending on the cells in which they are studied. As discussed above, a number of KLFs have different effects on axon growth in different neurons. Outside of the nervous system, KLF4 can arrest the cell cycle through upregulation of p21 and the inhibition of cyclins, resulting in a loss of proliferative abilities (reviewed in Black et al., 2001). In addition, it has been studied thoroughly in its role in differentiation of the skin cells of the epidermis (Segre et al., 1999). Recently, however, KLF4 has been shown to be important in the induction of pluripotency in somatic cells (Takahashi and Yamanaka, 2006; Zhao and Daley, 2008). This role in maintaining a proliferative, undifferentiated state seems to be in direct opposition to KLF4’s role in arresting the cell cycle, inhibiting proliferation and promoting differentiation, suggesting that KLF function may be cell-specific.

Furthermore, the specific endogenous expression profile of different modulating factors, co-factors, etc. in different neurons, ultimately affects the function of these transcription factors. For example, KLF1 knockout mice display a reduced level of KLF3 in erythroid cells, whereas there is no effect of KLF1 knockout on KLF3 expression levels in the brain, demonstrating a differential regulation between tissue type (Crossley et al., 1996). This implies that the endogenous components present in a
specific cell type greatly affect the transcriptional outcome. Thus, there is a need to study each KLF in the specific neuron of interest to understand function.

Relative Expression and Competition - KLF family members can compete for binding sites and regulation of the same target genes (Bieker, 2001; Black et al., 2001). KLF4 and KLF5, two highly related KLF family members, have opposing effects on shared target genes, such as transgelin for example (Adam et al., 2000), or the expression of KLF4 itself (Sun et al., 2001). In in vivo studies with KLFs -1, -3 and -8, the relative expression of the KLFs determines which KLF will outcompete the others at the binding site for specific genes. For example, KLF8’s two promoters contain multiple binding sites for KLFs. KLF1 can activate, and KLF3 can repress expression of KLF8. In vivo, KLF3 occupies both of the KLF8 promoters, repressing KLF8 expression, with little to no binding of KLF1 on these sites. In a KLF3 knockout animal, however, KLF1 binds these sites to activate the KLF8 promoter (Eaton et al., 2008).

KLF family members can also bind to different sites on a promoter to compete for functional transcriptional outcome. For example, KLF9 and -12 can both bind to a basic transcription element (BTE) site in the promoter of activating enhancer-binding protein 2 alpha (AP-2α) in separate locations. KLF9 activates and KLF12 represses AP-2α expression. After co-expression of KLF9 and -12 in equal amounts, the net expression of AP-2α transcription is unchanged (Imhof et al., 1999). Thus, all of these studies demonstrate that relative amounts of different KLFs present at a given time elicit different regulation of a target gene. This may be very important in studying the
developmental loss of axon growth ability in neurons, as the balance of expression of
different family members may shift at any specific developmental time period.

Compensation and Redundancy - Upon removal of one KLF, other KLFs can bind
to the same site and act similarly, compensating for the loss of the first. For example, in
embryonic stem cells, depletion of KLF4 did not lead to differentiation (Jiang et al.,
2008). Further studies showed that both KLF5 and KLF2 are also downregulated upon
differentiation. Knockdown of any of these KLFs singly, or in pairwise combinations
still did not enhance differentiation of stem cells. Only with depletion of all three KLFs
together did the stem cells finally differentiate. Detailed chromatin immunoprecipitation
(ChIP) analyses revealed that these three KLFs had many shared targets and upon
depletion of one factor, the others were able to bind to that target, compensating for the
loss of the removed KLFs (Jiang et al., 2008).

Redundancy has also been shown in retinal explants from zebrafish, where KLF6a
and KLF7a were identified as candidates for regeneration as they were upregulated after
injury. Knockdown of either KLF6a or KLF7a singly had no effect on axon growth;
however, concurrent knockdown of both factors resulted in a severe decrease in axon
growth (Veldman et al., 2007). Similarly, in erythroid cells, double knockouts for both
KLF1 and KLF2 yielded animals that died earlier, showed a greater reduction in
embryonic Ey- and βh1-globin gene expression, and manifested cells with more
morphological abnormalities when compared to single KLF1- or KLF2- knockout
animals (Basu et al., 2007). Thus, members of the KLF family can compensate for the
loss of one KLF. Whether such redundancy limits the regenerative ability of KLF4-knockout RGCs remains to be studied.

Effects of Post-Translational Modifications on Function - As has been shown with many other transcription factors, post-translational modifications of KLFs can affect transcriptional outcome. For example, sumoylation or acetylation of KLF5 in response to specific environmental stimulation can result in a complete switch of its function, likely due to the further recruitment of co-repressors or co-activators. These modifications can also affect the half-life of this protein in the cell (reviewed in Dong and Chen, 2009). Similarly, while KLF1 is required for β-globin expression, it is also expressed in erythroid cell lines that do not yet express β-globin. Post-translational phosphorylation of KLF1 allows it to bind and activate the β-globin promoter, revealing a functional requirement for post-translational modification in gene regulation (Bieker et al., 1998). These examples emphasize the importance of the expression levels of not only the transcription factors themselves, but also of those proteins that modify these transcription factors in a specific cell type and at a specific stage of development. We have characterized the mRNA expression profiles of multiple KLFs in RGCs (Figs. 2.4A-C, 5.1), but it is not known how their protein levels are regulated during development, or whether post-translational modifications affect their ability to enhance or suppress neurite growth.

Taken together, all of these studies suggest that the KLF family of transcription factors can function in unique ways that require an understanding of the simultaneous
functions of the whole family as opposed to single factors. Thus, further analysis of KLF overexpression in neurons should consider the endogenous expression of (1) all KLFs in these neurons at the time of overexpression, (2) enzymes that can post-translationally modify KLFs, and (3) co-factors that interact with the KLFs to activate or repress transcription. These considerations may help us to better understand the differences we have seen in the growth effects in cortical neurons versus RGCs, for example. KLF compensation or redundancy may also play an important role in axon regeneration of CNS neurons. By removing multiple suppressors which could be acting redundantly, we may increase axon regeneration after injury above that which we have seen with KLF4 knockout alone.

*Other transcription factors in neurite growth and regeneration*

A small number of other transcription factors have been studied for their ability to affect axon growth and regeneration (reviewed in Zhou and Snider, 2006), suggesting that transcriptional regulation may be a fundamental mechanism for the loss of axon regenerative ability in neurons. The transcription factors p53, c-Jun, ATF3, CREB, STAT3, NFATs, NFκB, Sox11, and SnoN all affect neurite growth and axon regeneration in varied ways. In general, the expression of the majority of these transcription factors is developmentally regulated, such that they are highly present early in development, when CNS axons are able to regenerate, and decrease soon after birth through adulthood, when CNS axons lose axonal regenerative ability. The expression or activation of many of these factors increases after injury in regenerating neurons, but is absent in non-
regenerating neurons. Interestingly, the majority of these other transcription factors enhance neurite growth, in contrast to our recent identification of multiple neurite growth-suppressing members of the KLF transcription factor family. Our studies suggest that in the presence of both growth-suppressors and growth-enhancers, the suppressors dominate the phenotypic outcome (Moore et al., 2009). Therefore, modulating only the levels of transcription factors which enhance axon growth may not be sufficient for functional regeneration without simultaneous repression or removal of growth-suppressing transcription factors.

**Future Directions**

The KLF family of transcription factors play a role in neurite growth and regeneration in the CNS, yet how these proteins function in neurons is still unclear. Very little is known about the downstream targets of these factors in neurons, and as discussed above, this list of targets may be different in different cell types. Thus, one first step to better understand how these transcription factors affect neurite growth is to identify their downstream gene targets in CNS neurons. Revealing similar targets between growth-suppressors and separately between growth-enhancers may identify mechanisms of neurite growth regulation. Additionally, studying how these KLFs regulate and interact with each other in neurons will greatly contribute not only to the greater field of KLF function, but also to understanding their neuron-specific effects. Further characterization and analysis of the relevant co-factors and their regulation also may be necessary for understanding the mechanisms behind KLF effects. As many of these KLFs can recruit
chromatin remodeling machinery, it will be important to determine the actual state of the chromatin at these different developmental ages, as KLFs may function to create more stable changes in gene expression. Finally, it will be interesting to more fully test the hypothesis that growth-suppressive KLFs are dominant over growth-enhancing KLFs.

Conclusions

Thus, the KLF family of transcription factors may be involved in the intrinsic loss of axon growth ability in RGCs. Specifically, KLF4 expression increases around the time that RGCs lose their intrinsic axon growth ability. Overexpression of KLF4 decreases neurite growth, and its deletion increases axon growth in vitro and regeneration in vivo after injury. Importantly, we have also shown that a number of KLF family members affect neurite outgrowth, with some acting to enhance neurite growth whereas others suppress neurite growth. The expression of many of these other KLFs is also developmentally regulated, further suggesting that this family could be involved in the intrinsic loss of axon growth ability in neurons. Combined overexpression of growth-suppressive KLFs and growth-enhancing KLFs suggests that the phenotypic effect of growth suppressive KLFs is dominant. In most cases, the expression of growth suppressive KLFs increases postnatally, while the expression of the growth enhancers decreases developmentally, again supporting a role for these family members in the decrease in regenerative potential of CNS neurons seen during development.

Very little is known about the KLF family in neurons. As other studies suggest that their function may be cell-type specific, there is a need for specific knowledge about
their function in neurons, and perhaps even in specific neuronal types. In other systems, KLFs have been shown to function in a “network,” such that the individual KLF contribution is only a part of the large interaction between family members which contributes to the phenotypic outcome. Any alteration in one KLF’s contribution could affect the final outcome. Therefore, it is clear that these unique functions in the KLF family will require a new way of analyzing and studying transcription factors in neurons, moving from an individual protein’s contribution to a more complete understanding of its place within the family interactions. Finally, our findings suggest that the long-held hypothesis that increasing the presence of growth-enhancing proteins can override the developmental loss of axon regenerative ability, may not be complete. In fact, the continued presence of growth-suppressive proteins may keep the brakes on the regenerative response, limiting functional recovery. There is still much to be determined to support this new hypothesis, but these contributions may lead to the ability to reverse the poor regenerative ability of adult mammalian CNS neurons to an embryonic growth potential, ultimately leading to therapeutic treatments for all types of CNS injuries.
MATERIALS AND METHODS

Where indicated, “Methods” contributions were made by Murray Blackmore (MGB), Ying Hu (YH), or William Feuer (WF).

Constructs for transfection

For the screen in hippocampal neurons, constructs in pEXPRESS-1, pSPORT or pCMV-SPORT6 (Open Biosystems) were co-transfected with pMAX (EGFP, Amaxa), and compared to an empty vector/pMAX co-transfection. The purchased constructs for the screen were full-length rat cDNAs (19/111, 17%), or else mouse (73%) or human (10%) when the full-length rat cDNA was unavailable.

Flag-tagged KLF4 constructs in a CS2+ vector were a generous gift of Chunming Liu (Univ of Texas). The flag control vector was purchased from Genecopoeia. Mouse KLF4 (Open Biosystems) and mCherry (gift of Roger Tsien, UCSD) were cloned into the pIRES2-eGFP vector (Clontech).

KLFs -1, -4, -5, -6, -7, -10, -12, -15, and -17 were obtained from Open Biosystems. KLF2 was a kind gift from Jerry Lingrel, Univ. of Cincinnati. The open reading frame of KLF9 was cloned from postnatal rat cortex, and KLFs -3, -8, -11, -13, -14, and -16 were cloned from mouse spleen or testis. All 17 KLFs were cloned into the CMV-pSPORT6 expression vector. pMAX (eGFP, Amaxa), or mCherry-pCMV-Sport6 were used as reporters in co-transfection experiments in cortical neurons. [MGB]

For combinatorial experiments, the EGFP coding region of pIRES2-EGFP was replaced with mCherry. KLFs 4, -6, -7, and -9, and truncated versions that lacked the C-
terminus zinc finger domain but maintained the adjacent NLS were cloned into both the IRES-EGFP and IRES-mCherry plasmids. [MGB]

**Culture and transfection of primary neurons**

*Hippocampal neurons* - Embryonic day 18 (E18) rat hippocampi (Brainbits, LLC) were placed into Hibernate E media (Brainbits, LLC) containing 0.25% trypsin and 0.008% DNAse for 15 min at 37 deg C, washed 5 times with Hibernate E containing B27 (1:50), and triturated with variable sized fire-polished pipettes. Cells (500,000/tube) were pelleted (5 min, 80g), resuspended in Rat Neuron Nucleofector solution (Amaxa) containing 3.5 µg DNA, and electroporated (Amaxa, program G-13). Immediately following transfection, 500µl of growth media (see below) were added to transfected cells. Cells were plated onto PDL- and laminin-coated plates. A full media change was performed 4 hours following transfection. Transfection efficiencies were typically ~60%, and co-transfection efficiencies using 3 µg “gene-of-interest” DNA/0.5 µg reporter DNA were typically ~99%.

*RGCs – Lipofectamine transfection* - 400,000 embryonic RGCs purified by immunopanning (Barres et al., 1988; Meyer-Franke et al., 1995), were incubated with 2 µl Lipofectamine 2000 (Invitrogen) and 0.8 µg DNA for 15 min at 37 deg C, and then plated on PDL- and laminin-coated plates in RGC media (see below Meyer-Franke et al., 1995). A full media change was performed 4 hours following transfection. Transfection efficiencies were typically ~2%.
**Electroporation** – 100,000 postnatal RGCs were purified by immunopanning. Final cell pellets were resuspended in an electroporation solution containing 2µg of total DNA (GFP reporter and gene of interest), placed in a small cell number cuvette (Amaxa) and electroporated using Amaxa program SCN#1. Immediately following electroporation, growth media was added to the mixture and the whole solution placed into a small Eppendorf tube. RGCs were centrifuged for 16 minutes at 1800 rpm prior to resuspension and plating.

**Lentiviral infection** – E20 RGCs were purified by immunopanning and plated in 6 well plates at a density of 4800 cells/well in RGC growth media. Lentivirus was diluted into media, applied to neurons with a final dilution of 1:550, and incubated for 5 ½ hours before a full media change. RGCs were cultured for 4 days prior to immunostaining and further analysis. Transduction efficiency was greater than 70%.

**Cortical neurons** - Frontal cortex from P5 rats was dissociated sequentially in papain and trypsin. Dissociated cells were co-transfected with plasmid DNA-encoding KLFs and mCherry reporter at a 1:6 ratio, using electroporation in a 96-well format (Buchser et al., 2006). Cells were plated in PDL- and laminin-coated 96-well plates in growth media conditioned overnight by astroglial cultures (De Hoop et al., 1998). Transfection efficiencies were typically ~20%, and co-transfection efficiencies were typically >90%. For experiments combining KLFs, 2µg of KLF-IRES-mCherry and 2µg of KLF-IRES-EGFP plasmid were co-transfected. Only neurons that expressed both mCherry and EGFP were included in the analysis of neurite lengths. [MGB]
**Growth media** - The culture media for RGCs, hippocampal and cortical neurons was modified from (Meyer-Franke et al., 1995), and included Neurobasal, penicillin/streptomycin, insulin (5µg/ml), sodium pyruvate (1mM), transferrin (100µg/ml), BSA (100µg/ml), progesterone (60ng/ml), putrescine (16µg/ml), sodium selenite (40ng/ml), triiodo-thyronine (T3, 1ng/ml), L-glutamine (1mM), N-acetyl cysteine (NAC, 5µg/ml), forskolin (5mM) and B27 (Chen et al., 2008). Media for RGCs and hippocampal neurons also contained BDNF (50ng/ml) and CNTF (10ng/ml); media for embryonic RGCs also contained GDNF (40ng/ml); media for electroporated RGCs also contained both GDNF and bFGF (10ng/ml).

**Immunostaining**

For cultured neurons, cultures were fixed using pre-warmed (37 deg C) 4% paraformaldehyde (PFA). Following rinses in PBS, cultures were blocked and permeabilized in 20% normal goat serum (NGS)/0.02% triton X-100 in antibody buffer (150mM NaCl, 50mM Tris base, 1% BSA, 100mM L-Lysine, 0.04% Na azide, pH 7.4) for 30 min to reduce non-specific binding. Cultures were incubated overnight at 4 deg C in antibody buffer containing primary antibodies, washed with PBS, incubated in antibody buffer containing secondary antibodies and DAPI for 4 hours at room temperature, washed with PBS, and left in PBS for imaging.

For whole-mount staining, retinas from PFA-perfused animals were immunostained as above with the following modifications: all incubations were performed on a rocker, and the secondary antibody incubation was performed overnight.
at 4 deg C. Retinas were mounted in mounting medium with DAPI (Vectashield) on coverslips for confocal imaging.

Primary antibodies used for these experiments included anti-Tau (1:200, Sigma, T6402) anti-FLAG (1:750, F1804, Sigma), anti-GFP (1:600, Aves Labs, GFP-1020), anti-MAP2 (1:10,000, Abcam, ab5392; 1:150, Sigma, M1406), anti-Turbo GFP (1:10,000, Evrogen, AB513), and anti-beta-III-tubulin (Tuj1, 1:400, Covance, MMS-435P; 1:500, Sigma, T3952). Secondary antibodies were Alexa Fluor-488, -594, or -647-conjugated, highly cross-adsorbed antibodies (Invitrogen).

**Quantification of neurite length**

For “High Content Analysis” (also called High Content Screening, or HCS) of neuronal morphology, including neurite length, dendrite length, neurite number and neurite branching, automated microscopes (Cellomics KSR or VTI) and image analysis software (Cellomics BioApplications) were used to image and trace neurons using a 5x or 10x objective following immunostaining. Cortical neurons were traced using βIII tubulin immunoreactivity to visualize neurites. RGCs were traced using antibody-amplified EGFP signal, which filled transfected neurites. In the case of RGCs, neurons with dim EGFP label in neurites were excluded from analysis, due to frequent tracing errors of faint processes; the threshold for exclusion was established using a population of control neurons. Images and tracing were spot-checked to verify that the algorithms were correctly identifying neurites and quantifying growth. [MGB]
For those experiments requiring hand tracing, including confirmations of automated quantification, surviving neurons were identified by nuclear morphology and DAPI intensity and imaged in multiple fluorescent channels using a Zeiss Axiovert 200M microscope. Hand tracing was performed using Axiovision software. MAP2+ neurites, which typically demonstrated thicker origins and tapering widths, were measured as dendrites; Tau+/MAP2- neurites, which typically demonstrated thinner, non-tapering profiles, were measured as axons.

**Quantification of neuronal survival**

Survival of neurons was determined using either an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983), identification of dead nuclei by the Cellomics software (see below), or Sytox staining. MTT (.5 mg/ml) was applied to at least 3 wells per condition and incubated at 37 deg C for 30 min. Surviving neurons produced a blue precipitate; dead neurons remained colorless. At least 3 wells per condition and multiple fields of view in identical well locations were counted for each sample using a grid overlay.

To determine survival using Cellomics HCS assays, DAPI nuclear staining morphology was used. Dead cells had a higher DAPI intensity per pixel and smaller nuclei; surviving cells had low DAPI fluorescence intensity per pixel and a larger nucleus. Multiple fields of view per well were counted for each sample, with a typical replicate being 6 wells within an experiment.
Cortical neuron survival after transfection was measured by simultaneous Hoechst and Sytox orange dye staining at 1 or 3 days after plating. Hoechst+/Sytox– (surviving) cells were quantified with the Cellomics KSR, with a minimum of 500 cells counted per treatment. [MGB]

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

RGCs were purified by immunopanning as above and the pellet from the final centrifugation (before any cell culture) was snap frozen in liquid nitrogen. In most cases, multiple pellets (preps) were combined for each sample. RNA was purified (RNeasy, Invitrogen), subjected to reverse transcription (RT, iScript, Bio-Rad), and the resulting cDNA was used as the template for a quantitative-PCR reaction (Sybr green, Bio-Rad) performed on an iCycler (Bio-Rad) with KLF-X and 18S primers. In most tests, 6 repeat wells (technical replicates) were used for each condition. “No RT” control samples were also tested. To determine fold change, an efficiency analysis was performed for each tissue type in combination with the specific primers being tested. Dilutions of a sample were made for 1:10, 1:100, and 1:1000, and the threshold counts graphed as a line, with the slope being used for the efficiency formula (Pfaffl, 2001). Each experiment was performed 2-3 times with different pools of RNA (biological replicates).

For non-quantitative RT-PCR, RGC RNA was purified and reverse-transcribed as described above. 1 µl of cDNA was used as a template for each PCR reaction (Phusion, NEB). 5 µl of this product was run on a 2% agarose gel containing Gel Red (Biotium, Hayward CA), and visualized using Gene Genius gel documentation system (Syngene,
Frederick, MD). The experiment was repeated to confirm initial band expression using RNA from a separate set of animals (biological replicates).

Primers used for genotyping N-Tg(Thy1-cre)1Vln/J mice (Jackson Laboratories) and Gt(Rosa)26Sor<sup>tm1(eYFP-Cos)</sup> mice (Jackson Laboratories), were according to the Jackson Laboratories recommendations (available on their website), as follows:

- Cre – oIMR0042 ctaggccacagaatggaagatct,
oIMR0043 gtaggtggaattctagcatcc,
oIMR1084 gcggtctggcagtaaaaactatc,
oIMR1085 gtaacagcattgtgctactt;
- Rosa – oIMR0316 ggagcgggagaaatggatat,
oIMR0883 aaagtcgctctgagttat,
oIMR0883 aagtctctctgagtgttat,
oIMR4982 aagaccggaagagtctgc.

fKLF4 mice were genotyped as described (Katz et al., 2002). The rd mutation was assayed through genotyping as described (Gimenez and Montoliu, 2001). For qRT-PCR, primers for KLF4 were as described (Suzuki et al., 2006); for rat KLF6 were forward: gagttccteggtcatttca, reverse: tgetttcaagttggagc; for rat KLF7 were forward: tgetctctctggagaagtt, reverse: gagctgagggagctt; for rat KLF9 were forward aacaatatccgacccatcc, reverse: agaactttccacagccttg; for rat Cyclin D1 were forward gtgacccggactgcctcgtt, reverse: agcctcccggggttgcaag; for rat p21 were forward: ccacgtggccctctgtcctgt, reverse: agcctccggtgggagcactt. For RT-PCR, primers for KLF1-KLF13 and KLF15-17 were as described (Eaton et al., 2008) and for KLF14 were as described (Scohy et al., 2000).
**Animals**

All use of animals conformed to the ARVO Statement for the Use of Animals in Research, and was approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the University of Miami.

Sprague-Dawley rats of varying ages were obtained from Harlan Laboratories.

Mice were bred from the following strains: floxed KLF4 (fKLF4) mice (Katz et al., 2002), B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J (Stock #006148, Jackson Laboratory), and FVB/N-Tg(Thy1-cre)1Vln/J (Stock#006143, Jackson Laboratory). The Thy1-cre background strain, FVB, was homozygous for retinal degeneration (rd) mutations, and this mutation was bred out using C57BL/6J as detected through genotyping (Gimenez and Montoliu, 2001). Once the rd mutation was bred out, we spot checked the rd genotype to confirm periodically that the mutation was absent.

**Intraorbital Optic Nerve Crush and Intravitreal Injection**

For all in vivo experiments, optic nerve crush, tissue processing, imaging and analysis were performed masked, such that the experimenters did not know the genotype of the animal at any stage until the analysis was complete. In separate experiments looking at shorter term post-crush survivals, we saw no spared axons greater than 0.2 mm beyond the crush site (Y. Hu, A. Peterson, J. Bixby and J. Goldberg, data not shown). In this manuscript, any axon sparing would be expected to be distributed randomly between groups, due to the masked design.
8-12 week old Thy1-cre+/KLF4+/+, Thy1-cre+/KLF4fl/+, Thy1-cre+/KLF4fl/fl littermate mice were used for optic nerve crush experiments. Following induction of anesthesia, the left intraorbital optic nerve was surgically exposed, the dural sheath was opened longitudinally, and the nerve was crushed 1 mm behind the eye with angled jeweler’s forceps (Dumont # 5) for 10 sec, avoiding injury to the ophthalmic artery. Nerve injury was verified visually at the crush site, while the vascular integrity of the retina was evaluated by fundoscopic examination. Mice with any significant postoperative complications (e.g., retinal ischemia, cataract) were excluded from further analysis. For anterograde axon labeling, intravitreal injections of 1 µl cholera toxin subunit B (CtB594, 10µg/µl; Molecular Probes) were performed just posterior to the pars plana with pulled glass pipette connected to a 50 µl Hamilton syringe. Care was taken not to damage the lens. One day later, at 2 weeks after the crush injury, mice were deeply anaesthetized and perfused with 4% PFA in 0.1 M phosphate buffer. Optic nerves and retinas were dissected and post-fixed in 4% PFA for one hour and subsequently washed in PBS. Optic nerves were incubated in 30% sucrose at 4 degrees overnight prior to mounting in OCT. Longitudinal sections (16 µm) were made of the entire optic nerve. All sections with an apparent crush site and CtB labelling were imaged with a 20x objective. Pictures were taken, starting with the furthest regenerating axons and working backwards toward the crush site. Lines were drawn perpendicular to the long axis of the optic nerve 0.2, 0.3, 0.5, 0.75, 1, and 1.5 past the crush site (as applicable), and CtB+ axons between these lines were counted. Analysis of the total sum of regenerating fibers
from all sections for each animal were performed as well as the average number of axons at each measurement location/distance per number of sections. Using either analysis, the data yielded the same results.

Statistical analysis. Distance and fiber-sum data were log transformed to effect linearity (a basic assumption of the statistical tests used) and approximate normality of residuals. As some fiber-sum measurements were zero, a small positive constant, 0.2, was added to all fiber-sum measurements prior to taking the logarithm. An analysis of covariance, with a mixed model component to account for multiple measurements in the same animals, was used to compare the distance relationship of fiber-sums by genotype between the groups. A second analysis in which zero values were excluded reached similar conclusions. [WF]

Retinal Survival Quantification

Eyes were dissected from PFA-perfused animals and left in 4% PFA for an additional hour. Retinas were then dissected into PBS to await immunostaining. Whole-mount immunostaining was performed (see above) using anti-beta-III tubulin (Tuj1) to visualize RGCs, and DAPI to detect nuclei. Retinas were mounted onto coverslips in mounting medium (Vectashield) and imaged on a Leica confocal microscope. Using a 40x oil objective, 4 stacked images were taken 2 fields of view from the optic disc in each perpendicular direction. RGCs were quantified by an observer masked to genotype using Metamorph software.
References


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