Small-molecule modulation of 14-3-3 adaptor proteins for axon regeneration

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1 Forward

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1.3 List of publications

[1] **Kaplan A#**, Ottmann C, and Fournier AE (2017). 14-3-3 adaptor protein-protein interactions as therapeutic targets for CNS diseases. *Pharmacological Research.* 2017 Nov;125:114-121. #corresponding author

[2] **Kaplan A#** and Fournier AE (2017). Targeting 14-3-3 adaptor proteins to stimulate CNS repair. *Neural Regeneration Research*. 12(7): 1040-1043. #corresponding author

[3] **Kaplan A#**, Bueno M, Fournier AE (2017). Extracellular functions of 14-3-3 adaptor proteins. *Cell Signaling*. 2017 Feb;31:26-30. #corresponding author

[4] **Kaplan A**, Bueno M, Hua L, Fournier AE (2017). Maximizing functional axon repair in the injured CNS: lessons from neuronal development. *Developmental Dynamics*. 2018 Jan;247(1):18-23.

[5] **Kaplan A**, Morquette B, Kroner A, Leong S, Madwar C, Sanz R, Banerjee SL, Antel J, Bisson N, David S, Fournier AE (2017). Small-Molecule Stabilization of 14-3-3 Protein-Protein Interactions Stimulates Axon Regeneration. *Neuron*. 2017 Mar 8;93(5):1082-1093.e5.
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[6] DeGeer J, **Kaplan A**, Mattar P, Morabito M, Stochaj U, Kennedy TE, Debant A, Cayouette M, Fournier AE, Lamarche-Vane N (2015). Hsc70 chaperone activity underlies Trio GEF function in axon growth and guidance induced by netrin-1. *Journal of Cell Biology*.

[7] Leong SY, **Kaplan A**, Wang LC, Almazan G, Fournier AE, Antel J (2015). Properties of human central nervous system neurons in a glia-depleted (isolated) culture system. *Journal of Neuroscience Methods*.

[8] **Kaplan A**, Ong Tone S, Fournier AE (2015). Extrinsic and intrinsic regulation of axon regeneration at a crossroads. *Frontiers in Molecular Neuroscience*.

[9] **Kaplan A** and Sanz RL (2014). Axotomy-induced ganglioside processing: a mediator of axon regeneration restricted to the PNS. *Journal of Neuroscience*. 2014 Jun 25;34(26):8659-61.

[10] **Kaplan A***, Sanz R*, Ferraro G*, Alchini R*, Fournier AE (2014) Neurite outgrowth and growth cone collapse assays to assess neuronal responses to extracellular cues. *Methods in Molecular Biology – Axon growth and regeneration: Methods and protocols*. 2014;1162:43-56. *equal contribution

[11] **Kaplan, A***, Kent CB*, Charron F, Fournier AE (2014). Switching Responses: Spatial and Temporal Regulators of Axon Guidance. *Molecular Neurobiology*. 2014 49(2):1077-86. *equal contribution

[12] Northey JJ, Dong Z, Ngan E, **Kaplan A**, Hardy WR, Pawson T, Siegel PM (2013). Distinct phosphotyrosine-dependent functions of the ShcA adaptor protein are required for transforming growth factor beta (TGFbeta)-induced breast cancer cell migration, invasion, and metastasis. *Journal of Biological Chemistry*. 288, 5210-22.

1.4 Author contributions

This thesis is presented as a manuscript-based thesis with three data chapters:

Chapter 2 is a published manuscript: "Small molecule stabilization of 14-3-3 protein-protein interactions stimulates axon regeneration"

Andrew Kaplan: Conception and design of project, data analysis, writing and editing of manuscript. Performed all experiments and analysis for Fig. 1-6, Fig. S2-S7 and Table S1, performed analysis for Fig. 7-8.
Barbara Morquette: Performed optic nerve injury experiments for Fig. 8 and Fig. S7.
Antje Kroner: Performed spinal cord injury experiments for Fig. 7.
SooYuen Leong: Provided human fetal neurons for Fig. 3.
Carolin Madwar: Performed chemical coupling of FC-A to beads for Fig. 4.
Ricardo Sanz: Performed experiments for Fig. S1.
Sara L. Banerjee: Prepared and analyzed samples for mass spectrometry for Table S1.
Jack Antel: Provided human fetal neurons for Fig. 3.
Nicolas Bisson: Supervision and analysis of mass spectrometry experiments for Table S1. Editing of manuscript.
Samuel David: Supervision of spinal cord injury experiments for Fig. 7. Editing of manuscript.
Alyson E. Fournier: Conception and design of project, data analysis, writing and editing of manuscript. Corresponding author.

Chapter 3 is a manuscript in preparation: "A novel fusicoccin-A derivative stimulates neurite outgrowth and axon regeneration"

Andrew Kaplan: Conception and design of project, data analysis, writing and editing of manuscript. Performed experiments and analysis for all figures.
Yusuke Higuchi: Synthesized FC-A derivatives for all experiments.
Anna S. van Regteren Altena: Performed experiments for Fig. 7.
Nobuo Kato: Provided FC-A derivatives for all experiments.
Alyson E. Fournier: Conception and design of project, data analysis, writing and editing of manuscript. Corresponding author.

Chapter 4 is a manuscript in preparation: “Exploring the role of GCN1 in axon regeneration”

Andrew Kaplan: Conception and design of project, data analysis, writing and editing of manuscript. Performed experiments and analysis for all figures.
Sara L. Banerjee: Prepared and analyzed mass spectrometry samples.
Evelyn Sattlegger: Provided GCN1 floxed mice.
Alan Hinnebusch: Provided GCN1 floxed mice.
Beatriz Castilho: Provided GCN1 floxed mice.
Nicolas Bisson: Supervision and analysis of mass spectrometry experiments.
Alyson E. Fournier: Conception and design of project, data analysis, writing and editing of manuscript. Corresponding author.

1.5 Abbreviations

AANAT	Arylkylamine N-acetyltransferase
AAV	Adeno-associated virus
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
Armcx1	Armadillo repeat containing, X-Linked 1
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
BDA	Biotinylated dextran amine
bpV	Bisperoxovanadium
Brn3a	Brain-specific homeobox/POU domain protein 3A
BV02	14-3-3 Antagonist II
C3	Clostridium botulinum C3 exoenzyme
cAMP	Cyclic adenosine monophosphate
chABC	Chondroitinase ABC
CHX	Cycloheximide
CN-A	Cotylenin-A
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CSPG	Chondroitin sulfate proteoglycan
CST	Corticospinal tract
Da	Dalton
Difopein	Dimeric fourteen-three-three peptide inhibitor
DIV	Days in vitro
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglion
EC50	Half maximal effective concentration
eIF2 alpha	Eukaryotic translation initiation factor 2 subunit alpha
FC-A	Fusicoccin-A
FC-THF	Fusicoccin-tetrahydrofuran
GAP43	Growth associated protein 43
GCN1	General control nonderepressible 1
GCN2	General control nonderepressible 2

GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HEAT	Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and TOR1
HEK	Human embryonic kidney
IMPACT	Imprinted and ancient gene protein homolog
IP	Intraperitoneal
ISP	Intracellular sigma peptide
JAK	Janus kinase
LAR	Leukocyte common antigen related
LC-MS/MS	Liquid chromatography–mass spectrometry
LINGO-1	Leucine rich repeat and immunoglobulin-like domain-containing protein 1
LRR	Leucine-rich repeat
LRRK2	Leucine-rich repeat kinase 2
MAG	Myelin-associated glycoprotein
MAI	Myelin-associated inhibitor
MDS	Miller-Dieker Syndrome
MOI	Multiplicity of infection
mTOR	Mechanistic target of rapamycin
NEP1-40	Nogo-66(1–40) antagonist peptide
NG2	Neural/glial antigen 2
NgR	Nogo receptor
OMgp	Oligodendrocyte myelin glycoprotein
ONC	Optic nerve crush
p75NTR	Low affinity neurotrophin receptor
PD	Parkinson's Disease
PDE4	Phosphodiesterase 4
PI3K	Phosphoinositide 3-kinase
PirB	Paired-immunoglobulin-like receptor B
PKA	Protein kinase A
PPI	Protein-protein interaction
PTEN	Phosphatase and tensin homolog

PTP sigma	Protein tyrosine phosphatase sigma
R18	14-3-3 peptide inhibitor
RAG	Regeneration associated gene
RGC	Retinal ganglion cell
ROCK	Rho-associated, coiled-coil-containing protein kinase
SAINT	Significance analysis of interactome
SAR	Structure activity relationship
SCI	Spinal cord injury
SEM	Standard error of the mean
SOCS3	Suppressor of cytokine signaling 3
SOD1	Superoxide dismutase
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
TROY	Tumor necrosis factor receptor superfamily, member 19
TUNEL	Terminal deoxynucleotidyl transferase dUTP nicked end labeling
YFP	Yellow fluorescent protein

1.6 Abstract

Axons are cellular projections that link neurons into functional circuits over long distances. Axonal tracts in the spinal cord transmit motor and sensory information between the brain and the body. Spinal cord trauma causes axonal damage and disrupts this communication. Damaged axons in the central nervous system (CNS) fail to spontaneously regenerate and re-connect with their targets. This fundamental problem is the direct underlying basis of permanent paralysis and sensory loss in spinal cord injury (SCI) patients. Drugs that can induce axon regeneration could be effective in restoring neurological function after SCI, nerve injuries, and in certain neurodegenerative conditions. We have discovered that a family of proteins in neurons called 14-3-3s can be targeted with small molecule drugs to induce axon regeneration. 14-3-3s are ubiquitously expressed adaptor proteins that regulate hundreds of functionally diverse 'client proteins.' In humans, there are seven isoforms with highly conserved sequence, structure and function. 14-3-3s bind to client proteins in a stereotyped fashion at phosphorylated motifs via a linear binding groove. Fusicoccanes are a class of natural small molecules that stabilize protein-protein-interactions (PPIs) between 14-3-3s and a subset of their client proteins. These compounds bind to a pocket created by the interface of 14-3-3 and the client motif within the groove, forming a stable tri-partite complex. In chapter 2, we show that stabilization of 14-3-3 PPIs with fusicoccin-A (FC-A) stimulates neurite outgrowth, alleviates axonal die-back in a mouse model of SCI, and induces axon regeneration in a mouse model of optic nerve injury (Kaplan et al, Neuron, 2017). We show that FC-A stabilizes a PPI between 14-3-3 and GCN1, a regulator of protein translation, leading to GCN1 turnover and enhanced neurite outgrowth. In chapter 3, we explore structure activity relationship (SAR) of FC-A with a library of novel semi-synthetic derivatives. Using a high-content image-based screen for neurite outgrowth, we identify a series of potent derivatives. Following subsequent SAR and pharmacokinetic analyses, we select a lead compound that penetrates the blood-brain-barrier for further testing in mouse models of CNS injury. In chapter 4, we explore the role of GCN1 in axon regeneration by conditionally deleting GCN1 from neurons in GCN1 floxed mice. In this thesis, we discover and develop small molecules with a unique mechanism of action, suggesting an entirely novel approach to stimulate axon regeneration after CNS injury.

1.7 Résumé

Les axones sont des projections cellulaires qui relient les neurones dans des circuits fonctionnels sur de longues distances. Les réseaux d'axones dans la moelle épinière transmettent des informations motrices et sensorielles entre le cerveau et le corps. Traumatisme de la moelle épinière provoque des dommages axonaux et perturbe cette communication. Les axones endommagés dans le système nerveux central (SNC) ne se régénèrent pas spontanément et ne se reconnectent pas à leurs cibles. Ce problème fondamental est la cause directe de la paralysie permanente et de la perte sensorielle chez les patients atteints d'une lésion de la moelle épinière. Les médicaments qui peuvent induire la régénération des axones pourraient être efficaces pour restaurer la fonction neurologique après les lésions de la moelle épinière, les lésions nerveuses et pour certaines conditions neurodégénératives. Nous avons découvert qu'une famille de protéines dans les neurones appelées 14-3-3s peuvent être ciblées avec de petites molécules pour induire la régénération des axones. Les 14-3-3s sont des protéines adaptatrices exprimées de façon omniprésente qui régulent des centaines de «protéines clientes» fonctionnellement diverses. Chez l'homme, il y a sept isoformes avec une séquence, une structure et une fonction hautement conservées. Les 14-3-3s se lient de manière stéréotypée aux protéines clientes par des motifs phosphorylés dans une rainure linéaire. Les fusicoccanes sont une classe de petites molécules naturelles qui stabilisent les interactions protéine-protéine (IPP) entre les 14-3-3s et un sous-ensemble de leurs protéines clientes. Ces composés se lient à une poche créée par l'interface de 14-3-3s et le motif du client dans la rainure, formant un complexe tripartite stable. Dans le chapitre 2, nous montrons que la stabilisation de 14-3-3 PPI avec la fusicoccine-A (FC-A) stimule la croissance des neurites, allège le dépérissement axonal dans un modèle murin de lésion de la moelle épinière, et induit la régénération des axones dans un modèle de lésion du nerf optique chez la souris (Kaplan et al, Neuron, 2017). Nous montrons que FC-A stabilise une IPP entre 14-3-3 et GCN1, un régulateur de la traduction des protéines, conduisant à la baisse du GCN1 et une croissance accrue des neurites. Dans le chapitre 3, nous explorons la relation structure-activité de FC-A avec une collection de nouveaux dérivés semi-synthétiques. En utilisant un test basé sur des images à haute teneur pour l'excroissance des neurites, nous identifions une série de dérivés actifs. Suite à des analyses additionnelles de structure-activité et pharmacocinétiques, nous sélectionnons un composé primaire qui pénètre dans la barrière hémato-encéphalique pour des tests supplémentaires dans des modèles murins de lésion du SNC. Dans le chapitre 4, nous explorons le rôle de GCN1 dans la

régénération des axones en supprimant conditionnellement GCN1 dans les neurones de souris avec GCN1 « floxé ». Dans cette thèse, nous découvrons et développons de petites molécules avec un mécanisme d'action unique qui suggère une approche entièrement nouvelle pour stimuler la régénération des axones après une lésion du SNC.

1.8 Preface to introduction

In the introduction, we provide an overview of the core literature and historical context of the work presented in this thesis. We first describe the fundamental problem posed by the failure of damaged axons to regenerate after injury in the CNS. We then review recent work, which has implicated both extrinsic and neuron-intrinsic factors that contribute to this problem. We introduce the 14-3-3 family of adaptor proteins as key regulators of neuronal biology. We go on to describe historical and recent work, which has shown how 14-3-3 proteins can be targeted with small molecule drugs. We suggest that these pharmacological approaches could have important applications in CNS injury.

Introduction

2 Chapter 1. Introduction

This chapter contains text modified from three publications:

14-3-3 adaptor protein-protein interactions as therapeutic targets for CNS diseases
Andrew Kaplan, Christian Ottmann, Alyson E. Fournier. *Pharmacological Research*, 2017; 125B:114-121.

Maximizing functional axon repair in the injured CNS: lessons from neuronal development
Andrew Kaplan, Mardja Bueno, Luyang Hua, Alyson E. Fournier. *Developmental Dynamics.* 2018; 247(1):18-23.

Extrinsic and intrinsic regulation of axon regeneration at a crossroads
Andrew Kaplan, Stephan Ong Tone, Alyson E. Fournier. *Frontiers in Molecular Neuroscience*, 2015; 8:27.

2.1 General introduction

Our ability to experience and interact with our environment is made possible by trillions of connections between billions of neurons in the nervous system. The central nervous system (CNS), composed of the brain and spinal cord, processes sensory input and generates motor output to control how we sense and respond to our surroundings. The CNS communicates with the body through the nerves of the peripheral nervous system (PNS). The spinal cord functions as the central relay of motor and sensory information between the CNS and PNS. Neurons must often communicate with each other over long distances. This is achieved by axons, 'wire-like' projections that grow out from the neuronal cell body to form these circuits during nervous system development. Axonal outgrowth is directed by the growth cone, a highly motile tip at the growing end of the axon. Once growth cones reach their destinations, they form synapses, the intercellular junctions that transmit chemical signals between neurons. This is ensued by a fundamental, but poorly understood, transformation in the biology of the neuron, which must now stop growing its axon and instead function as a receiving and transmitting unit within a fixed circuit. After injury in the mature PNS, axons spontaneously re-form growth cones at the damaged tip and regenerate. Although slow and imperfect, this can lead to partial or full recovery of motor and sensory function. In sharp contrast, axons in the CNS do not regenerate after injury. These fundamental observations were first documented by the neuroscientist Santiago Ramón y Cajal over 100 years ago. Once a CNS axon has been severed, the damaged tip becomes what Cajal termed a 'sterile end-bulb,' a bulbous inanimate swelling at the end of the axon(Ramón y Cajal et al., 1991). The failure of CNS axons to regenerate is the basis of permanent paralysis and sensory loss in spinal cord injury (SCI) patients, making this a key target for therapies aimed at restoring neurological function. Here we provide an overview of our current knowledge surrounding axon regeneration in the CNS and potential therapeutic approaches to address this unmet need.

In many of the studies discussed herein, the effect of genetic and/or pharmacological perturbation of a candidate molecule is first tested in cultured neurons, where neurite outgrowth serves as a common readout. Rodent models of SCI are then used to assess outcomes in a more translational paradigm, where axonal tract tracing and motor behavior serve as readouts. The corticospinal tract (CST), composed of bundles of axons from cortical neurons that synapse onto motor neurons in the spinal cord, is often lesioned and examined for regeneration in these models because of its central role in

controlling voluntary motor behavior. Rodent models of optic nerve injury are also commonly used in this field. The optic nerve is part of the CNS and is composed of axons from retinal ganglion cells (RGCs), which convey visual information from the retina to the brain. RGC axons also fail to regenerate after injury. These models all provide critical insight into the cellular and molecular biology of axon regeneration after CNS injury and serve as important tools for preclinical development of candidate therapeutics.

2.2 Extrinsic regulation of axon regeneration

“ A pioneering study conducted by David and Aguayo suggested that differences in the microenvironments of the CNS and PNS underlie the disparity in axon regeneration after injury(David and Aguayo, 1981). They hypothesized that providing CNS axons with a PNS milieu might permit CNS axon regeneration. To test this idea, peripheral sciatic nerve sheaths were grafted onto the injured spinal cord in adult rats. The spinal cord axons were then labeled with a tracer, revealing extensive long-distance regenerative growth into the peripheral nerve graft. This provided evidence that CNS axons possess the intrinsic ability to regenerate, but are prevented from doing so by factors within the CNS microenvironment. This led to the hypothesis that the failure of axons to regenerate is attributed to the presence of inhibitory factors and/or the absence of permissive factors in the CNS(David and Aguayo, 1981; Yiu and He, 2006). It is now known that CNS glia express specific molecules that are inhibitory to axonal growth. Oligodendrocyte proteins termed myelin-associated inhibitors (MAIs) include Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp). These molecules possess growth cone collapsing activity and inhibit axon growth. Chondroitin sulfate proteoglycans (CSPGs) expressed by reactive astrocytes also hamper axon regeneration through sites of CNS injury (Yiu and He, 2006). Targeting these extrinsic inhibitory factors has led to improvements in axon regeneration and functional recovery after CNS injury (see Fig. 1 for summary schematic).

2.2.1 Myelin-associated inhibitors

The identification of the inhibitory ligands and neuronal receptors that mediate myelin-dependent inhibition of regeneration has facilitated the development of selective antagonists to neutralize the inhibitory effect of myelin proteins. MAIs in CNS myelin bind to either paired immunoglobulin repeat B (PirB) (Atwal et al., 2008) or Nogo receptor (NgR), which forms a complex with LINGO-1 (LRR and Ig containing Nogo Receptor

interacting protein) and p75NTR (p75 neurotrophin receptor) or TROY (tumor necrosis factor receptor superfamily member 19)(Yiu and He, 2006). One strategy to target these interactions is with peptide mimics of ligands to compete for receptor binding. An example is the Nogo extracellular peptide (NEP1-40), a competitive peptide that blocks the action of MAIs (GrandPre et al., 2002). NEP1-40 was shown to abrogate Nogo-dependent neurite outgrowth inhibition and promote corticospinal tract regeneration(GrandPre et al., 2002; Li and Strittmatter, 2003). Vaccines(Huang et al., 1999) and monoclonal antibodies(Zorner and Schwab, 2010) have also been developed to neutralize MAIs. However, deletion of NgR or Nogo, MAG and OMgp in mice has had more variable effects on regeneration and recovery (Cafferty et al., 2010; Lee et al., 2009; Lee et al., 2010; Zheng et al., 2005; Zheng et al., 2003). Deletion of NgR stimulates collateral sprouting of intact CST axons after a unilateral injury at the level of the medullary pyramids(Cafferty and Strittmatter, 2006). Furthermore, a soluble NgR decoy receptor, NgR(310)ecto-Fc that neutralizes Nogo, MAG and OMgp, shows promise in promoting axonal sprouting and functional recovery in acute and chronic SCI in rodents(Ji et al., 2005; Li et al., 2005; Wang et al., 2011).

2.2.2 The astrocytic scar

The astrocytic response to CNS injury is also thought to impair axon regeneration. After injury, astrocytes upregulate CSPGs, which inhibit axonal growth(Yiu and He, 2006). The inhibitory action of CSPGs has been attributed to the negatively charged glycosaminoglycan (GAG) chains that decorate the protein core. Chondroitinase ABC (chABC), a bacterial enzyme that digests the GAG chains, abrogates CSPG-dependent neurite outgrowth inhibition *in vitro* and improves functional recovery after SCI (Alilain et al., 2011; Garcia-Alias et al., 2011; Lee et al., 2012). The GAG chains have been thought to electrostatically repel growth cones, however recent studies suggest that CSPGs may in fact tightly adhere to and confine growth cones (Filous et al., 2014; Lang et al., 2015). After SCI, axons closely associate with NG2, a transmembrane CSPG expressed by a subset of oligodendrocyte progenitor cells. These sites of apposition stain positive for pre-synaptic markers, suggesting the formation of ‘synapse-like’ contacts between axons and NG2+ cells(Filous et al., 2014). Furthermore, DRG axons preferentially adhere to CSGP substrates in *in vitro* stripe assays. Silver and colleagues propose a model whereby CSPGs capture and arrest regenerating growth cones after injury instead of repelling them (Filous et al., 2014). In support of this view, a recent report shows that the astrocytic scar facilitates axonal growth proximal to the lesion. Multiple methods were employed to ablate the scar, all of which worsened axonal die-back after injury.

Moreover, ablation of the scar impaired axon regeneration induced by neurotrophins(Anderson et al., 2016). These studies suggest that axonal growth after injury may be quite sensitive to the adhesive nature of the cells and substrates of the scar tissue. Too much adhesion may impair growth cone formation and too little can prevent sufficient engagement with the matrix to support regenerative growth.

In addition to the physical interactions between axons and CSPGs, multiple receptors including protein tyrosine phosphatase sigma (PTPsigma), leukocyte common antigen related phosphatase (LAR) and NgR have been shown to functionally interact with and mediate CSPG-dependent inhibition of growth(Dickendesher et al., 2012; Fisher et al., 2011; Shen et al., 2009). Pharmacological targeting of these receptors is another strategy that might improve axon regeneration after injury. Systemic administration of cell-penetrating peptide mimetics of the extracellular and intracellular regions of LAR have been shown to enhance serotonergic axon sprouting and functional recovery after SCI in mice(Fisher et al., 2011). PTPsigma can be targeted in a similar fashion with a cell–permeable peptide, termed intracellular sigma peptide (ISP), to enhance functional recovery after SCI in rats(Lang et al., 2015). In cultured DRG neurons, CSPGs maintain growth cones in a dystrophic state, reminiscent of Cajal's end-bulbs, and treatment with ISP allows for growth cone release. Daily injection of ISP following spinal cord contusion in rats resulted in delayed beneficial outcomes, particularly in resumption of bladder function(Lang et al., 2015). The idea that CSPGs entrap growth cones at the lesion suggests that these approaches may also be efficacious in chronic SCI, where it might be possible to reanimate persistent end-bulbs in the spinal cord.

2.3 Intrinsic regulation of axon regeneration

A poor intrinsic capacity for growth is also thought to be a major barrier to axon regeneration in the mature CNS. Targeting intracellular signaling molecules is a strategy that has gained traction more recently in the field. Extensive regeneration can be elicited by targeting master regulators of cellular growth(Liu et al., 2010; O'Donovan et al., 2014). These include tumor suppressors and oncogenes, transcription factors, and regulators of various post-translational modifications. Work from the He group first showed that knockout of the tumor suppressor PTEN results in extensive long-distance axon regeneration in SCI and optic nerve injury models(Park et al., 2010; Park et al., 2008; Sun et al., 2011). This provided proof of concept that switching on a growth state in CNS

neurons can enable axon regeneration, even in the presence of an inhibitory extracellular milieu. Although PTEN knockout was the first intervention to show extraordinarily robust regeneration, targeting neuron-intrinsic growth regulators was also suggested by earlier studies reviewed herein.

2.3.1 The conditioning lesion

Injury to PNS axons is followed by neuronal expression of regeneration-associated genes (RAGs), a response that is quite limited in CNS neurons(Mar et al., 2014a). The peripheral conditioning lesion is an injury model in which the peripheral axonal processes of DRG axons are lesioned prior to lesioning the central processes in the spinal cord. This stimulates the regeneration of ascending DRG axons within the spinal cord. This effect on CNS regeneration has been attributed to the engagement of a RAG expression response from the peripheral lesion(Blesch et al., 2012). One of these RAGs, c-Jun, has been shown to be critical for axon regeneration, as its deletion impairs axon regeneration and results in exacerbated cell death after peripheral nerve injury in mice(Raivich et al., 2004). The conditioning lesion also enhances the axonal transport of a multitude of signaling and cytoskeleton regulators into the central process(Mar et al., 2014b). Elevation of cAMP is a critical component of the conditioning lesion effect. Injection of cAMP into dorsal root ganglia can mimic the pro-regeneration effect of a conditioning lesion(Qiu et al., 2002). Interestingly, cAMP also regulates the sensitivity of neurons to MAIs and CSPGs(Cai et al., 2001). The Filbin group demonstrated that an age-associated decline of neuronal cAMP correlates with an acquisition of responsiveness to myelin-mediated inhibition of growth and that pharmacological elevation of cAMP renders neurons insensitive to myelin and CSPGs(Cai et al., 2001). This suggests that extrinsic inhibitors rely on the intrinsic sensitivity of the neuron to inhibit growth and suggests that targeting neuron-intrinsic signaling could also relieve the influence of the inhibitory microenvironment. Rolipram, a phosphodiesterase 4 (PDE4) inhibitor that elevates intracellular cAMP, has been shown to enhance regeneration and recovery after SCI in rodents, suggesting therapeutic approaches to harness this pathway(Costa et al., 2013; Kadoya et al., 2009; Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004).

2.3.2 RhoA signaling

The small GTPase RhoA and downstream effector Rho kinase (ROCK) are extensively studied regulators of neurite outgrowth. Inhibition of ROCK with the small molecule inhibitors Y-27632 or fasudil stimulates neurite outgrowth and improves recovery in SCI

models(Fournier et al., 2003; Sung et al., 2003). RhoA can be specifically inhibited with the *Clostridium botulinum* exoenzyme C3 transferase, which ADP-ribosylates and inactivates RhoA. Treatment of animals with C3 improves locomotor recovery in SCI models(Boato et al., 2010). C3 has a dual effect of enhancing neurite outgrowth(Dergham et al., 2002) and promoting cell survival(Dubreuil et al., 2003). Interestingly, C3 has also been shown to stimulate neurite outgrowth and induce Erk and Akt phosphorylation independent of its ability to inhibit RhoA, suggesting that its mechanism-of-action has yet to be fully elucidated(Auer et al., 2012). The positive outcomes with C3 have translated into clinical trials with a cell-permeable form of C3 that is delivered locally onto the spinal cord in a fibrin sealant during spinal decompression surgery after acute SCI. Results from one trial have shown improvements in locomotor function compared to historical statistics(McKerracher and Anderson, 2013).

2.3.3 Tumor suppressors and oncogenes

Recent studies have provided a strong rationale for targeting intracellular growth regulators to both drive axon growth and relieve sensitivity to extrinsic inhibition of growth. Initial studies explored strategies to stimulate intrinsic growth potential by genetically manipulating the expression of classical tumor suppressors or oncogenes. PTEN is a tumor suppressor that antagonizes the PI3K-Akt-mTOR pathway(Song et al., 2012). mTOR activity decreases during development in cortical neurons. Conditional knockout of PTEN and consequent increase in mTOR activity in mouse cortex promotes regeneration of CST axons(Liu et al., 2010). Another study showed that combined deletion of PTEN and SOCS3, a negative regulator of the JAK/STAT pathway, synergize to promote axon regeneration in a mouse model of optic nerve crush injury(Sun et al., 2011). A complementary strategy is to enhance the activity or expression of growth-promoting molecules. A recent study showed that expression of a point-mutated kinase activated version of the B-Raf oncogene (V600E B-Raf) results in robust regeneration of RGC axons after optic nerve injury(O'Donovan et al., 2014). Further enhancement of regeneration was observed with combined knockout of PTEN. These studies were noted for extraordinarily extensive long-distance axon regeneration that had not been observed with prior interventions. These positive outcomes have resulted in a greater focus on manipulation of intrinsic regulation of neuron growth as an approach for drug development.

2.3.4 Axonal trafficking

As the nervous system matures, there is a selective exclusion of growth molecules, including integrins and TrkB, from CNS axons(Cheah et al., 2016; Franssen et al., 2015; Hollis et al., 2009). This developmental decline in the localization of growth molecules to the axonal compartment may underlie the loss of intrinsic growth competency in the adult CNS. Enhancing the expression of these molecules or stimulating their trafficking into the axon could re-establish growth competence in damaged axons after injury. Several trafficking regulators including Arf6, Rab11, and Kif4A have been implicated in regulating the localization of integrins in axons(Eva et al., 2010; Franssen et al., 2015), which could lead to the conception of pharmacological strategies to favor axonal growth. Importantly, restoring these molecules to the axonal compartment could allow for more precise navigation and functional integration of regenerating axons. For instance, knockout of the tumor suppressor protein PTEN induces extensive long-distance axon regeneration after CNS injuries, but this does not translate into substantial functional recovery(Geoffroy et al., 2015; Park et al., 2008). This may be due haphazard growth that does not discriminate and connect with post-synaptic partners in the target tissue. Interestingly, a recent study found that the mitochondrial transport regulator Armcx1 (Armadillo Repeat Containing, X-Linked 1) is upregulated in RGCs after knockout of PTEN and SOCS3. Overexpression of Armcx1 enhanced mitochondrial transport in RGC axons and knockdown completely blocked axon regeneration induced by PTEN and SOCS3 knockout(Cartoni et al., 2016). These studies highlight the importance of the axonal trafficking machinery in ensuring sufficient support for regenerative axon growth.

2.3.5 Transcriptional regulation

Developmental changes in transcriptional programs may be a key process that underlies the low intrinsic growth capacity of CNS neurons. Profiling the expression of transcription factors during development may help identify central regulators of axon growth. For instance, the KLF (Kruppel-like factor) family of transcription factors have been shown to regulate axon growth and regeneration in the CNS. Intriguingly, the expression levels of KLFs are developmentally regulated in RGCs. KLF4 increases between the transition from embryogenesis to postnatal life and its deletion in adult mice enhances axon regeneration after optic nerve injury(Moore et al., 2009). On the other hand, KLF7 expression decreases with age and overexpression of a KLF7 chimera with a transactivation domain promotes CST regeneration after SCI(Blackmore et al., 2012). In another study, a histone acetylase called PCAF was found to be an important mediator of regeneration induced by conditioning lesions to the sciatic nerve and its

overexpression promoted the transcription of a pro-growth gene network. Turning on this gene expression switch promoted sensory axon regeneration in the spinal cord after dorsal column lesions(Puttagunta et al., 2014). Another study showed that hypoxia inducible factor 1 alpha (HIF1α) turns on multiple pro-growth genes and is critical for peripheral nerve regeneration(Cho et al., 2015). STAT3, STAT6 and c-Jun are additional transcription factors that promote axon growth after CNS injury(Lerch et al., 2014; Luo et al., 2016). Manipulation of these factors likely induces wide-ranging alterations in gene expression. This lack of selectivity may in fact underlie the robust effects elicited by these interventions, in that a coordinated network of genes may address multiple intrinsic growth deficiencies as well as sensitivities to extrinsic inhibitors in the injury microenvironment. Screening of transcription factors for axon growth activity may help identify new targets for the eventual development of drugs to induce broad changes in gene expression to boost axon regeneration.

2.3.6 Post-translational modifications

Global regulators of cell signaling may be attractive targets for inducing a growth state in mature CNS neurons. A recent study showed that the class of cholesterol-lowering drugs, statins, potently stimulate neurite outgrowth(Li et al., 2016; Whitlon et al., 2015). Statins inhibit HMGCo-A reductase, a rate-limiting enzyme in the synthesis of cholesterol. Intriguingly, the mechanism underlying statin-induced neurite outgrowth involves the downstream enzymes geranylgeranyl transferase and farnesyl transferase, which are responsible for protein prenylation. Small molecule inhibitors of these enzymes enhance neurite outgrowth comparably to statins(Li et al., 2016). Inhibition of prenylation likely has a multitude of effects on many proteins. Rho GTPases are a well-known example of prenylated proteins that are potentially implicated in these effects on neurite outgrowth. Although further study into downstream substrates may yield more specific targets, the efficacy of statins in stimulating axon growth may be rooted in their ability to have actions on multiple substrates.

Poly-ADP ribosylation is another post-translational modification pathway that regulates axon regeneration. Inhibition of poly-ADP ribose-polymerases (PARPs) with small molecule drugs stimulates regeneration after axotomy in *c. elegans*(Brochier et al., 2015; Byrne et al., 2016). Like inhibiting prenylation, PARP inhibition likely has manifold effects across multiple substrates. Unfortunately, the potential of PARP inhibitors or PARP knockout to promote axon regeneration and functional recovery in mouse models of

injury did not show any benefit(Wang et al., 2016). This highlights an important consideration for developing therapeutic strategies based on multi-action therapies, which is the potential for detrimental effects on other cell types in the complex *in vivo* environment. For instance, although PARP inhibition may promote axon outgrowth in cultured neurons, it could also influence secondary events such as astrogliosis or the immune response when administered after injury. These effects could offset the positive effects on axon growth. For example, ROCK inhibition with the small molecule inhibitor Y27632 stimulates neurite outgrowth, but also induces astrocyte reactivity and expression of CSPGs(Chan et al., 2007). The net outcome after injury *in vivo* depends on a highly complex balance of effects on multiple cellular types and behaviors. A major challenge will also be developing multi-action drugs that have a reasonably large therapeutic window, meaning that high enough doses can be administered to induce beneficial effects without causing toxicity.

2.3.7 Neuronal activity

During development, activity dependent-processes play a critical role in the targeting and fine-tuning of the axonal arbor and its synaptic connections with the post-synaptic cell(Katz and Shatz, 1996). A role for neuronal activity in mediating axon regeneration had not been appreciated until recently, since long-distance axon regeneration has been achieved. There is now evidence that regenerated axons can reach their original target tissue and re-form functional synapses with some topographic organization, the ultimate goal of regenerative therapies. For instance, deletion of PTEN and SOCS3 induces regeneration of RGC axons to targets in the superior colliculus after injury of the optic tract. Evidence for functional connectivity was found by showing that optogenetic stimulation of regenerated axons produced responses in the superior colliculus. However, when subjected to visual behavior assays, mice failed to demonstrate significant recovery of optomotor function. It was found that regenerated axons were unmyelinated, suggesting poor conduction may underlie the absence of functional recovery. Administration of the voltage-gated potassium channel blocker 4-aminopyridine (4-AP) significantly improved conduction and ameliorated the visual behavior defect(Bei et al., 2016). Drug combinations that enhance both axon regeneration and myelination could therefore be critical to the development of therapeutic strategies to improve functional outcomes.

Beneficial effects of activity on axon regeneration are also supported by a recent study using DREADD technology (designer receptors exclusively activated by a designer

drug) and visual stimulation to show that enhancing RGC activity after optic nerve crush promotes axon regeneration. Moreover, when combined with increased mTOR activity, axons regenerated in a target-specific manner to multiple brain regions, aiding the restoration of visual behaviors(Lim et al., 2016). These exciting results in the visual system could guide therapeutic strategies for maximizing functional outcomes in SCI. Drugs that enhance axon growth combined with rehabilitative training could help optimize the functional gains afforded by regenerated axons.

2.3.8 The search for new targets and drugs

Both the extrinsic and intrinsic aspects of CNS injury forge the basis for regeneration failure. The Bradke group has shown that local administration of low dose taxol, a potent microtubule-stabilizing drug, improves outcomes by reducing CSPG production and fibrosis, inhibiting meningeal fibroblast migration and stimulating axon extension(Hellal et al., 2011). A recent report from the same group shows that systemic administration of another microtubule stabilizer, epothilone B, similarly inhibits scarring and promotes axon regeneration(Ruschel et al., 2015b). Both drugs are approved chemotherapeutics, however epothilone B, but not taxol, is CNS-penetrant. The repurposing of approved drugs for SCI is an attractive approach to achieve accelerated approval. However, the identification of new targets and drugs that hit multiple pathways may offer superior efficacy in stimulating CNS repair.

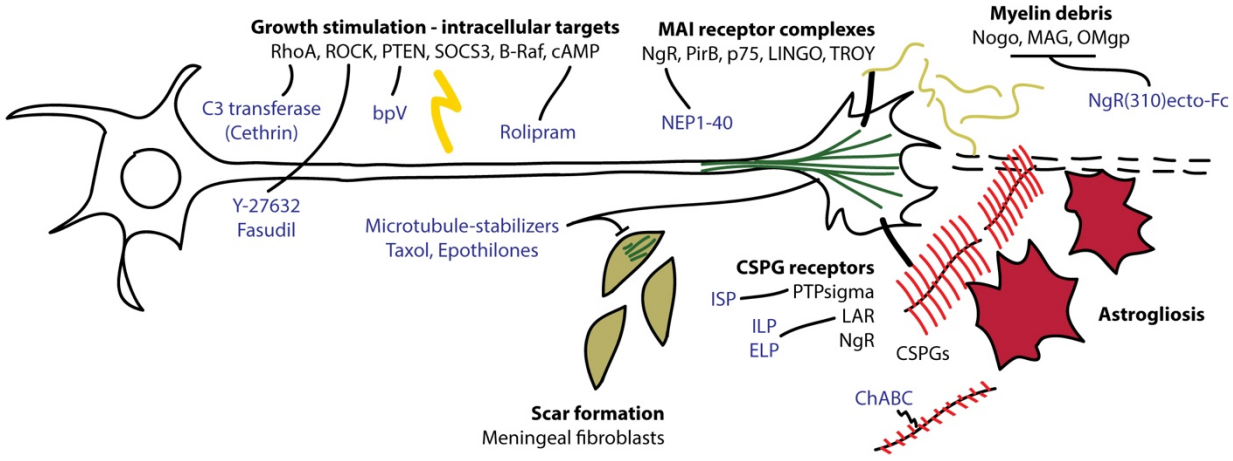

Figure 1. Overview of intrinsic and extrinsic molecules involved in axon regeneration after CNS injury. Myelin debris from damaged oligodendrocytes and chondroitin sulfate proteoglycans (CSPGs) produced by reactive astrocytes interact with receptors on the growth cones to interfere with axon extension. Intracellular signaling molecules regulate the ability of the neuron to regenerate its damaged axon through the lesion environment. C3 transferase inhibits RhoA to promote axon extension and cell

survival. Fasudil and Y-27632 inhibit Rho kinase (ROCK) to promote axon extension. Bisperoxovanadium (bpV) inhibits phosphatase and tensin homologue (PTEN) to promote neuroprotection. Rolipram inhibits phosphodiesterase 4 (PDE4) to stabilize cAMP and promote axon extension. Microtubule stabilizers, taxol and epothilones, reduce fibrotic scarring and promote axon extension. Chondroitinase ABC (ChABC) digests CSPG glycosaminoglycan chains (in red), relieving CSPG-dependent growth inhibition. Intracellular sigma peptide (ISP) inhibits protein tyrosine phosphatase sigma (PTPsigma) and intracellular/extracellular LAR peptides (ILP/ELP) inhibit leukocyte antigen-related receptor (LAR) to neutralize the inhibitory effect of CSPGs and promote axon sprouting. NgR(310)ecto-Fc competes for binding to Nogo, MAG and OMgp to relieve myelin-dependent growth inhibition. Nogo extracellular peptide 1-40 (NEP1-40) competes for NgR binding to neutralize inhibitory signaling through NgR. (Modified from Kaplan et al, 2015)

2.4 14-3-3 adaptor proteins

14-3-3s are a family of cytosolic adaptor proteins expressed in all eukaryotic organisms. In 1967, Moore and Perez first identified these proteins in bovine brain homogenate and named them 14-3-3 because they eluted in the 14th fraction and migrated to positions 3.3 in 2D gel electrophoresis. 14-3-3 proteins are ubiquitously expressed, but are especially abundant in the CNS, where they play critical roles in development and disease (see Table 1 for summary). In humans, there are seven isoforms (β, θ, ε, η, γ, ξ, σ), encoded by separate genes, that form homo and hetero-dimers. Each monomer is composed of a bundle of 9 alpha helices that form a central groove, which is highly conserved through evolution and among the seven isoforms (Figs. 2 and 3) (Kaplan et al., 2017a). 14-3-3s use this groove to bind hundreds of functionally diverse 'client proteins' at phospho-serine/threonine motifs. 14-3-3 binding can influence client protein activity, localization, stability or protein-protein-interactions (PPIs)(Bridges and Moorhead, 2005). 14-3-3s are generally regarded as neuroprotective, and they are dysregulated in neurodegenerative diseases, including Parkinson's Disease. Deletions in the gene encoding 14-3-3epsilon cause Miller-Dieker syndrome (MDS), a congenital disease characterized by a smooth brain(Cheah et al., 2012; Toyo-oka et al., 2003; Toyo-oka et al., 2014). Disruption of 14-3-3 function or knockout of 14-3-3s in mice leads to neuronal apoptosis, migration defects, axonal outgrowth defects, synaptic dysfunction, cognitive deficits, and schizophrenia-like behavioral phenotypes(Cheah et al., 2012; Foote et al., 2015; Jaehne et al., 2015; Qiao et al., 2014; Ramshaw et al., 2013; Xu et al., 2015). Protein-protein interactions (PPIs) between 14-3-3s and their clients are druggable with small molecules. Targeted manipulation of 14-3-3 interactions with client

proteins could offer an entirely new therapeutic approach to treat CNS injury and disease.

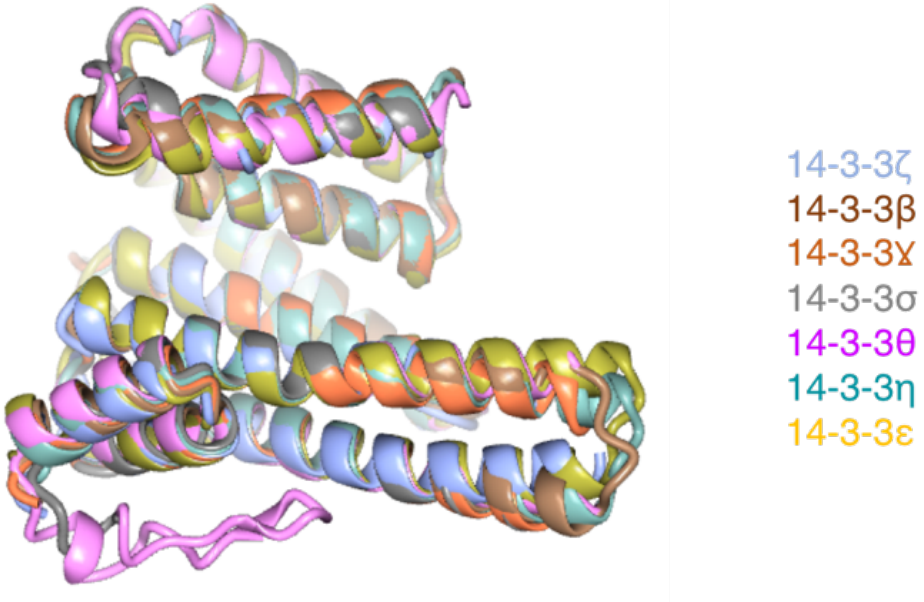

Figure 2. Superimposed crystal structures of all seven human 14-3-3 isoforms. Superimposed ribbon models of all seven human 14-3-3 isoform crystal structures. Note the high conservation of structural conformation between the isoforms. (PDB: 1A4O, 2BR9, 3UZD, 4DAT, 2BTP, 2C63, 2BQ0)

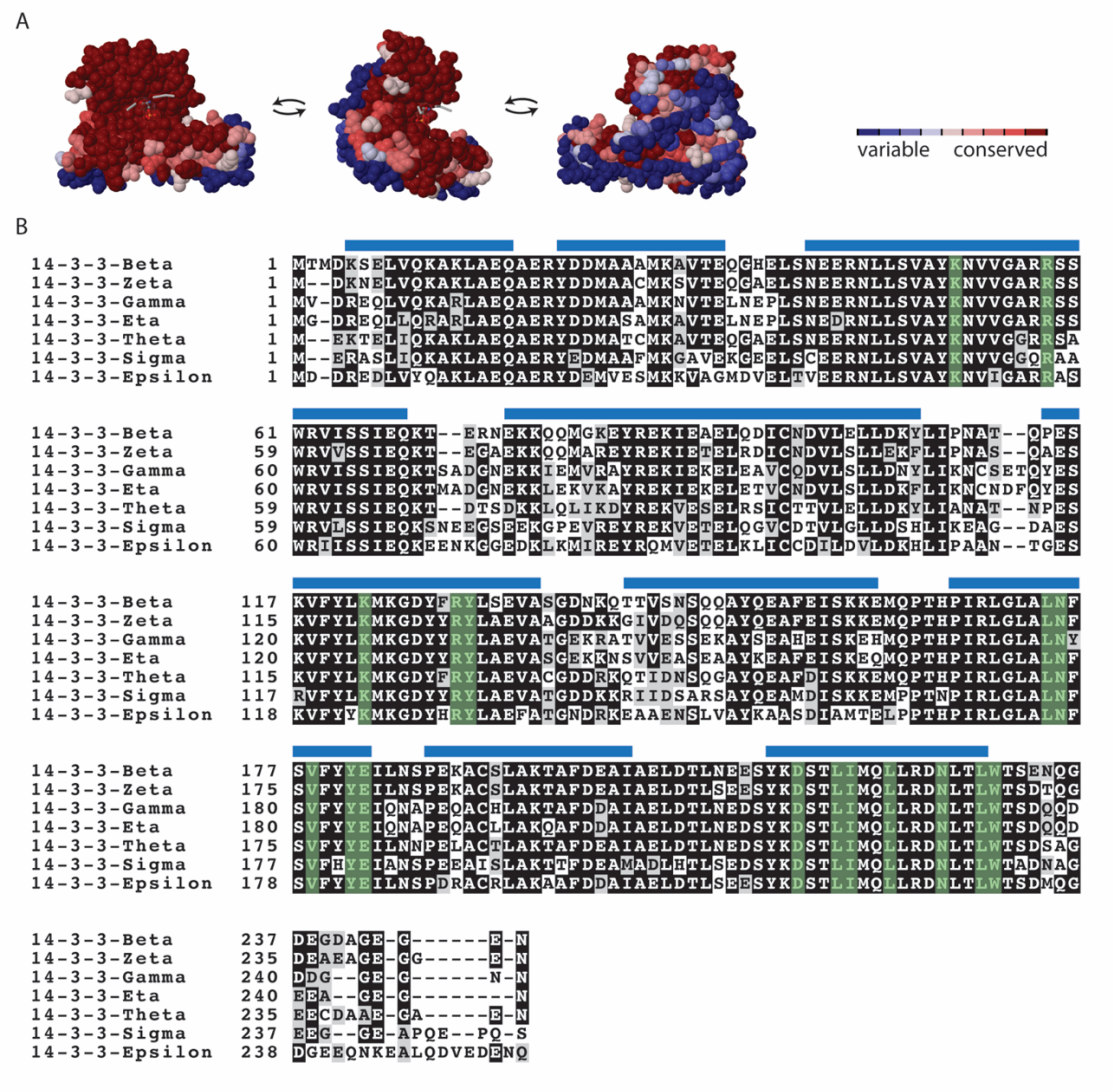

Figure 3. Structure and sequence conservation of human 14-3-3 isoforms. A) Color-coded evolutionary sequence conservation of human 14-3-3epsilon complexed to a peptide (PDB: 2BR9, generated using ConSurf). Note the high conservation of the client-binding groove. B) Multiple sequence alignment of human 14-3-3 isoforms. Note the high degree of sequence identity between isoforms. Blue bars above sequence indicate the 9 alpha helices. Residues highlighted in green indicate residues that directly contact the phosphopeptide of the client protein as described in (Rittinger et al. 1999). (Kaplan et al, 2017)

2.4.1 Neuroprotective functions of 14-3-3s

A fundamental cellular function of 14-3-3s is suppression of apoptosis via sequestration of pro-apoptotic factors including Bcl-2 family members Bad and Bax(Masters and Fu, 2001). 14-3-3 binding to these proteins is thought to restrain their activity by preventing translocation to their site of action at the mitochondria. Moreover, 14-3-3s also suppress

the activity of pro-apoptotic transcription factors including Foxo3a and c-Abl by preventing their shuttling to the nucleus. These activities have been exploited in the use of 14-3-3 PPI inhibitors that compete for the client-binding groove such as the R18 peptide or difopein (dimeric version of R18) as anti-cancer agents(Masters and Fu, 2001; Porter et al., 2006; Zhao et al., 2011). However, in the context of neurodegenerative diseases or CNS injury, the reverse strategy of stabilizing these activities may promote neuronal survival. For instance, in models of Parkinson's Disease (PD), where the loss of dopamine neurons leads to progressive motor and cognitive impairments, exogenous overexpression of 14-3-3s, especially the theta isoform, confers protection against neuronal death(Ding et al., 2015; Slone et al., 2011; Yacoubian et al., 2010). Intriguingly, 14-3-3 expression is reduced in mouse models of PD and multiple isoforms are found in Lewy bodies, toxic protein aggregates that are a histopathological hallmark of PD(Masliah et al., 2000). It is thought that reduced expression of 14-3-3 as well as sequestration within Lewy bodies renders neurons more vulnerable to degeneration and death. Consistent with this idea, inhibition of 14-3-3 PPIs with difopein exacerbates neuronal loss in multiple cell-based and mouse models of PD(Ding et al., 2015; Yacoubian et al., 2010). The mechanisms underlying these neuroprotective effects are not fully understood, but are thought to involve 14-3-3-mediated sequestration of Bax(Slone et al., 2011). A similar mechanism has recently been shown to occur in models of amyotrophic lateral sclerosis (ALS), where aggregates of mutant superoxide dismutase (SOD1) were shown to sequester 14-3-3s and promote cell death via Bax activation. Moreover, overexpression of 14-3-3s protected neurons against mutant SOD1-induced cell death(Park et al., 2017). Together, these results suggest that enhancing 14-3-3 expression or stabilizing their binding to pro-apoptotic factors could have therapeutic value in the treatment of neurodegenerative diseases where 14-3-3 function is compromised.

2.4.2 14-3-3s in axon growth and regeneration

14-3-3s are found in abundance in isolated growth cones and perturbation of 14-3-3s by genetic and pharmacological means impairs growth cone turning responses to guidance cues during CNS development(Kent et al., 2010; Yam et al., 2012). In cancer, 14-3-3 proteins are generally considered as growth-promoting, pro-migration molecules(Hermeking, 2003). 14-3-3s are thought to play an analogous function during the outgrowth of axonal projections. In *Xenopus* embryos, expression of R18 impairs the growth of RGC axons as they project from the eye to the brain. This may occur through activation of cofilin, an actin-depolymerizing factor. 14-3-3 binding to cofilin protects its

phosphorylation and maintains it in an inactive state, whereas loss of 14-3-3 binding allows for dephosphorylation and activation of cofilin, resulting in actin depolymerization(Yoon et al., 2012). 14-3-3 inhibition also impairs axon growth from DRG neurons, cortical neurons and hippocampal neurons, suggesting a general role in promoting axon outgrowth across multiple neuronal populations (Kaplan et al., 2017b; Lavalley et al., 2016; Mar et al., 2014b). In models of PD, the interaction of 14-3-3 with leucine-rich repeat kinase 2 (LRRK2) has been shown to regulate neurite extension. Interestingly, multiple PD-causing mutations in LRRK2 decrease 14-3-3 binding(Muda et al., 2014; Nichols et al., 2010). The most common LRRK2 mutation, G2019S, lies within the kinase domain. This increases its kinase activity, impairs neurite outgrowth, and induces neuronal degeneration(West et al., 2005). 14-3-3s bind to LRRK2 at multiple sites and are thought to play an important role in restraining LRRK2 activity. Consistent with this idea, overexpression of 14-3-3 theta protects against neurite outgrowth inhibition induced by G2019S LRRK2, whereas expression of difopein exacerbates the defect. However, expression of difopein on its own impairs neurite outgrowth, even in LRRK2-null neurons, suggesting that 14-3-3s also have a general function of promoting neurite growth independently of LRRK2(Lavalley et al., 2016).Together these studies indicate that inhibiting 14-3-3s has a negative effect on growth, suggesting that the opposite strategy of stabilizing 14-3-3s or promoting their interactions with client proteins could stimulate growth. Intriguingly, when certain client proteins bind to 14-3-3, they leave an empty pocket as they terminate within or turn out of the client-binding groove. For instance, the structure of 14-3-3 bound to the pS935 motif within LRRK2 reveals a druggable pocket created by the LRRK2 motif as it turns out of the groove (Fig. 4)(Stevers et al., 2017a).

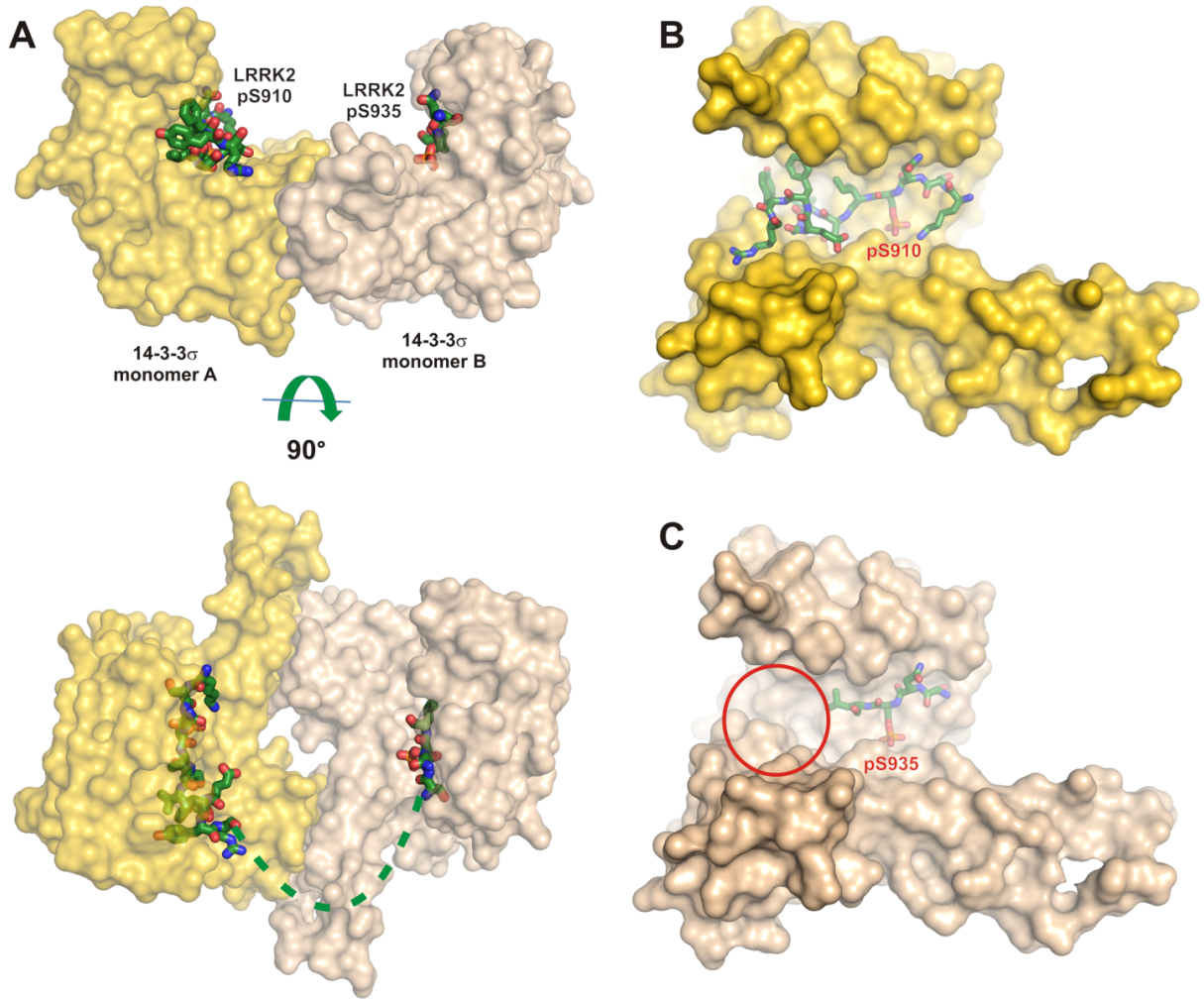

Figure 4. Crystal structure of 14-3-3σ in complex with the LRRK2pS910pS935 peptide. A) Synthetic LRRK2pS910 and LRRK2pS935 peptides (green sticks) bound to the 14-3-3σ dimer (golden and sand surface). The green dotted line indicates the position of the linker region not visible in the electron density. B) 14-3-3σ monomer accommodating the LRRK2pS910 peptide motif in the amphipathic binding groove. C) 14-3-3σ monomer accommodating the LRRK2pS935 peptide motif in the amphipathic binding groove. The peptide sequence exits the binding groove C-terminal of the +1 position after pS935, revealing a druggable pocket (red circle) that might allow for binding of a 14-3-3/LRRK2 PPI stabilizer. (Kaplan et al, 2017)

2.4.3 Fusicoccanes: from botany to drug development

Fusicoccanes are a family of natural small molecules that are well characterized for their ability to stabilize the binding between 14-3-3s and client proteins. Fusicoccin-A (FC-A), the most widely studied member of this family, is produced by certain plants and fungi, most notably *Phomopsis amygdali*. First isolated and described by the group of Ballio in 1964, FC-A captured significant interest in the fields of natural product chemistry and

botany due to its intriguing effects on plants during infection with *Phomopsis amygdali*(Ballio et al., 1964). FC-A causes rapid and irreversible opening of stomata, pore-like structures in plant leaves that regulate gas and water exchange across the plasma membrane. Stomatal opening induced by FC-A results in water loss and leaf wilting. This correlates with massive proton flux across the plasma membrane(de Boer and de Vries-van Leeuwen, 2012). In 1994, a series of three independent back-to-back publications reported that FC-A binds to 14-3-3 proteins(Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). Affinity-purified preparations also contained a protein identified as a plasma membrane H+ ATPase. Subsequent studies led to the discovery that FC-A stabilizes 14-3-3 binding to H+ ATPase, but the mechanism underlying this effect remained unknown. Nearly a decade later in 2003, the crystal structure of FC-A bound to the 14-3-3:H+ ATPase complex was solved by Wurtele, Ottmann, Wittinghofer and Oecking. The structure revealed that FC-A binds to a pocket in the 14-3-3 groove created by the docking of the H+ ATPase motif. FC-A displaces water molecules from this exposed hydrophobic pocket and causes a 100-fold increase in binding affinity of the two proteins for one another, relieving an auto-inhibition of the ATPase. FC-A has a low affinity for un-cliented 14-3-3 and only binds appreciably to the complex(Wurtele et al., 2003). More recently, this fascinating binding mechanism has been explored in the context of the human 14-3-3 interactome, which does not include this plant-specific H+ ATPase protein. FC-A also binds mammalian 14-3-3 protein complexes because of the extraordinarily high conservation of sequence and structure between plants and animals. Structural and biochemical analyses indicate that FC-A can stabilize a growing repertoire of human 14-3-3:client interactions. Many of these include disease-relevant molecules. For instance, FC-A stabilizes 14-3-3 binding to CFTR (cystic fibrosis transmembrane conductance regulator) and promotes its trafficking to the plasma membrane, a key therapeutic strategy for the treatment of cystic fibrosis(Stevers et al., 2016a). Targeted stabilization of 14-3-3 protein-protein interactions is an innovative approach in the development of drugs for many indications.

2.4.4 Stabilization of 14-3-3 PPIs

Many studies, particularly in the cancer field, have employed competitive inhibitors of 14-3-3 PPIs including the R18 peptide, dimeric R18 (difopein), and small molecules that bind to the client-binding groove(Milroy et al., 2013). These compounds globally interfere with all 14-3-3 isoforms because of the high conservation of the groove, and they are not selective for specific 14-3-3:client PPIs. In many cases, such as the interaction between 14-3-3 and LRRK2, stabilization of the 14-3-3:client complex may have a desirable

therapeutic activity(Stevers et al., 2017a). Additionally, compounds like fusicoccanes, which stabilize 14-3-3 PPIs, do not bind in a competitive fashion. Moreover, the residues downstream of the phosphorylation site in the client motif determine whether the pocket can accommodate these compounds, meaning that the variable sequences in different clients can be leveraged to tailor these compounds for specific 14-3-3:client complexes.

The consensus motifs for 14-3-3 binding are characterized as mode I (RSXpS/TXP), mode II (RXSXpS/TXP) or C-terminal mode III (pS/TX$_{1-2}$-COOH)(Kaplan et al., 2014). The +2 proline in mode I and mode II motifs causes the peptide to turn out of the groove, while binding at mode III motifs leaves an empty pocket as the peptide terminates within the groove before spanning its entire length. However, many 14-3-3 clients do not strictly adhere to these consensus sequences and do not have a +2 proline. FC-A was previously thought to only stabilize mode III binding, however it has recently been shown that FC-A can also bind and stabilize internal mode I/II motifs that do not have a +2 proline(Doveston et al., 2017; Stevers et al., 2016a). In these cases, residues with small hydrophobic side-chains like leucine, isoleucine and valine are thought to create a compatible pocket downstream of the phosphorylation site. On the other hand, cotylenin-A (CN-A), another fusicoccane, can stabilize classical mode I/II 14-3-3 binding to motifs with a +2 proline (Fig. 5)(Molzan et al., 2013).

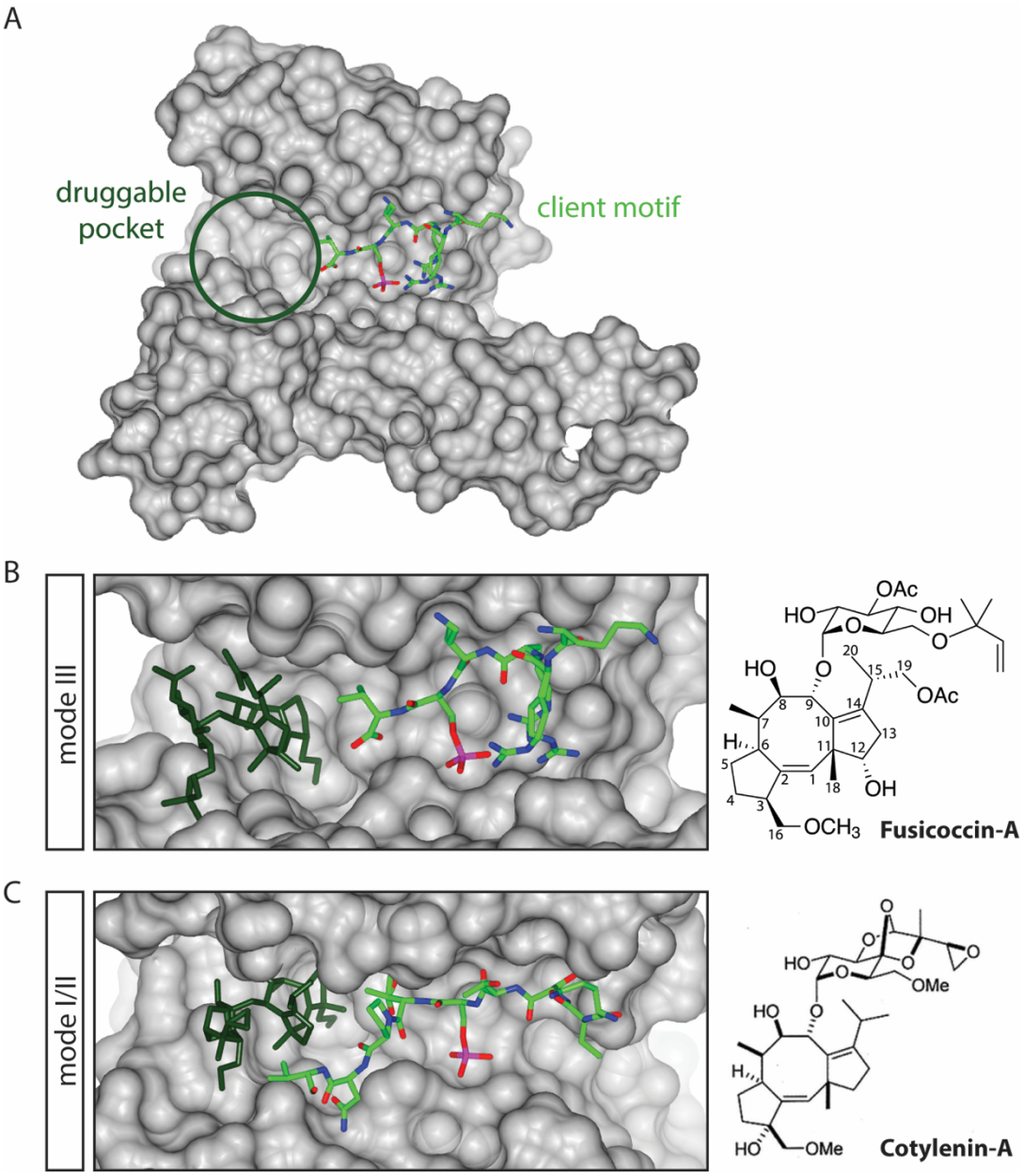

Figure 5. Crystal structures of fusicoccanes bound to 14-3-3:client complexes. A) 14-3-3 monomer bound to a mode III motif derived from TASK3 potassium channel, revealing a druggable pocket as the motif terminates in the groove. B) Fusicoccin-A (FC-A) (dark green sticks) bound to 14-3-3:client complex (mode III motif derived from TASK3, PDB: 3P1Q). C) Cotylenin-A (CN-A) (dark green sticks) bound to 14-3-3:client complex (mode II motif derived from Raf, PDB: 4IHL). Note the how the proline in the Raf peptide induces a turn out of groove. Note the apposition of the compounds with the back wall of the groove and the side of the client motif.

Structural analyses suggest that the C12 hydroxylation of FC-A, which is absent in CN-A, causes a steric clash with the proline residue, making it incompatible with the pocket

(Fig. 6). It is therefore likely that FC-A stabilizes a smaller repertoire of 14-3-3 PPIs, compared to CN-A. This key difference between these two fusicoccanes has provided important insight into how chemical modification of the scaffold could achieve selectivity for specific 14-3-3:client complexes. The feasibility of this has been shown by the design of a semisynthetic FC-A derivative with a fused tetrahydrofuran ring (FC-THF), which ensures steric conflicts with downstream residues, resulting in a mode III-specific 14-3-3 PPI stabilizer (Fig. 6) (Anders et al., 2013). High throughput screening for compounds that can stabilize a mode III 14-3-3 PPI have also resulted in the discovery of synthetic stabilizers with different scaffolds, epibestatin and pyrrolidone 1, serving as proof of concept that the 14-3-3:client pocket is druggable with synthetic small molecules(Rose et al., 2010). Moreover, pyrrolidone 1 was further modified to yield derivatives with higher potency(Richter et al., 2012), suggesting that these molecules could serve as starting points for stabilizers with higher potency and perhaps selectivity for specific 14-3-3:clients. "

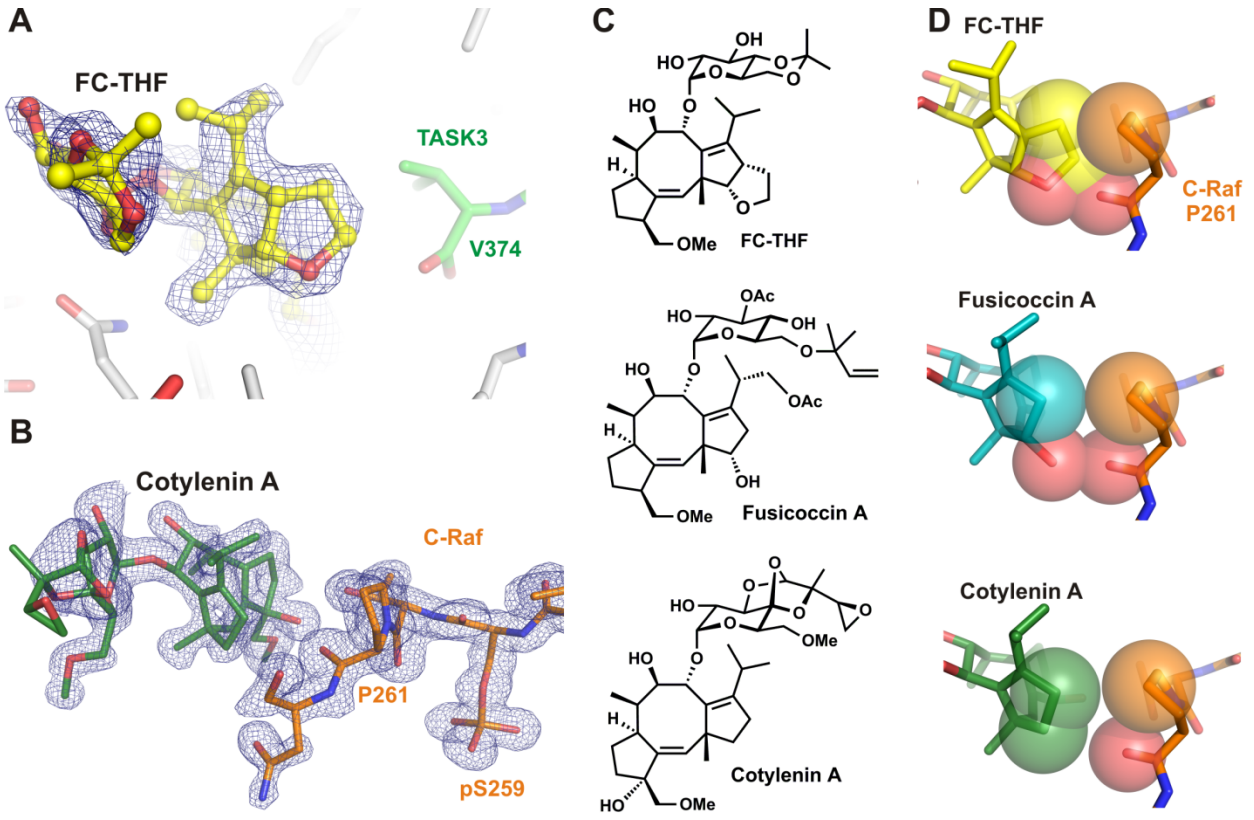

Figure 6. Fusicoccanes as mode-specific stabilizers of 14-3-3 PPIs. A) FC-THF (yellow sticks) bound to 14-3-3σ (white sticks) in complex with a TASK3-derived, mode III peptide (green sticks). The $2F_o$-F_c electron density (contoured at 1σ) is shown as blue mesh. B) Cotylenin A (dark green sticks) bound to 14-

3-3σ (not shown) and the C-RafpS259 peptide (orange sticks). The $2F_o$-F_c electron density (contoured at 1σ) is shown as blue mesh. C) Structures of fusicoccanes. D) Steric clashes (FC-THF, FC-A) and steric compatibilities (Cotylenin A) of different fusicoccanes with binding of 'internal' mode I and mode II 14-3-3 recognition motifs, for example C-RafpSer259. (Kaplan et al, 2017)

2.5 Thesis overview

CNS axons fail to spontaneously regenerate, but studies in rodent models of SCI and optic nerve injury have shown that targeting both extrinsic and intrinsic factors can coax neurons to regrow their axons and, in some cases, reconnect with appropriate targets and restore function. Most of these promising outcomes have largely been based on genetic means of manipulating candidate molecules. There is still a need for drugs that can stimulate regeneration. 14-3-3s are a multi-functional family of adaptor proteins that play important roles in axon growth and guidance. In this thesis, we explore the role of 14-3-3s in neurite outgrowth and axon regeneration using genetic and pharmacological approaches in cell-based assays and rodent models of SCI and optic nerve injury. In chapter 2, we discover that stabilization of 14-3-3 PPIs with FC-A stimulates neurite outgrowth and axon regeneration. We use affinity chromatography and mass spectrometry to identify client proteins that bind to FC-A, resulting in the discovery of a translational regulator called GCN1 as a novel target for axon regeneration. In chapter 3, we embark on a structure activity relationship campaign to enhance the potency and improve the properties of FC-A. We identify a series of potent derivatives that cross the blood-brain-barrier (BBB), suggesting routes for further development of these compounds. In chapter 4, we explore the role of GCN1 in axon regeneration using cell-based assays and a mouse model of optic nerve injury. Our work uncovers new targets and new drugs which could be used to stimulate axon regeneration.

Experimental manipulation	Phenotypes	Refs.
14-3-3 PPI inhibitor R18	Reduced neurite outgrowth in cultured cortical, hippocampal and dorsal root ganglion neurons	(Kaplan et al., 2017b; Lavalley et al., 2016; Mar et al., 2014b)
14-3-3 zeta knockout mice	Diffuse axonal projections, neuronal migration impairments, reduced dendritic spine density (Balb/c), increased dopamine levels, locomotor hyperactivity, increased exploratory behavior (Sv/129) (dependent on background)	(Cheah et al., 2012; Jaehne et al., 2015; Ramshaw et al., 2013; Xu et al., 2015)
14-3-3 zeta + 14-3-3 epsilon double knockout mice	Impaired neuronal migration, increased neuronal differentiation and apoptosis	(Toyo-oka et al., 2014)
14-3-3 zeta overexpression	Increased tau phosphorylation and aggregation, increased neurite outgrowth	(Kaplan et al., 2017b; Li and Paudel, 2016; Qureshi et al., 2013)
14-3-3 PPI inhibition in Thy1-YFP-difopein mice (neuronal specific 14-3-3 inhibition beginning in perinatal period)	Impaired learning and memory, impaired long-term potentiation, impaired sensorimotor gating, increased dopamine levels, reduced dendritic complexity, reduced dendritic spine density, increased vulnerability to neurotoxicant MPTP	(Ding et al., 2015; Foote et al., 2015; Qiao et al., 2014)
In vivo and in vitro overexpression of 14-3-3 theta in neurons	Neuroprotection (against MPTP, rotenone, alpha synuclein, mutant SOD1), protection against mutant LRRK2-induced neurite shortening	(Ding et al., 2015; Park et al., 2017; Slone et al., 2011;

		Yacoubian et al., 2010)
14-3-3 epsilon overexpression	Reduced neurite formation, increased axon regeneration in cultured cortical neurons	(Cornell et al., 2016a; Kaplan et al., 2017b)
14-3-3 epsilon knockout mice	Increased neurite formation, locomotor hyperactivity, increased sociability	(Cornell et al., 2016b; Wachi et al., 2017)
14-3-3 PPI stabilizer fusicoccin-A (FC-A)	Increased axon regeneration after optic nerve crush, reduced axonal dieback after spinal cord injury, increased axon outgrowth and regeneration in cultured cortical neurons	(Kaplan et al., 2017b)

Table 1. CNS phenotypes resulting from experimental manipulation of 14-3-3s (Kaplan et al, 2017)

Results

3 Chapter 2. Small molecule stabilization of 14-3-3 protein-protein interactions stimulates axon regeneration

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3.1 Preface

In this chapter, we employ a variety of genetic and pharmacological approaches to probe the function of 14-3-3 proteins in axon growth and regeneration. We show that 14-3-3s promote neurite outgrowth and report a new strategy to harness the pro-growth activity of these proteins using a small molecule called fusicoccin-A (FC-A). Our results suggest that FC-A could be a starting point for the development of drugs aimed at inducing axon regeneration after CNS injury.

3.2 Abstract

Damaged central nervous system (CNS) neurons have a poor ability to spontaneously regenerate, causing persistent functional deficits after injury. Therapies that stimulate axon growth are needed to repair CNS damage. 14-3-3 adaptors are hub proteins that are attractive targets to manipulate cell signaling. We identify a positive role for 14-3-3s in axon growth and uncover a developmental regulation of the phosphorylation and function of 14-3-3s. We show that fusicoccin-A (FC-A), a small molecule stabilizer of 14-3-3 protein-protein interactions, stimulates axon growth *in vitro* and regeneration *in vivo*. We show that FC-A stabilizes a complex between 14-3-3 and the stress response regulator GCN1, inducing GCN1 turnover and neurite outgrowth. These findings show that 14-3-3 adaptor protein complexes are druggable targets and identify a new class of small molecules that may be further optimized for the repair of CNS damage.

3.3 Introduction

Neurons in the central nervous system (CNS) have a poor capacity to spontaneously repair after injury, limiting restitution of motor and sensory function. Stimulation of axon growth is an important strategy to encourage repair of damaged connections. Robust axon regeneration after injury can be elicited by genetic manipulation of oncogenes and tumor suppressors(Belin et al., 2015; O'Donovan et al., 2014; Park et al., 2008; Sun et al., 2011). These studies have suggested that a global alteration of the cell-intrinsic growth state is required to achieve extensive axon regeneration. However, the current challenge is to identify druggable targets and treatments to repair CNS injury.

Adaptor proteins facilitate many important molecular processes and are interesting targets to manipulate the intrinsic growth state. 14-3-3s are a seven-isoform (β, γ, ε, ζ, η, θ, σ) family of dimeric cytosolic adaptor proteins highly expressed in the CNS. 14-3-3s have pervasive roles in cell signaling and are important regulators of neurodevelopment and axon guidance(Cheah et al., 2012; Kent et al., 2010; Toyo-oka et al., 2003; Toyo-oka et al., 2014; Yam et al., 2012), however their roles in axon growth and regeneration after injury are unknown. Here we report that 14-3-3s enhance axon growth. Moreover, we find that a developmental increase in 14-3-3 phosphorylation antagonizes 14-3-3 function.

14-3-3s bind to serine and threonine –phosphorylated client proteins via a highly-conserved binding groove (Fig. 1a). Intriguingly, a small molecule called fusicoccin-A (FC-A) can be used as a chemical tool to stabilize 14-3-3:client complexes. FC-A binds to a solvent-exposed hydrophobic pocket created by the interface of the 14-3-3 binding groove and certain client proteins(de Boer and de Vries-van Leeuwen, 2012). Pharmacological stabilization of 14-3-3 PPIs is a potentially powerful approach to alter the cell-intrinsic growth state through modulation of multiple signaling pathways. We discover that FC-A stimulates neurite outgrowth *in vitro,* reduces corticospinal axon dieback after spinal cord injury and stimulates optic nerve regeneration. We demonstrate that FC-A acts through a 14-3-3-dependent mechanism and identify FC-A-binding proteins using bead-immobilized FC-A and mass spectrometry. We show that FC-A stabilizes a complex between 14-3-3 and the stress response regulator GCN1, leading to GCN1 turnover and enhanced neurite outgrowth. These results identify a novel strategy and class of small molecules to enhance axon growth after injury.

3.4 Results

3.4.1 14-3-3s promote neurite outgrowth and axon regeneration *in vitro*

To assess the role of 14-3-3 adaptor proteins in neurite outgrowth, we treated E18 rat cortical neurons with BV02, a cell-permeable small molecule inhibitor of 14-3-3 PPIs (Corradi et al., 2010; Gomez-Suarez et al., 2016; Mohammad et al., 2013). BV02 significantly reduced neurite growth at low micromolar concentrations and at 10μM many of the neurons failed to initiate neurites or only sprouted few very short processes, with an overall ~60% reduction in average total neurite length per neuron (Fig. 1b,c). This was not accompanied by any significant increase in TUNEL-positive apoptotic cells (Fig. 1d). Expression of dimeric R18 (difopein), a peptide inhibitor of 14-3-3s(Masters and Fu, 2001), similarly impaired neurite outgrowth (Fig. 1e).

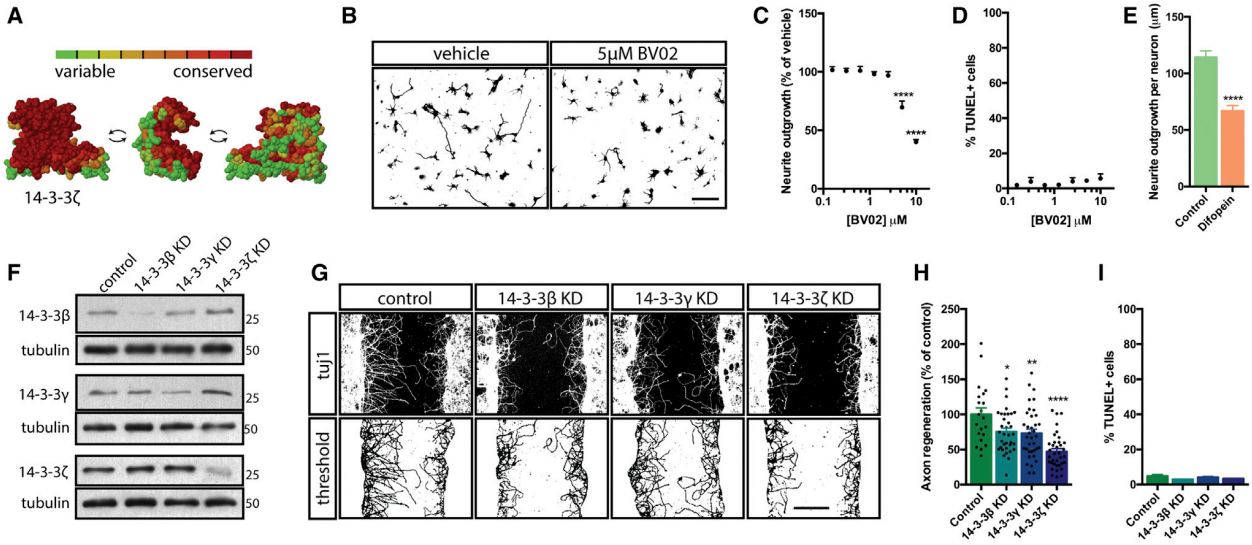

Figure 1. 14-3-3 loss of function impairs neurite outgrowth and regeneration in vitro. (A) Crystal structure of human 14-3-3ζ (PDB: 4HKC) color-coded for evolutionary sequence conservation using ConSurf. (B,C) Treatment of E18 rat cortical neurons with BV02 small molecule 14-3-3 inhibitor reduces neurite outgrowth and (D) does not induce apoptosis (n=3, ****p<0.0001 Fisher's LSD, scale bar = 100μm). (E) Expression of dimeric R18 (difopein) impairs neurite outgrowth (n=60 neurons from 3 experiments, ****p<0.0001, t-test). (F) Knockdown of 14-3-3 β, γ, ζ (G,H) impairs axon regeneration and (I) does not induce apoptosis after scratch injury (n=21-36 scratches from 3 experiments, *p<0.05, **p<0.01, ***p<0.001, One-way ANOVA Dunnett's post-test, scale bar = 200μm). Data are presented as mean +SEM.

We next investigated the effect of knocking down individual 14-3-3 isoforms on axon regeneration *in vitro*. We employed a scratch assay where rat cortical neurons are grown to confluence for 10 days and mechanically injured with a pipet tip(Huebner et al., 2011). The day after injury, nearly all the regenerating neurites are tau-positive axons (Fig. S1a).

14-3-3s are present throughout the soma and neurites in mature sparse cultures (Fig. S1b), however there is an enrichment at the growth cone tips after scratch injury, suggesting a role in axon regrowth (arrowheads, Fig. S1c). Knockdown of 14-3-3 isoforms β, γ, or ζ significantly reduced axon regeneration after scratch injury (Fig. 1f-h) and did not impact cell survival (Fig. 1i). We confirmed the specificity of these effects in a neurite outgrowth assay by expressing RNAi-resistant myc-tagged 14-3-3s and found that this was sufficient to restore baseline levels of growth (Fig. S2). Further supporting a positive role for 14-3-3s in axon growth, overexpression of myc-tagged 14-3-3 β, γ, or ζ significantly enhanced regeneration after scratch injury (Fig. 2a-c).

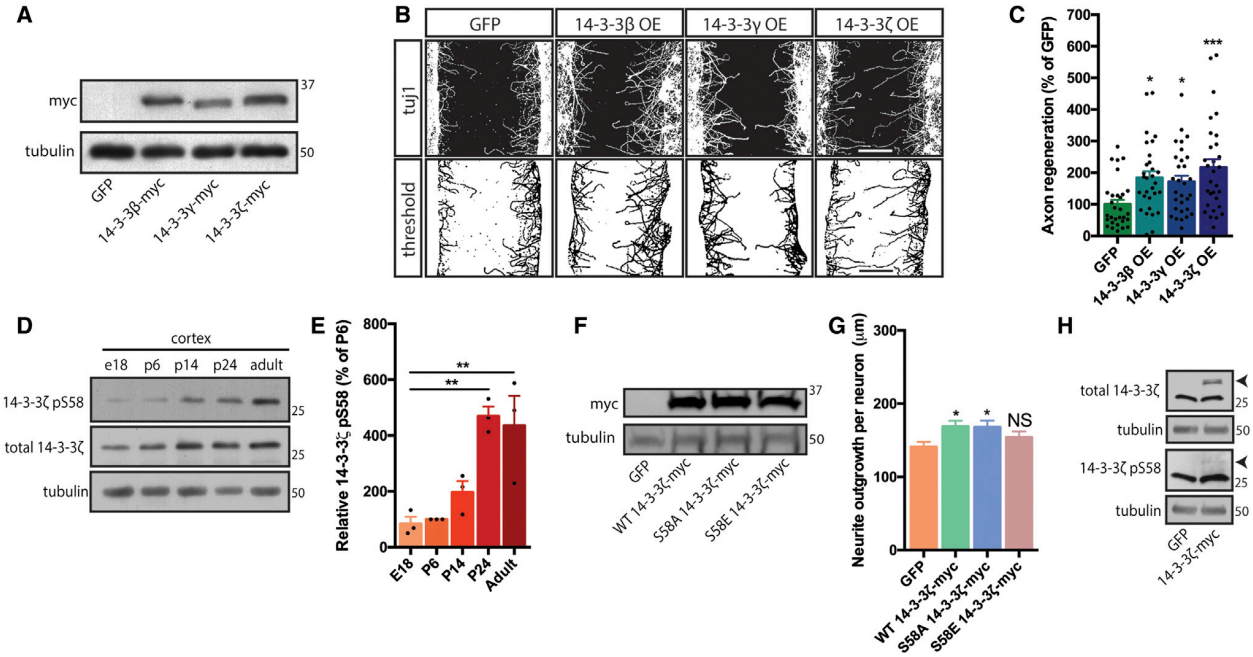

Figure 2. 14-3-3 overexpression enhances axon regeneration in vitro. (A) Western blot of overexpressed myc-tagged 14-3-3 β, γ, ζ in cortical neurons. (B,C) 14-3-3 overexpression (OE) enhances axon regeneration after scratch injury (n=30 scratches from 3 experiments, *p<0.05, ***p<0.001, One-way ANOVA Dunnett's post-test, scale bar = 200μm). (D,E) Western blots and densitometry of 14-3-3ζ pS58 from rat cortex (normalized to P6 total 14-3-3ζ and tubulin, n=3, **p<0.01, One-way ANOVA Dunnett's post-test). (F) Overexpression of myc-tagged wild type, S58A and S58E 14-3-3ζ. (G) S58E 14-3-3ζ mutant fails to enhance neurite outgrowth (n=69-74 neurons from 3 experiments, *p<0.05, One-way ANOVA Dunnett's post-test). Data are presented as mean +SEM. (H) Exogenously expressed 14-3-3ζ is largely unphosphorylated relative to endogenous 14-3-3ζ pS58 (arrowheads, overexpressed myc-14-3-3ζ).

3.4.2 Phosphorylated 14-3-3 is elevated in adulthood and fails to promote neurite outgrowth

Phosphorylation of a conserved serine (S58) in the 14-3-3 dimerization interface impairs dimerization and binding to client proteins(Powell et al., 2003; Shen et al., 2003; Woodcock et al., 2003; Zhou et al., 2009). Intriguingly we found that 14-3-3 phosphorylation in the cortex is upregulated between the second and third week of life and high levels persist into adulthood (Fig. 2d, e). We tested the effect of 14-3-3 phosphorylation on neurite outgrowth and found that overexpression of both wild type 14-3-3ζ and a non-phosphorylatable S58A mutant enhanced neurite outgrowth, while overexpression of a phosphomimetic S58E mutant was ineffective (Fig. 2f,g). We also found that overexpressed 14-3-3 is largely unphosphorylated relative to endogenous 14-3-3 (arrowheads, Fig. 2h), suggesting that the process mediating 14-3-3 phosphorylation is saturated. Together this supports a model wherein an increase in phosphorylation-mediated antagonism of 14-3-3 function contributes to a decline in the intrinsic capacity of neurons to grow.

3.4.3 Fusicoccin-A (FC-A), a small molecule stabilizer of 14-3-3 PPIs, enhances neurite outgrowth and improves axon regeneration *in vitro*

Fusicoccin-A (FC-A) is a fusicoccane small molecule produced by the *Phomopsis amygdali* fungus that stabilizes the binding between 14-3-3s and client proteins(de Boer and de Vries-van Leeuwen, 2012). Crystal structures of FC-A bound to 14-3-3:client complexes show that FC-A binds to a pocket created by the interface of the 14-3-3 binding groove and the client peptide docked within the groove(Wurtele et al., 2003) (Fig. 3a). FC-A has a very low affinity for “un-cliented” 14-3-3 and binds to a limited set of 14-3-3:client complexes, dependent on client motif sequences(Wurtele et al., 2003). We reasoned that FC-A, by its ability to stabilize 14-3-3 PPIs, might be a means to pharmacologically harness the pro-growth activity of 14-3-3s in neurons. Treatment of cortical neurons with FC-A produced a striking dose-dependent enhancement of neurite outgrowth on day 1 of culture (Fig. 3b, c). Stimulation of neurite outgrowth was apparent at low micromolar concentrations with an EC50 of 29μM (Fig. 3d). A maximal effect of more than doubling neurite outgrowth per cell was reached at ~200μM. The EC50 is comparable to biologically active concentrations of FC-A that have been reported in other mammalian cell types(Bury et al., 2013; Camoni et al., 2011; De Vries-van Leeuwen et al., 2013). FC-A also markedly enhanced axon regeneration after scratch injury (Fig. 3e,f). Although no outward toxicity was noted, axon regeneration peaked at 25μM and

began to decline at higher doses, suggesting that injured aged cortical neurons may be more sensitive to high doses of FC-A. Importantly, FC-A also promoted outgrowth from primary human fetal neurons, indicating a conservation of activity from rodent to human (Fig. 3g, h).

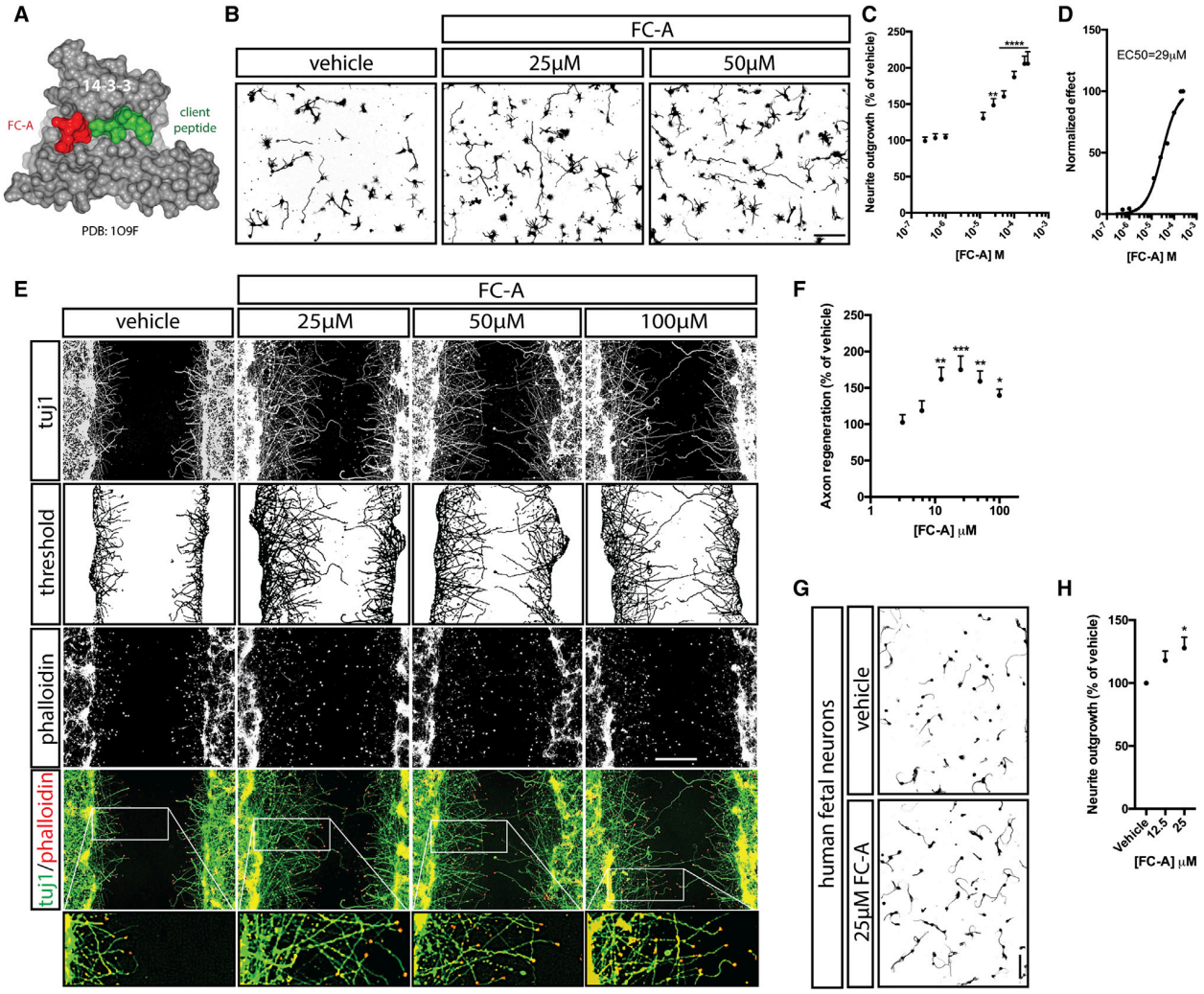

Figure 3. 14-3-3 modulator fusicoccin-A (FC-A) enhances neurite outgrowth and regeneration in vitro. (A) Crystal structure of FC-A in ternary complex with 14-3-3 and a client peptide (PDB: 1O9F). (B-D) FC-A stimulates neurite outgrowth from cortical neurons with an EC50 of 29µM (n=6, **p<0.01, ****p<0.0001, One-way ANOVA Fisher's LSD, scale bar = 100µm). (E,F) FC-A enhances axon regeneration after scratch injury (n=18 scratches from 3 experiments, **p<0.01, ***p<0.001, One-way ANOVA Fisher's LSD, scale bar = 200µm). (G,H) FC-A enhances neurite outgrowth from primary human fetal neurons (n=3, *p<0.05, One-way ANOVA Fisher's LSD, scale bar = 100µm). Data are presented as mean +SEM.

3.4.4 FC-A requires 14-3-3 PPIs to stimulate neurite outgrowth

To confirm that FC-A targets 14-3-3s to induce neurite outgrowth, we treated cortical neurons with a combination of FC-A and the cell-permeable 14-3-3 PPI inhibitor BV02.

FC-A failed to elicit a significant increase in neurite outgrowth in the presence of BV02, which severely impaired baseline neurite outgrowth as expected (Fig. 4a, b). In parallel as a control, we cultured neurons in the presence of the rho kinase inhibitor Y-27632, a known neurite outgrowth inducer, and found that BV02 had no effect on the ability of Y-27632 to stimulate outgrowth (Fig. 4a, b). Interestingly, although Y-27632 is more potent in the neurite outgrowth assay, it does not significantly stimulate regeneration after scratch injury of aged cultures, consistent with another report showing no effect of Y-27632 in the same scratch injury paradigm (Fig. S3; (Huebner et al., 2011)). To further test the 14-3-3-dependence of FC-A-induced neurite outgrowth, we knocked down 14-3-3 β, γ, and ζ, which resulted in a substantial depletion of total 14-3-3 (Fig 4c). This significantly impaired FC-A-induced neurite outgrowth, further supporting that FC-A functions through a 14-3-3-dependent mechanism (Fig. 4d). We have previously shown that 14-3-3s regulate axon guidance through modulation of protein kinase A (PKA)(Kent et al., 2010; Yam et al., 2012). However, inhibition of PKA with KT5720 had no effect on FC-A-induced neurite outgrowth (Fig. S4), suggesting that FC-A stimulates neurite outgrowth in PKA-independent manner.

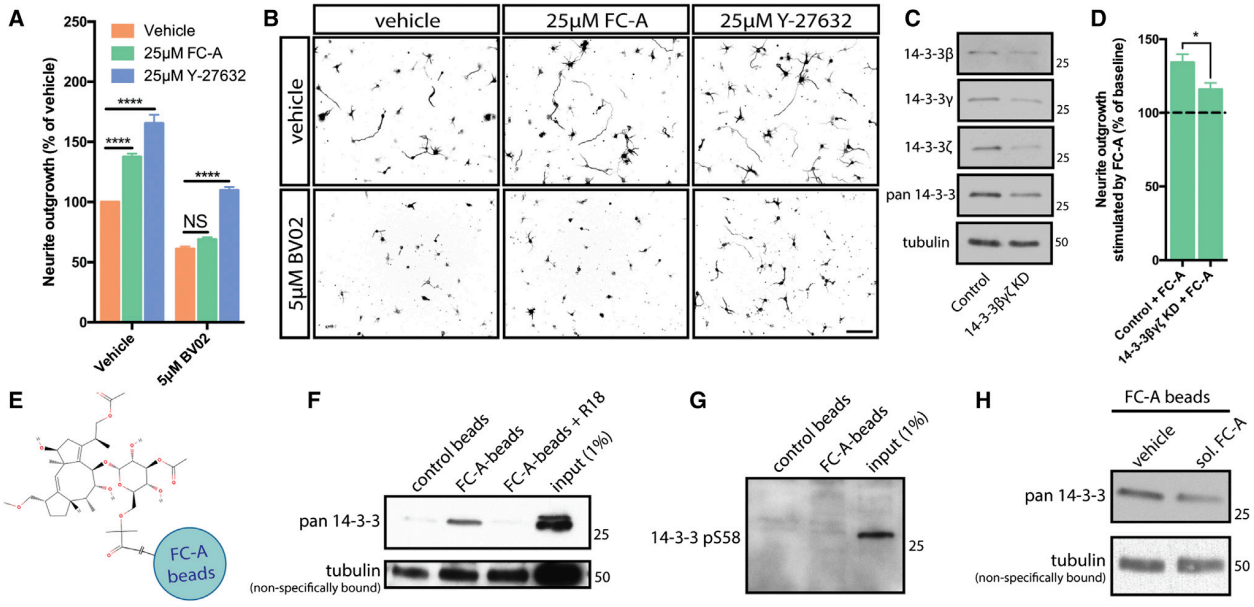

Figure 4. FC-A binds to 14-3-3 proteins in neuron lysate and induces neurite outgrowth in a 14-3-3-dependent manner. (A,B) Inhibition of 14-3-3s with BV02 blocks FC-A-induced stimulation of neurite outgrowth without affecting Y-27632-induced neurite outgrowth (n=3, ****p<0.0001, Two-way ANOVA Bonferroni's post-test, scale bar = 100μm). (C,D) Knockdown of 14-3-3 β, γ and ζ blocks FC-A-induced outgrowth (n=4, *p<0.05, unpaired t test). Data are presented as mean +SEM. (E) Coupling of FC-A to beads. (F) FC-A-beads selectively precipitate 14-3-3s from cortical neuron lysate and R18 blocks binding. (G) FC-A beads do not precipitate 14-3-3 pS58. (H) Soluble FC-A blocks binding of 14-3-3 to FC-A-beads.

3.4.5 Identification of FC-A targets in cortical neurons

To identify FC-A targets, we chemically coupled FC-A to magnetic beads (Fig. 4e) to use as an affinity reagent for pull-downs from cortical neuron lysate. We first confirmed that FC-A-beads selectively precipitate 14-3-3 proteins (Fig. 4f). Interestingly, FC-A beads precipitated a small fraction of total 14-3-3, suggesting a small pool of “cliented” 14-3-3s. The 14-3-3 PPI inhibitor R18, which spans the binding groove, blocked the binding of 14-3-3 to the beads, indicating that 14-3-3 binds to FC-A at the client-binding groove and not at another surface of the protein (Fig. 4f). The client-binding deficient phospho-S58 species was not precipitated by FC-A beads, again indicating that FC-A selectively binds cliented 14-3-3s (Fig. 4g). As a control for binding specificity, soluble FC-A impaired 14-3-3 binding to the FC-A-beads (Fig. 4h).

To gain insight into the mechanism underlying FC-A-induced neurite outgrowth, we used FC-A-beads for affinity chromatography followed by mass spectrometry to identify the complement of FC-A-binding proteins. Pull-downs from cortical neuron lysate using FC-A-beads or control beads were performed in three independent experiments. Using ‘significance analysis of interactome’ (SAINT)(Choi et al., 2011), we identified 14 high-probability interactors (cutoff of 0.9). Remarkably, 13 of these 14 proteins contain potential FC-A/14-3-3 binding motifs (Table S1), defined by the characteristics of known binding motifs which can either be C-terminal serines or threonines (S/T) or internal S/T preceded by an arginine and followed by residues with small hydrophobic side-chains. Of these proteins, ‘general control non-derepressible 1’ (GCN1), a regulator of translation in response to cell stress, possesses 11 potential binding sites, one of which has identical sequence to a known binding site(Stevers et al., 2016b). We therefore focused our efforts on querying a potential role for GCN1 in FC-A-dependent neurite outgrowth.

3.4.6 FC-A stabilizes a GCN1:14-3-3 complex and induces GCN1 turnover in cortical neurons

We first confirmed that FC-A-beads selectively precipitate GCN1 and that soluble FC-A blocks the interaction by western blot(Fig. 5a,b). FC-A beads precipitated a relatively large proportion of GCN1 compared to 14-3-3, likely reflecting the selective precipitation of a large pool of GCN1 which is incorporated into a relatively small pool of cliented 14-3-3s. Supporting this idea, R18 blocked the binding of GCN1 to the FC-A-beads,

demonstrating the 14-3-3-depndence of FC-A binding to GCN1. Moreover, we immunoprecipitated GCN1 and found that FC-A stimulates the co-precipitation of 14-3-3 (Fig. 5c). As 14-3-3s are known to sequester and affect the stability of client proteins(Tzivion et al., 2001), we assessed the effect of FC-A treatment on GCN1 expression in cortical neurons. Interestingly, we found a dose-dependent decrease in GCN1 expression levels (Fig. 5d,e). 14-3-3s can mediate proteasome-dependent turnover of client proteins(Qureshi et al., 2013; Toyo-oka et al., 2014; Wang et al., 2010) so we therefore assessed the effect of proteasome inhibition with MG132. This increased GCN1 levels and attenuated the FC-A-mediated decrease, suggesting that FC-A induces proteasomal degradation of GCN1 potentially via stabilization of a GCN1:14-3-3 complex(Fig. 5f,g).

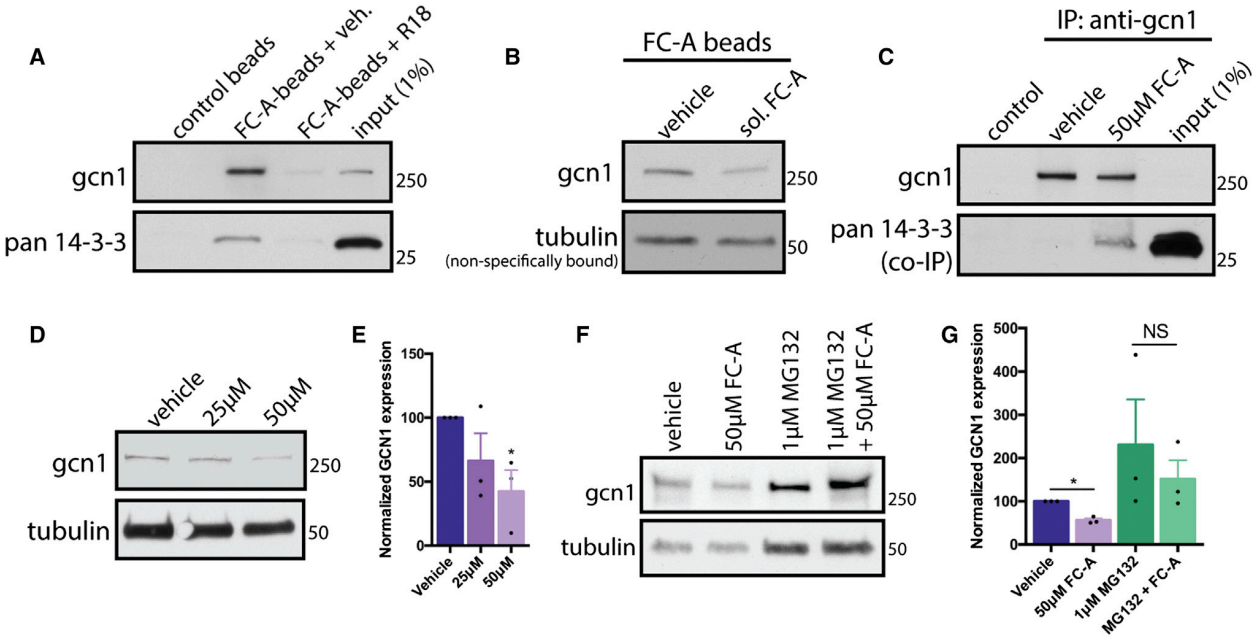

Figure 5. FC-A binds to GCN1 in a 14-3-3-dependent manner and induces GCN1 turnover. (A) GCN1 selectively binds to FC-A-beads and R18 blocks binding. (B) Soluble FC-A blocks the binding of GCN1 to FC-A-beads. (C) FC-A induces a co-immunoprecipitation of 14-3-3 with GCN1. (D,E) Western blot and densitometry showing that 24hr treatment of cortical neurons with FC-A induces a dose-dependent decrease in GCN1 expression (n=3, *p<0.05, Fisher's LSD). (F,G) Western blot and densitometry showing that co-treatment of cortical neurons with FC-A and the proteasome inhibitor MG132 attenuates FC-A-mediated GCN1 turnover (n=3, *p<0.05, One-way ANOVA Bonferroni's post-test). Data are presented as mean +SEM.

3.4.7 GCN1 loss-of-function contributes to FC-A-induced neurite outgrowth

To determine whether loss of GCN1 underlies FC-A-induced neurite outgrowth, we electroporated cortical neurons with a GCN1-targetting shRNA together with GFP and cultured the neurons in the presence of vehicle or FC-A. Knockdown of GCN1 significantly enhanced basal neurite outgrowth (Fig. 6a-c). The improved outgrowth of GCN1 knockdown neurons occluded any further enhancement by 25μM FC-A, but the neurons did respond to a higher dose of 100μM (Fig. 5c). This indicates that FC-A acts in part through GCN1 loss-of-function, releasing an intrinsic 'brake' on neurite outgrowth, but that other molecular pathways are also engaged by higher doses. Further supporting this model, 25μM FC-A is also capable of stimulating neurite outgrowth from a population of neurons with more than ~100μm baseline outgrowth (Fig. 6d). GCN1 is solely known for its role in reducing global translation by binding and activating the eIF2α kinase GCN2 in response to cell stress. GCN2-dependent phosphorylation of the eIF2α subunit on a conserved serine (S51) converts eIF2 to a dominant negative, reducing global translation(Sattlegger and Hinnebusch, 2000). We therefore postulated that because FC-A reduces GCN1 expression, this might lead to an increase in global translation that could be responsible for stimulating neurite outgrowth. We treated neurons with a combination of 25μM FC-A and cycloheximide (CHX) or anisomycin to block translation. Both CHX and anisomycin severely impaired neurite outgrowth, but did not fully block the stimulation of neurite outgrowth by 25μM FC-A (Fig. 6e,f), suggesting a mechanism that may be independent from protein synthesis. Moreover, we found that FC-A does not have any reproducible effect on eIF2α phosphorylation (Fig. 6g,h). Rapamycin also failed to block outgrowth induced by both FC-A and GCN1 knockdown (Fig. S5), suggesting a mechanism independent of mTOR, a well-established pro-regeneration pathway. Combined, these results indicate that 14-3-3 PPI stabilization with FC-A stimulates axon growth in part by stabilizing a 14-3-3:GCN1 complex, resulting in GCN1 downregulation (Fig. 6i).

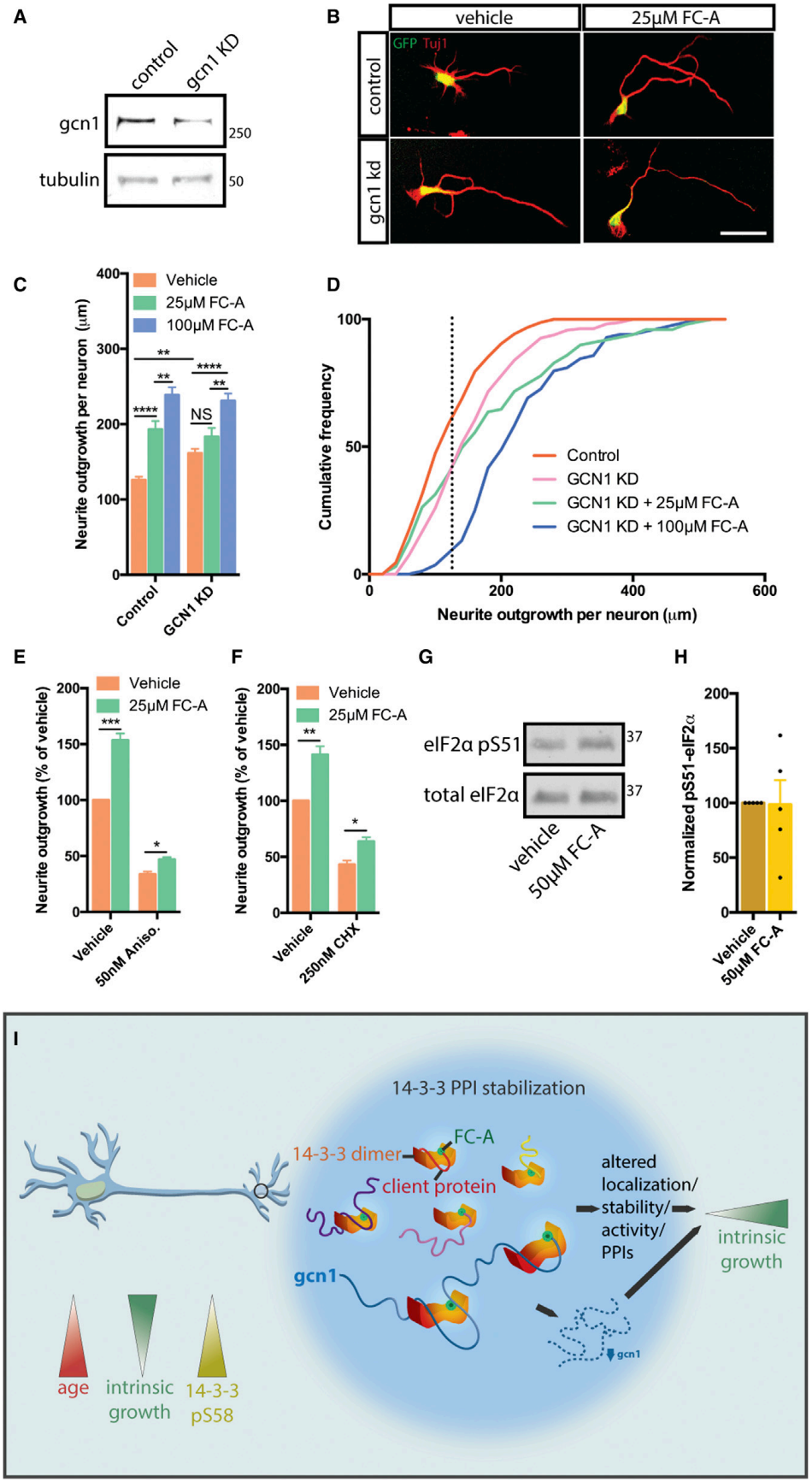
A
control
gcn1 KD
gcn1
250
tubulin
50
B
vehicle
25μM FC-A
control
gcn1 kd
GFP Tuj1
C
Vehicle
25μM FC-A
100μM FC-A
Neurite outgrowth per neuron (μm)
Control
GCN1 KD
NS
D
Cumulative frequency
Control
GCN1 KD
GCN1 KD + 25μM FC-A
GCN1 KD + 100μM FC-A
Neurite outgrowth per neuron (μm)
E
Neurite outgrowth (% of vehicle)
Vehicle
25μM FC-A
50nM Aniso.
F
Neurite outgrowth (% of vehicle)
Vehicle
25μM FC-A
250nM CHX
G
eIF2α pS51
total eIF2α
vehicle
50μM FC-A
H
Normalized pS51-eIF2α
Vehicle
50μM FC-A
I
14-3-3 PPI stabilization
14-3-3 dimer
FC-A
client protein
gcn1
altered localization/ stability/ activity/ PPIs
intrinsic growth
age
intrinsic growth
14-3-3 pS58

Figure 6. GCN1 knockdown enhances neurite outgrowth and contributes to FC-A-dependent growth. (A) Knockdown of GCN1 in cortical neurons (B,C) enhances baseline neurite outgrowth and blocks a further increase in outgrowth induced by 25μM FC-A, but not by 100μM FC-A (scale bar = 50μm). (D) Cumulative frequency distribution of neurite outgrowth, dashed line indicates separation of GCN1 KD and 25μM FC-A curves (n= 84-163 neurons, **p<0.01, ****p<0.0001, Two-way ANOVA Tukey's post-test). (E,F) Treatment of cortical neurons for 24hr with FC-A has no effect on eIF2α phosphorylation. (G,H) Anisomycin and cycloheximide (CHX) severely impair neurite outgrowth, but do not block FC-A-dependent outgrowth (n=3, *p<0.05, **p<0.01 Two-way ANOVA Bonferroni's post-test). Data are presented as mean +SEM. (I) Model of FC-A stabilization of 14-3-3 PPIs and stimulation of axon growth. Increase in 14-3-3 S58 phosphorylation during development contributes to a decline in intrinsic growth capacity. FC-A stimulates intrinsic growth capacity through downregulation of GCN1 and stabilization of 14-3-3:client protein complexes.

3.4.8 A single application of FC-A reduces corticospinal axon die-back after dorsal hemisection spinal cord injury

The damaged CNS is characterized by the presence of growth-inhibitors including chondroitin sulfate proteoglycans (CSPGs) produced by reactive glia. FC-A significantly improved neurite outgrowth on aggrecan substrates, suggesting it may be efficacious in improving growth after injury *in vivo* (Fig. S6). We therefore sought to determine whether FC-A could improve axon growth after a dorsal hemisection spinal cord injury, which transects the entire corticospinal tract (CST). Mice received an immediate local application of FC-A in a rapidly polymerizing fibrin gel onto the injury site. CST axons were then anterogradely traced by injecting biotinylated dextran amine (BDA) into the motor cortex and the mice were analyzed on day 21 (Fig. 7a). Mice that received a single treatment of FC-A had a significant reduction in axonal die-back away from the injury site compared to control mice treated with vehicle-containing fibrin gel. While the cut axons of control mice retracted an average distance of ~72μm, the end-bulbs of FC-A-treated mice were within the lesion or in close proximity to the lesion (Fig. 7b,c). These results indicate that transient exposure to FC-A is sufficient to diminish the collapse and retraction of severed axons *in vivo*.

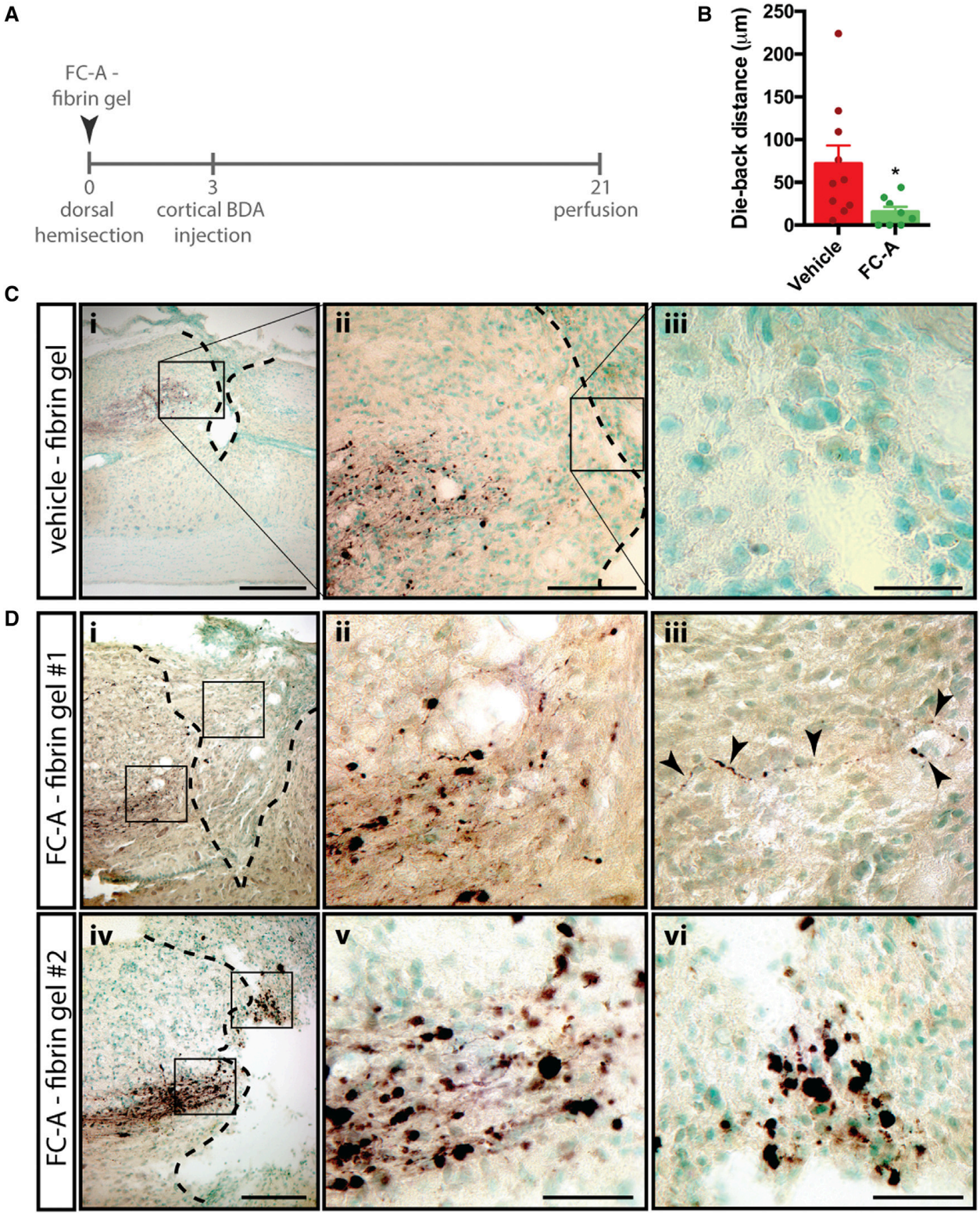

Figure 7. A single application of FC-A in a fibrin gel reduces corticospinal axon die-back after dorsal hemisection spinal cord injury. (A) Experimental timeline. (B) FC-A reduces average axon die-back distance (n=10 control, 8 FC-A-treated mice, *p<0.05, unpaired t-test). Data are presented as mean

+SEM. (C.i) Longitudinal section from a control vehicle-treated mouse spinal cord showing BDA-labelled corticospinal axons stopping proximal to the lesion (dotted line). Boxed area in C.i is shown at higher magnification in C.ii, and boxed area in C.ii is shown at higher magnification in C.iii (scale bar C.i = 400µm, C.ii = 100µm, C.iii = 50µm). (D.i-vi) Two FC-A –treated mice with axons and end bulbs within lesion or in close proximity to lesion border (dotted line). Boxed areas in D.i and D.iv are shown at higher magnification in D.ii, D.iii, and D.v, d.vi. Note BDA labeled axons that extend into the lesion (one is indicated by arrowheads in D.iii, scale bar D.i, D.iv = 200µm, D.ii, D.iii, D.v and D.vi = 50µm).

3.4.9 FC-A stimulates optic nerve regeneration

Next we determined whether FC-A could stimulate CNS axon regeneration past the lesion with longer therapeutic exposure using the optic nerve crush (ONC) model and intravitreal injections. To visualize FC-A distribution after intravitreal injection, we generated an Alexa-488 FC-A conjugate. 488-FC-A was enriched in the retinal ganglion cell layer 1 day after injection, suggesting that it is readily taken up by retinal ganglion cells (RGCs) (Fig. S7a). We also examined retinal GCN1 expression by western blot as a readout for activity. GCN1 was unchanged at 1 day post-injection, but significantly downregulated 3 days post-injection. Intriguingly, GCN1 levels remained suppressed 7 days post-injection, suggesting a sustained effect (Fig. S7b,c). We next determined whether FC-A could induce axon regeneration after ONC. Mice received either 1 or 2 intravitreal injections of FC-A(Fig. 8a). Cholera toxin β (CTβ) was used to trace RGC axons and GAP43 staining was used to visualize and quantify actively growing fibers. Treatment with a single injection of FC-A was insufficient to stimulate axon regeneration, however a second injection on day 7 resulted in significant regeneration at 100, 200 and 500µm from the lesion compared to control animals (Fig. 8b,c). Nearly all CTβ+ axons were also GAP43+, indicating that the axons were in a growth-state (Fig. 8d). We also assessed RGC density in the retina using Brn3a as a marker. As expected, ONC caused a massive loss of RGCs (~70% loss of RGCs). Interestingly, FC-A-did not improve RGC survival, indicating that FC-A stimulates axon regeneration independent of cell survival (Fig. 8e,f). These findings show that therapeutic administration of FC-A can stimulate axon regeneration after CNS injury and open the door to a new class of small molecules that could be further optimized to repair CNS damage.

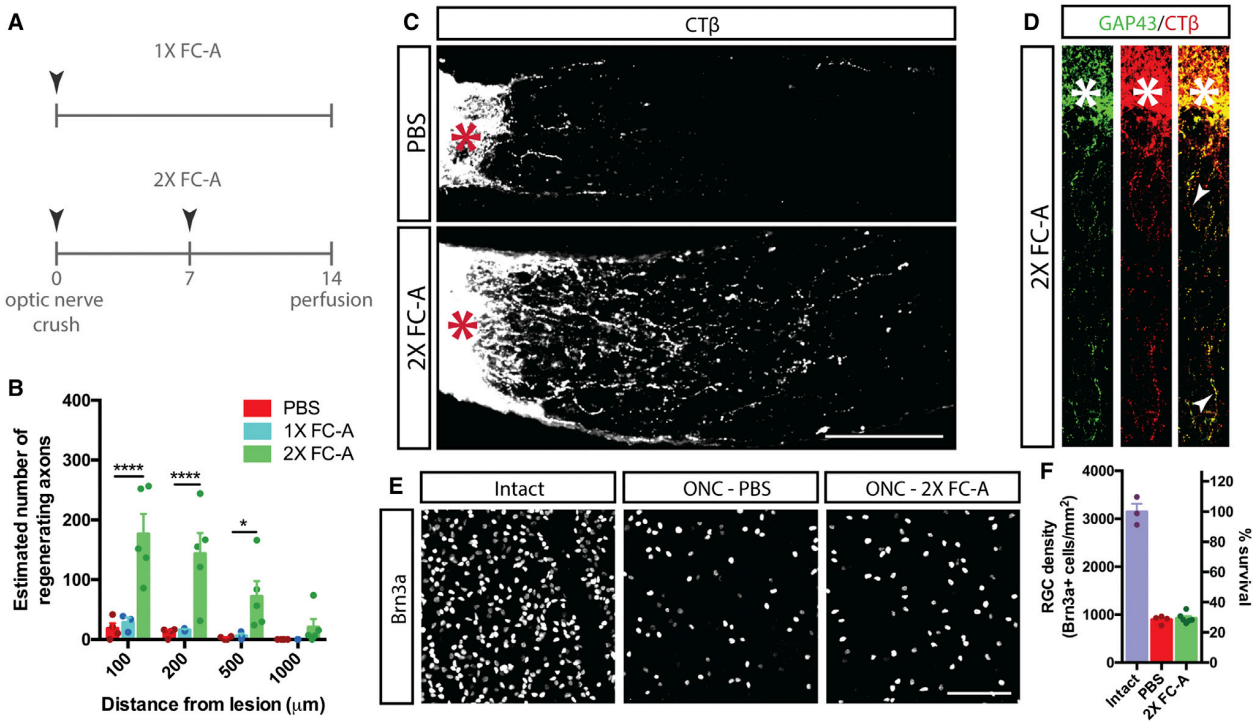

Figure 8. FC-A stimulates optic nerve regeneration. (A) Experimental timeline of optic nerve injury study. (B) Counts of GAP43+ regenerating axons (n=4 PBS control, 3 1X FC-A treated and 5 2X FC-A-treated mice, *p<0.05, ****p<0.0001, Two-way ANOVA, Dunnett's post-test, scale bar = 100μm). (C) Intravitreal injections of FC-A stimulate optic nerve regeneration (lesion site marked with asterisk, scale bar = 100μm). (D) GAP43 and CTB staining show double labeling of regenerating axons (lesion site marked with asterisk, arrowheads indicate double-positive axons). (E,F) Quantification of Brn3a+ retinal ganglion cell density in retinal flat mounts (n=3 intact, n=4 PBS control and 6 2X FC-A-treated mice, scale bar = 100μm). Data are presented as mean +SEM.

3.5 Discussion

We have found that 14-3-3 adaptor proteins facilitate neurite outgrowth and that 14-3-3 function is dynamically modulated by phosphorylation. Antagonism of 14-3-3 function by phosphorylation increases during development of the cortex, suggesting that this may dampen the intrinsic growth capacity of neurons. The similar effects we observed on neurite outgrowth by manipulating the expression of 14-3-3 β, γ, or ζ suggest functional redundancy. Moreover, because manipulating the expression of single isoforms was sufficient to elicit effects on neurite outgrowth, we speculate that there is a limited pool of 14-3-3 proteins that are competent to bind to clients and that there is an excess of clients available for 14-3-3 binding. Small changes in 14-3-3 abundance could therefore

have major impacts on cell signaling. While a neuroprotective role for 14-3-3s has been reported in nervous system injury models(Shimada et al., 2013; Yacoubian et al., 2010; Zhu et al., 2014), these results suggest that the promotion of 14-3-3 expression or activity may also enhance repair of damaged axons.

We have discovered that pharmacological stabilization of 14-3-3 PPIs with FC-A stimulates axon regeneration. A single treatment of the injured spinal cord prevents corticospinal fiber die-back, a mild but encouraging result that serves as proof-of-concept that even a brief exposure to FC-A after injury can provide benefit. We then show that more sustained dosing with FC-A is sufficient to induce axon regeneration past the lesion using the optic nerve crush model. Although modest, it is important to note that the effect on optic nerve regeneration is independent of RGC survival. A recent interesting study reported a benefit of similar magnitude with genetic overexpression of Armcx1, a regulator of mitochondrial motility, and this was accompanied by enhanced RGC survival(Cartoni et al., 2016). Moreover, extensive long-distance axon regeneration elicited by PTEN loss or overexpression of oncogenes is also accompanied by enhanced RGC survival(Belin et al., 2015; Sun et al., 2011). Our results suggest that manipulation of 14-3-3 PPIs combined with neuroprotective drugs could maximize axon regeneration. FC-A is a member of the fusicoccanes, a family of small molecules produced by plants and fungi. Cotylenin A, another member of this family, as well as semi-synthetic FC-A derivatives also act on 14-3-3 protein complexes(Anders et al., 2013; de Boer and de Vries-van Leeuwen, 2012). This new activity of FC-A may motivate the exploration of other fusicoccanes and derivatives for axon regeneration activity and the subsequent optimization of these small molecules.

We have identified GCN1 as an important target for FC-A-induced neurite outgrowth. Our results suggest that FC-A “glues” 14-3-3 to GCN1, mediating its degradation by the proteasome. It is also possible that FC-A-induced 14-3-3 binding to GCN1 impairs its interactions with other binding partners. The role of the interaction between 14-3-3 and GCN1 under baseline physiological conditions remains unknown. We found that GCN1 loss-of-function promotes neurite outgrowth, suggesting that it is an intrinsic inhibitor of neurite extension. GCN1 is solely known for its involvement in nutrient sensing and regulation of translation in response to stress by activating the eIF2α kinase GCN2(Sattlegger and Hinnebusch, 2000). It is interesting to speculate that GCN1 could also be engaged by injury-induced stress and that stress response pathways could be targeted to promote axon regeneration. It is also possible that GCN1 has an important

function in axon outgrowth and guidance during nervous system development, but this remains to be explored. Interestingly, it has been shown that knockdown of GCN2 enhances spontaneous neuritogenesis and that cultured neurons from GCN2 knockout mice have enhanced neurite outgrowth. Moreover, IMPACT, a protein that binds to GCN1 and antagonizes its interaction with GCN2, promotes neurite outgrowth(Roffe et al., 2013). However, the mechanisms by which these pathways regulate neurite outgrowth are unknown. Our results suggest that protein synthesis may not be required for FC-A-induced neurite outgrowth as both CHX and anisomycin failed to fully block neurite outgrowth induction. There are no known functions of GCN1 or GCN2 outside of the eIF2 pathway. Intriguingly, GCN1 harbors a series of HEAT repeats that span the length of the protein. HEAT repeats are domains which are thought to serve as a surface for PPIs and scaffolding(Neuwald and Hirano, 2000). It is also possible that GCN1-dependent activation of GCN2 leads to the phosphorylation of other substrates involved in axon growth and regeneration.

The full repertoire of 14-3-3 PPIs that are stabilized by FC-A has yet to be defined. Because FC-A persisted in stimulating neurite outgrowth from GCN1 knockdown neurons at a high 100μM concentration, it is likely that FC-A targets other 14-3-3:client complexes. Given the additional client proteins for which FC-A has been shown to stabilize 14-3-3 binding, we speculate that FC-A alters the 14-3-3 PPI network and has effects on multiple clients. FC-A selectively binds to both C-terminal and internal 14-3-3:client interfaces that create a compatible pocket, dependent on client protein motifs (Stevers et al., 2016b). Importantly, further selectivity for specific motifs can be achieved by chemically modifying the FC-A scaffold. For instance, a semisynthetic derivative of FC-A with a fused tetrahydrofuran ring (FC-THF) has been devised to be selective for 14-3-3:client complexes that form an empty pocket in the binding groove when 14-3-3 binds to the C-terminus of client proteins(Anders et al., 2013). The ability of such modifications to attain specificity for 14-3-3:client complexes raises the exciting possibility of designing compounds that can target specific 14-3-3 PPIs. Further discovery and optimization of compounds to target these interactions could result in the development of new tools to study 14-3-3s and new leads for drug development.

3.6 Author Contributions

Conceptualization, A. Kaplan and A.E. Fournier; Experiments, A. Kaplan, B. Morquette, A. Kroner, S.Y. Leong, C. Madwar, S.L. Banerjee and Ricardo Sanz; Writing, A. Kaplan and A.E. Fournier; Supervision, A.E. Fournier, S. David, N. Bisson and J. Antel.

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3.8 Competing financial interests

The authors declare no competing financial interests.

3.9 Materials and methods

3.9.1 Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-pan-14-3-3	Santa Cruz	Cat#sc-1657; RRID: AB_626618
anti-pan-14-3-3-HRP	Santa Cruz	Cat#sc-1657 HRP; RRID: AB_626618
anti-14-3-3β	Santa Cruz	Cat#sc-25276; RRID: AB_626617
anti-14-3-3γ	Santa Cruz	Cat#sc-731; RRID: AB_2217962
anti-14-3-3ζ	Santa Cruz	Cat#sc-1019; RRID: AB_2218378
anti-14-3-3ζ phospho-S58	Abcam	Cat#ab51109; RRID: AB_867443
anti-myc (clone 9E10)	Sigma	Cat#M4439; RRID: AB_439694
anti-GCN1L1	Abcam	Cat#ab86139; RRID: AB_1925025
anti-Brn3a	Santa Cruz	Cat#sc-31984; RRID: AB_2167511
anti-GAP43	Novus	Cat#NBP1-41123; RRID: AB_10005026
Anti-Tau-1 (clone PC1C6)	Millipore	Cat#IHCR1015-6; RRID: AB_2139842
Anti-betaIII tubulin (clone Tuj1)	BioLegend	Cat# 801201 or 801202; RRID: AB_2313773 or RRID: AB_10063408
Alexa 488 anti-mouse	Thermo Fisher	Cat#A11029; RRID: AB_138404
Alexa 568 anti-mouse	Thermo Fisher	Cat#A11031; RRID: AB_144696
Alexa 647 anti-mouse	Thermo Fisher	Cat#A21235; RRID: AB_141693
Alexa 488 anti-rabbit	Thermo Fisher	Cat#A11034; RRID: AB_2576217
Alexa 568 anti-rabbit	Thermo Fisher	Cat#A11011; RRID: AB_143157
Alexa 647 anti-rabbit	Thermo Fisher	Cat#A21244; RRID: AB_141663
Alexa 568 anti-goat	Thermo Fisher	Cat#A11057; RRID: AB_14258
Alexa 488 anti-sheep	Thermo Fisher	Cat#A11015; RRID: AB_141362
Anti-mouse HRP	Jackson ImmunoResearch	Cat#115-035-071; RRID: AB_2338506
Anti-Rabbit HRP	Jackson ImmunoResearch	Cat#111-035-046; RRID: AB_2337939
Bacterial and Virus Strains		
3rd generation lentiviral system	Kent et al., 2010	N/A
Biological Samples		
Human fetal brain tissue	Human Fetal Tissue Repository (Albert Einstein College)	N/A
Chemicals, Peptides, and Recombinant Proteins		
Fusicoccin-A	Santa Cruz or Enzo	Cat#BML-EI334 or Cat#sc-200754
BV02 (14-3-3 antagonist II)	Millipore	Cat#100082
Y27632	Millipore	Cat#688001
R18 peptide	Enzo	Cat#BML-P214
Alexa 594 Cholera toxin beta	Thermo Fisher	Cat#C22842
SiMAG-hydrazide beads	Chemicell	N/A
Aggrecan	Sigma	Cat#A1960
KT5720	Sigma	Cat#K3761
Rapamycin	Sigma	Cat#R0395
Alexa 568 phalloidin	Thermo Fisher	Cat# A12380
Biotinylated dextran amine (10kDa MW)	Molecular probes	Cat#D-1956
DAB tablets	Sigma	Cat#D-5905
ABC Kit	Vector Laboratories	Cat#PK-6100
Alexa 488 hydrazide	Thermo Fisher	Cat#A10436

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
Evicel Fibrin Sealant Kit	Ethicon	Cat#3901
In situ cell death detection kit TMR red	Roche	Cat#12156792910
Experimental Models: Cell Lines		
Primary cortical neurons – E18/19 Sprague Dawley rat	Charles River Labs	N/A
Primary cortical neurons – E16/17 CD-1 mouse	Charles River Labs	N/A
Experimental Models: Organisms/Strains		
C57BL/6 mice	Charles River Labs	N/A
Recombinant DNA		
pRRLsinPPT- 14-3-3β shRNA	Kent et al., 2010	N/A
pRRLsinPPT- 14-3-3γ shRNA	Kent et al., 2010	N/A
pRRLsinPPT- 14-3-3ζ shRNA	Kent et al., 2010	N/A
pRRLsinPPT- myc-14-3-3β	Kent et al., 2010	N/A
pRRLsinPPT- myc-14-3-3γ	Kent et al., 2010	N/A
pRRLsinPPT- myc-14-3-3ζ	Kent et al., 2010	N/A
pMDLg	Kent et al., 2010	N/A
pMD2g	Kent et al., 2010	N/A
pRSV-Rev	Kent et al., 2010	N/A
pCS4- WT 14-3-3ζ	Gu et al., 2006	N/A
pCS4- 14-3-3ζ S58A	Gu et al., 2006	N/A
pCS4- 14-3-3ζ S58E	Gu et al., 2006	N/A
pEYFP-C1-Difopein	Masters and Fu, 2001	N/A
pLKO- GCN1 shRNA	Sigma MISSION library	Cat#TRCN0000251695
pRRL-EGFP	Kent et al., 2010	N/A
Software and Algorithms		
MetaXpress – Automated Neurite Outgrowth Module	Molecular Devices	N/A
Prism 6	GraphPad	RRID: SCR_002798
ImageJ	NIH	RRID: SCR_003070
Other		
ImageXpress – Automated High Content Imaging System	Molecular Devices	N/A
Nucleofector	Lonza	AAB-1001

3.9.2 Primary rodent neuron cultures

All studies were approved by the McGill University Animal Care and Use Committee. For cortical neuron cultures, cortices were dissected from E18-19 Sprague Dawley rat or E16-17 CD-1 mouse in cold Leibovitz L15 medium (Thermo Fisher). Isolated cortices were incubated in 0.25% trypsin-EDTA and dissociated into a single cell suspension by gentle trituration. Neurons were seeded onto culture dishes coated with 100μg/mL poly-l-lysine (PLL). Culture medium consisted of Neurobasal medium supplemented with 2% B27, 1% N2, 1% penicillin/streptomycin and 2mM L-glutamine.

3.9.3 Human fetal neuron culture

Human fetal brain tissue (16–18 gestational weeks) was acquired from the Human Fetal Tissue Repository (Albert Einstein College of Medicine, Bronx, NY) and was dissociated and cultured as previously described(Leong et al., 2015).

3.9.4 Optic nerve injuries, intravitreal injections and tissue processing

Adult male and female C57BL/6 mice (8-14 weeks of age) were anesthetized with isoflurane and an incision was made above the left orbit. Under a surgical microscope, the extra-ocular muscles were resected to expose the underlying optic nerve. The optic nerve was crushed 0.5-1mm behind the optic nerve head with fine forceps (Dumont #5) for 10 seconds. Care was taken to avoid damaging the ophthalmic artery. Vascular integrity of the retina was assessed by a fundus examination. For intravitreal injections, a small puncture was made in the sclera with a 30-gauge needle and 2μL of PBS or FC-A (1μg/μL solution in PBS) was injected into the wound using a Hamilton syringe. The needle was held in place for ~2 minutes to avoid reflux and the puncture was sealed with surgical glue. Animals that had significant reflux were excluded from the study. Injections of PBS or FC-A were performed immediately after injury and again 7 days after injury. Mice were transcardially perfused with 4% PFA on day 14. Optic nerves and eyes were harvested and post-fixed in 4% PFA for 2hr. Retinal flat mounts were prepared and stained with anti-Brn3a as previously described(Morquette et al., 2015). Optic nerves were cryoprotected in 30% sucrose-PBS at 4°C overnight and embedded in OCT. Longitudinal sections (14μm thickness) were collected on slides, permeabilized and blocked with 0.3% Triton-X in 5% BSA for 1hr at room temperature, stained with anti-GAP43 overnight at 4°C, washed 3 times with PBS and stained with FITC-conjugated secondary antibody.

3.9.5 Dorsal hemisection spinal cord injuries

Adult female C57BL/6 mice (8-10 weeks of age) were deeply anesthetized with ketamine:xylazine:acepromazine (50:5:1mg/kg) and a laminectomy was done to expose the T9 thoracic spinal cord. Dorsal hemisection of the spinal cord was performed with spring micro-scissors to cut through the central canal. 200μg FC-A solubilized in a 1:3 stock of ethanol:PBS was diluted in a thrombin solution into a final volume of 25μL, quickly mixed with 25μL of a fibrinogen solution and immediately applied directly onto the injury site, forming a viscous gel. Thrombin and fibrinogen solutions were purchased

as a kit (Evicel Fibrin Sealant). Cortical injections of biotinylated dextran amine (BDA) and processing of tissue to analyze corticospinal tract axons was performed as previously described(Fry et al., 2010). Scar borders were determined from the methyl green stain as previously shown (Fry et al., 2010). Quantification of axonal die-back distance was assessed blinded to the experimental condition by measuring and averaging the distances between the 5 closest end-bulbs from the edge of the lesion.

3.9.6 Lentiviral production and infection

Lentiviruses were produced in HEK 293T cells as previously described(Ritter et al., 2007). Rat cortical neurons were infected overnight at a multiplicity of infection (MOI) of 5 for knockdown or 2-3 for overexpression of 14-3-3 isoforms. The following day, the medium was replaced and half was replenished every 3-4 days thereafter.

3.9.7 Neurite outgrowth assays

Cortical neurons were seeded in 96-well plates at 7,000 cells per well. After 2hr, medium was replaced with fresh culture medium containing vehicle or indicated doses of FC-A, Y-27632, BV02, KT5729, or Rapamycin. On day 1 of culture (DIV1) the neurons were fixed with 4% paraformaldehyde/20% sucrose, permeabilized with 0.2% Triton-X, blocked with 5% BSA, and stained with anti-βIII tubulin, Hoechst 33342 (1:10000 dilution, Sigma) and fluorescent secondary antibodies. For human fetal neurite outgrowth, cultures were treated with FC-A on DIV2 and fixed and stained on DIV4. For re-seeding of neurons infected with lentiviruses, neurons in 6-well plates were incubated with 0.125% trypsin in Neurobasal medium for 10min at 37°C, gently triturated, replated in 96-well plates at 7,000 cells per well, treated with vehicle or 25μM FC-A and grown for an additional 3 days before fixing and staining. For overexpression of WT, S58A and S58E 14-3-3ζ, dissociated rat cortical neurons were electroporated with a 2μg:1μg mixture of 14-3-3 plasmid and a plasmid encoding EGFP and fixed and stained on DIV2; for knockdown of GCN1, mouse cortical neurons were electroporated with a 2μg:1μg mixture of GCN1 shRNA plasmid and a plasmid encoding EGFP and fixed and stained on DIV3. Electroporations were performed using the Nucleofector kit, Lonza, program O-003 for rat or O-005 for mouse neurons in Mirus electroporation solution (MIR50111). Automated image acquisition and analysis were performed using an ImageXpress system and the neurite outgrowth module of the MetaXpress software (Molecular Devices).

3.9.8 Scratch assays

Rat cortical neurons were seeded in 96-well plates at 30,000 cells per well, half the medium was replenished every 3-4 days and on DIV10, a scratch was made across the center of each well with a plastic P10 pipet tip. Immediately after scratching, compounds were added to the wells. The following day, the cultures were fixed with 4% paraformaldehyde/20% sucrose, permeabilized with 0.2% Triton-X, blocked with 5% BSA, and stained with anti-βIII tubulin, Alexa-568-phalloidin (Thermo Fisher, A12380, 1:1000) and fluorescent secondary antibodies. Images of βIII tubulin stained axons were acquired in the center of each scratch and thresholded in ImageJ. The outline of each scratch was traced and the percent area of βIII tubulin stained neurites in the central ~70% of the scratch was measured in ImageJ.

3.9.9 FC-A-bead production

For conjugation of FC-A to beads, the vinylic carbon on the sugar group of FC-A was oxidized to generate an aldehyde as previously described(Feyerabend and Weiler, 1987). Briefly, FC-A dissolved in tetrahydrofuran (THF) was oxidized with OsO_4 and $NaIO_4$ to generate FC-A-aldehyde. The following day, the product was loaded onto a Sep Pak C18 cartridge (Millipore Waters) and eluted with methanol. The efficiency of FC-A-aldehyde formation was determined by thin layer chromatography and the FC-aldehyde was then incubated with magnetic SiMAG hydrazide-beads (Chemicell) to couple the FC-A-aldehyde to the beads through the formation of hydrazone bonds. Unreacted sites were blocked with glyceraldehyde. Magnetic beads coupled to glyceraldehyde were used as control beads.

3.9.10 Affinity chromatography with FC-A-beads

Rat cortical neurons were plated in 10cm dishes and on DIV7 the neurons were lysed in ice cold lysis buffer composed of 100mM NaCl, 50mM Tris-HCl, 5mM EDTA, 2mM $MgCl_2$, 10% glycerol and 1% NP-40 supplemented with protease inhibitors (Complete protease inhibitor cocktail, Roche) and phosphatase inhibitors (10mM sodium fluoride and 1mM sodium orthovanadate). Lysates were clarified by centrifugation and protein concentration was determined by DC protein assay (Bio-Rad). 1.5mg of lysate was added to 30μL pre-washed control beads or FC-A-beads in 500μL lysis buffer. Lysates were incubated with the beads and vehicle or 10μM R18 or 100μM FC-A for 2hr at 4°C

with rotation and subsequently washed 3 times with lysis buffer and boiled in 2X SDS sample buffer for western blot analysis.

3.9.11 Mass spectrometry analysis

For mass spectrometry, FC-A beads were washed 5 times and resuspended in 50mM ammonium bicarbonate pH 8.5 with 1µg of trypsin (Promega) and incubated at 37°C overnight with agitation. The next day, an additional 1 µg of trypsin was added and samples were incubated for 3 h at 37°C. Beads were pelleted and the supernatant was transferred to a fresh tube. Beads were then rinsed two times with 100 µl of MS-grade water. All supernatants were combined and dried in a vacuum centrifuge. Tryptic peptides were resuspended in 15µL of 5% formic acid; 5µL were used per analysis.

An Orbitrap Fusion mass spectrometer equipped with a nanoelectrospray ion source (Thermo Scientific) and coupled to a UltiMate 3000 nanoRSLC (Dionex/Thermo) was used for peptide analyses. Mass spectra were acquired using a data dependent acquisition mode using Thermo XCalibur software version 3.0.63. Full scan mass spectra (350 to 1800m/z) were acquired in the orbitrap using an automatic gain control target of 4e5, a maximum injection time of 50ms and a resolution of 120 000. Selected ions were isolated using the quadrupole analyzer in a window of 1.6m/z and fragmented by higher energy collision-induced dissociation (HCD) with 35% of collision energy. The resulting fragments were detected by the linear ion trap at a rapid scan rate. Dynamic exclusion of previously fragmented peptides was set for a period of 20sec and a tolerance of 10ppm.

All MS/MS peak lists were generated using Thermo Proteome Discoverer version 1.4.0.288 (Thermo Scientific). MGF sample files were then analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1). Both were set up to search Uniprot Rattus norvegicus database (November 2014 release, 37218 entries) assuming the digestion enzyme trypsin. They were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10ppm. Carbamidomethyl of cysteine was specified as a fixed modification. Deamidated of asparagine and glutamine, oxidation of methionine and phospho of serine, threonine and tyrosine were specified as variable modifications. Two miscleavages were allowed.

3.9.12 MS data analysis

Scaffold (version 4.0.1, Proteome Software Inc.) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve an FDR less than 1.0% and contained at least 1 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (PMID 14632076). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

MS data were analyzed with SAINTexpress, a simplified version of the Significance Analysis of INTeractome method described previously (PMID 24513533) via the CRAPome website (PMID 23921808). SAINT probabilities were calculated using MS data from control beads. The average probability is reported as the final SAINT score in the table. Preys with an average greater or equal to 0.9 were considered "true" interactors (Table S1).

3.9.13 Co-immunoprecipitation

Rat cortical neurons were lysed in ice cold lysis buffer composed of 100mM NaCl, 50mM Tris-HCl, 5mM EDTA, 2mM $MgCl_2$, 10% glycerol and 0.5% NP-40 supplemented with protease inhibitors phosphatase inhibitors. 1.5mg of pre-cleared total cell lysate was incubated with protein A/G beads (Santa Cruz, sc-2003) and 1μg of anti-GCN1L1 and vehicle or 50μM FC-A for 2hr at 4°C with rotation and subsequently washed 3 times with lysis buffer and boiled in 2X SDS sample buffer for western blot analysis. Blots were probed with anti-GCN1L1 and anti-pan-14-3-3-HRP.

3.9.14 Optic nerve regeneration and RGC density quantification

The number of regenerating axons visualized by GAP43 staining were counted at distances of 100, 200, 500 and 1000μm from the lesion. The estimated total number of regenerating axons Σa_d per nerve with a radius r, in a section with thickness t, at a distance d from the lesion was determined using the formula $\Sigma a_d = \pi r^2$ x (axons/mm width)/t, as previously described(Yin et al., 2003). Axon counts were performed blinded to the experimental condition from 3-4 sections per mouse. Mice that had GAP43+ axons near the optic chiasm reflecting spared fibres were excluded from the study. To

determine the RGC density per mm^2, Brn3a+ RGCs were counted as previously described(Morquette et al., 2015)

3.9.15 Quantification and Statistical Analysis

All statistical tests were performed with GraphPad Prism 6. As indicated in figure legends, the following statistical tests were used: unpaired student's t-test with Welch's correction, one-way ANOVA with Dunnett, Bonferroni or Fisher's LSD post-test, Two-way ANOVA with Dunnett or Bonferroni post-test. Significance was defined as $p<0.05$.

3.10 Supplementary Figures

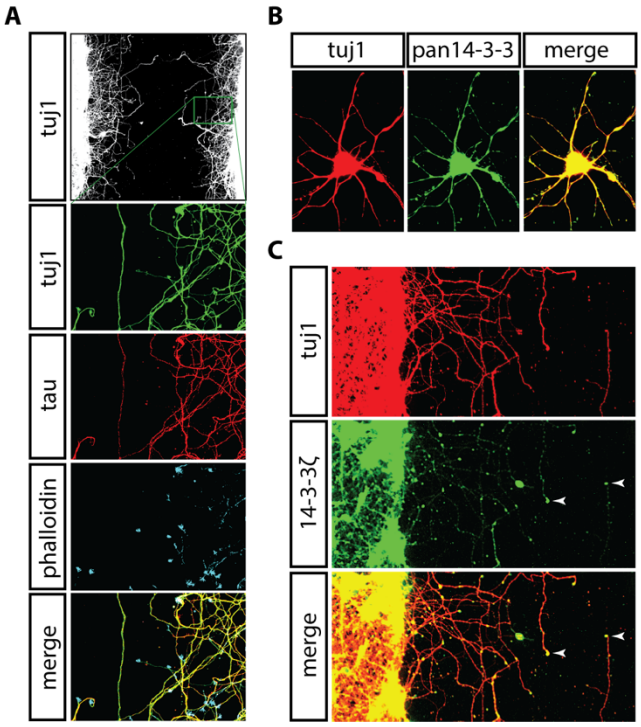

Figure S1. Regenerating neurites after scratch injury are Tau-positive axons and 14-3-3 is enriched at re-growing tips. (A) 1day following scratch injury, the majority of regenerating cortical neurites are Tau positive, with actin-rich growth cone tips. (B) 14-3-3s are localized throughout the soma and neurites in mature cortical neuron cultures. (C) 14-3-3ζ is enriched at growing tips of axons after scratch injury.

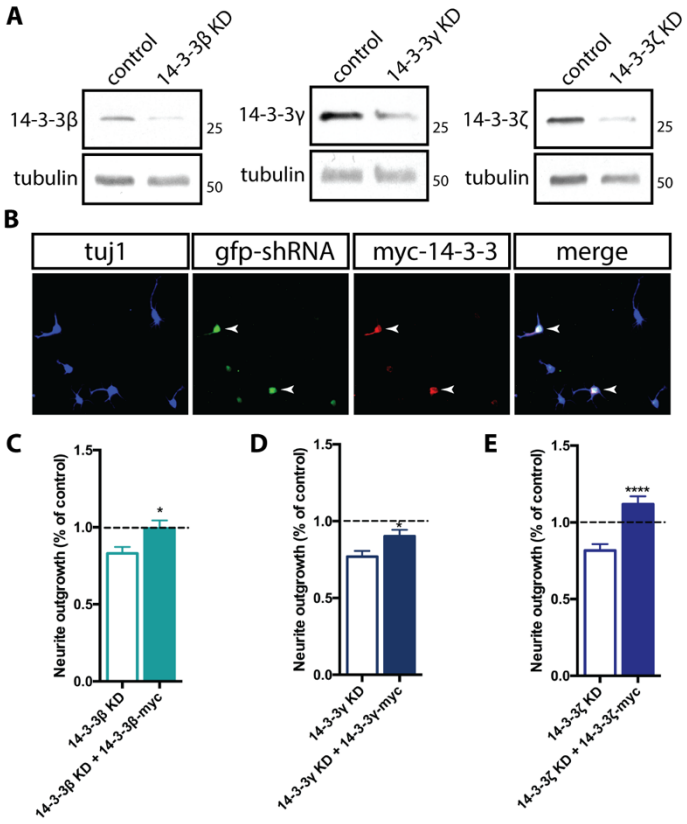

Figure S2. Expression of RNAi-resistant myc-tagged 14-3-3s rescues outgrowth defect in 14-3-3 knockdown neurons. (A) Western blots showing 14-3-3 knockdowns in cortical neurons. (B) Co-expression of GFP-tagged shRNA plasmid and myc-tagged 14-3-3s in cortical neurons. (C-E) Expression of myc-tagged 14-3-3s restores baseline levels of neurite outgrowth in 14-3-3 knockdown neurons (n=87-102 neurons from 3 experiments, *p<0.05, ****p<0.0001, unpaired t-test). Data are presented as mean +SEM.

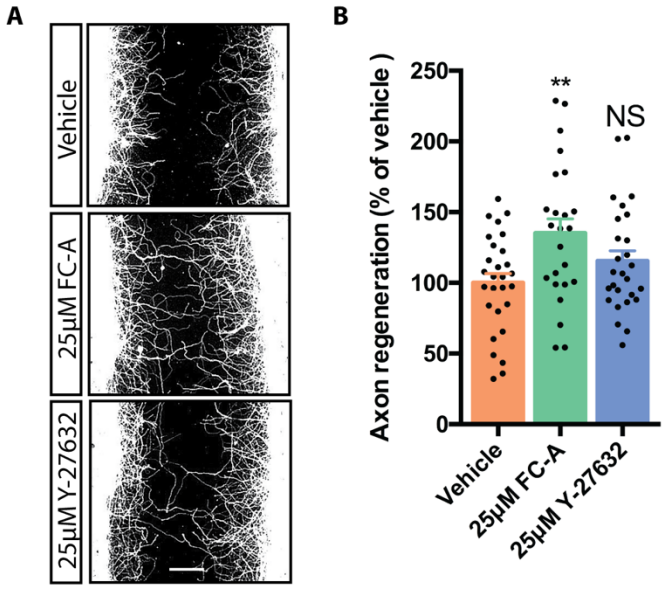

Figure S3. FC-A stimulates axon regeneration more potently that Y-27632 in scratch assay. (A,B) Cortical neurons were scratch injured and treated with 25μM FC-A or 25μM Y-27632 (n=24-28 scratches from 3 experiments, **p<0.01, One-way ANOVA Dunnett's post-test, scale bar = 200μm). Data are presented as mean +SEM.

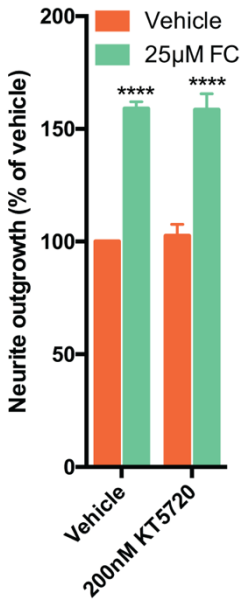

Figure S4. PKA inhibition does not affect FC-A-induced neurite outgrowth. Cortical neurons were cultured in the presence of 25μM FC-A with vehicle or 200nM KT5720 for 24hr (n=3, ****p<0.0001, Two-way ANOVA Bonferroni's post-test). Data are presented as mean +SEM.

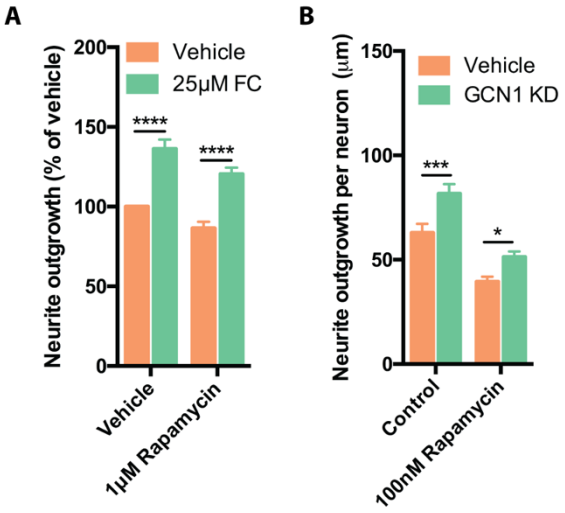

Figure S5. FC-A and GCN1 regulation of neurite outgrowth are independent of mTOR. (A) Co-treatment of cortical neurons with FC-A and rapamycin for 24hr has no effect on FC-A-stimulated growth (n=5 experiments, ****p<0.0001, Two-way ANOVA Bonferroni's post-test). (B) Treatment of neurons with rapamycin for 3days impairs neurite outgrowth, but does not block outgrowth induced by GCN1 knockdown (n=48 neurons from 3 experiments, *p<0.05, ***p<0.001, Two-way ANOVA Bonferroni's post-test). Data are presented as mean +SEM.

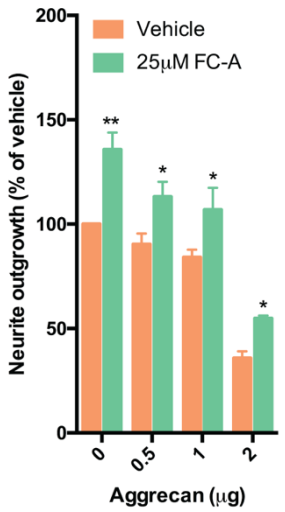

Figure S6. FC-A promotes cortical neurite outgrowth on aggrecan substrates. 7DIV cortical neurons were re-seeded onto aggrecan coated substrates in the presence of vehicle or 25μM FC-A (n=3, *p<0.05, **p<0.01, Two-way ANOVA Bonferroni's post-test). Data are presented as mean +SEM.

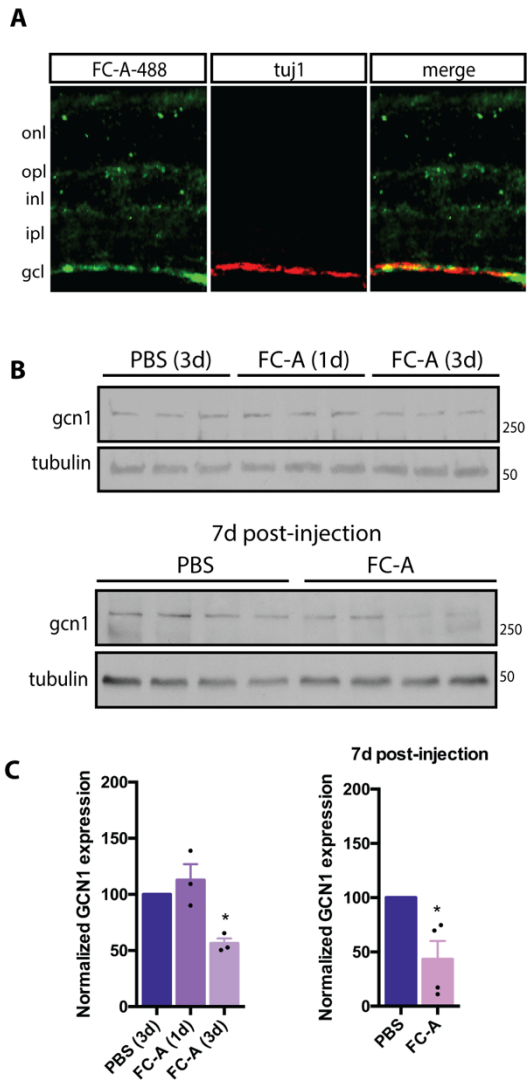

Figure S7. FC-A reduces GCN1 expression in the retina after intravitreal injection. (A) Alexa-488 conjugated FC-A is enriched in the retinal ganglion cell layer (marked by Tuj1) 24hr after intravitreal injection (gcl: ganglion cell layer, ipl: inner plexiform layer, inl: inner nuclear layer, opl: outer plexiform layer, onl: outer nuclear layer). (B,C) Western blot and densitometry showing that intravitreal injection of FC-A induces GCN1 downregulation in retinas 3d and 7d post-injection (n=3-4, *p<0.05, One-way ANOVA Fisher's LSD). Data are presented as mean +SEM.

	Accession #	Protein	SAINT	Total peptide counts Expt. 1 FC	Expt. 1 C	Expt. 2 FC	Expt. 2 C	Expt. 3 FC	Expt. 3 C	C-terminal amino acids	# of internal $RX_{1-3}[ST][ILVA]_2$
1	P62632	Elongation factor 1 alpha-2 (EEF1A2)	1.0	50	0	18	0	17	0	QKAGK	1
2	P16970	ATP-binding cassette D3 (ABCD3)	1.0	7	1	12	0	9	0	VEFG**S**	1
3	Q7TT49	MRCK beta Kinase (CDC42BPB)	1.0	28	6	22	1	23	4	PACDA	1
4	Q5XIH7	Prohibitin 2 (PHB2)	1.0	40	0	6	0	5	0	IKGKK	5
5	P67779	Prohibitin (PHB)	0.99	34	1	6	1	7	0	LQLPQ	0
6	Q9Z214	Homer 1 (HOMER1)	0.99	10	1	6	0	7	1	LLEC**S**	0
7	G3V846	Amino acid transporter EAAT1 (SLC1A3)	0.97	9	2	10	2	10	2	SE**T**KM	1
8	Q6JAM9	Transmembrane protein 35 (TMEM35)	0.96	6	1	3	0	4	0	KVKV**S**	1
9	Q5U2N3	Phosphatidylinositol transfer protein 1 (PITPNM1)	0.94	9	1	14	1	10	4	LD**S**EE	1
10	B2GUY8	Peroxisomal membrane protein (SLC25A17)	0.93	2	0	2	0	3	0	S**S**HKH	1
11	F1LRI5	Translational activator GCN1 (GCN1L1)	0.92	49	1	4	1	9	1	D**T**IL**T**	10
12	M0R6E0	Phospholipid-transporting ATPase (ATP9A)	0.9	6	1	11	1	7	2	SKL**TS**	2
13	F1LV44	Teneurin 3 (TENM3)	0.9	2	0	2	0	2	0	EIGKR	1
14	P38718	Mitochondrial pyruvate carrier 2 (MPC2)	0.9	2	0	2	0	2	0	SKGIQ	1

Putative internal motif(s) only
Putative C-terminal motif
Known motif

Table S1. Mass spectrometry identification of FC-A-binding proteins in cortical neuron lysate. Pull-downs from cortical neuron lysate with FC-A and control beads were performed in 3 independent experiments. Bound proteins were identified by mass spectrometry and analyzed by 'significance analysis of interactome' (SAINT).

4 Chapter 3. A novel fusicoccin-A derivative stimulates neurite outgrowth and axon regeneration

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This is a manuscript in preparation for publication.

4.1 Preface

In this chapter, we follow up on our previous study by embarking on a structure activity relationship (SAR) campaign to enhance the potency and improve the properties of FC-A for axon regeneration. Using high content screening technology, we identify a series of potent novel derivatives. We further explore the effects of these compounds on neurite outgrowth and assess their pharmacokinetics after systemic administration in mice. Our results suggest new avenues for further development of these compounds for CNS injury applications.

4.2 Abstract

Axons are cellular projections that link neurons into functional circuits. In the central nervous system (CNS), axons do not spontaneously regenerate after injury. This underlies persistent deficits in neurological function after traumatic spinal cord injuries, nerve injuries or in certain neurodegenerative conditions. Therapeutic stimulation of axon regeneration could be an effective means to treat these conditions. We have previously shown that the natural product fusicoccin-A (FC-A) stimulates neurite outgrowth in cultured neurons and induces axon regeneration upon local administration after traumatic optic nerve injury in mice. FC-A targets 14-3-3 proteins, a cytosolic family of adaptor proteins with a multitude of roles in cell signaling. Here we explore structure activity relationship of FC-A using a high content neurite outgrowth screen. We identify a series of potent derivatives that cross the blood-brain-barrier, suggesting new routes for further development of these compounds for CNS injury indications.

4.3 Introduction

Neurological dysfunction resulting from spinal cord and nerve trauma is due to axonal damage and the disruption of neural circuitry. Damaged axons in the central nervous system (CNS) fail to spontaneously regrow, making this a key target for therapies aimed at restoring motor and sensory function. A major culprit in the failure of axons to regenerate is thought to be a low neuron-intrinsic capacity for initiating and sustaining a regenerative response after injury(He and Jin, 2016). Strategies aimed at enhancing intrinsic growth by knockout of tumor suppressor genes or overexpression of oncogenes result in robust axon regeneration in mouse models of spinal cord or optic nerve injury(Belin et al., 2015; Liu et al., 2010; Park et al., 2008). This suggests that switching on a growth state could aid the repair of damaged axons. However, there is an ongoing need for the identification of specific molecular targets and drugs that can induce axon regeneration.

14-3-3s are a family of adaptor proteins that promote axon outgrowth and regulate axon pathfinding during development(Kent et al., 2010; Yam et al., 2012; Yoon et al., 2012). These proteins are thought of as signaling hubs because they bind hundreds of 'client proteins' to regulate their functions in several different ways, including altering their stability, activity and localization(Kaplan et al., 2017c). 14-3-3s bind to client proteins at phosphorylated serine/threonine motifs via a central groove, which is highly conserved through evolution and among the seven isoforms in humans(Kaplan et al., 2017a). Pharmacological inhibition of 14-3-3 binding to client proteins with peptides or small molecules that compete for the groove impairs neurite outgrowth, whereas overexpression promotes growth(Kaplan et al., 2017b; Lavalley et al., 2016; Mar et al., 2014b; Yoon et al., 2012). This suggests that stimulating the function of these proteins could be used to stimulate axon growth.

Fusicoccanes are a family of natural product small molecules that stabilize the binding of 14-3-3 to client proteins(Stevers et al., 2017b). These compounds are characterized by a 5-8-5 fused ring scaffold (Fig. 1). Fusicoccanes bind to a pocket in the 14-3-3 groove that is created when certain client proteins bind within the groove. The simultaneous binding of the compound to both proteins forms a stable tri-partite complex, enhancing the affinity of the 14-3-3:client interaction. We previously showed that fusicoccin-A (FC-A) stimulates neurite outgrowth, alleviates axonal die-back in a mouse model of spinal cord injury, and induces axon regeneration in a mouse model of

optic nerve injury(Kaplan et al., 2017b). Further optimization of FC-A through chemical modification could result in higher potency compounds with improved properties for therapeutic development. Here we explore the structure activity relationship of FC-A and describe the effects of novel derivatives on neurite outgrowth, suggesting potential routes for further development.

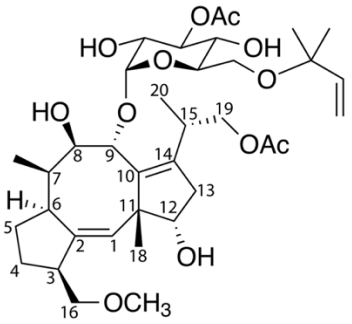


Figure 1. Fusicoccin-A (FC-A).

4.4 Results

4.4.1 Enhanced potency of FC-A 19-amide derivatives in neurite outgrowth

To explore structure activity relationship (SAR) of FC-A in neurite outgrowth, we screened a library of 35 semisynthetic derivatives prepared using FC-A purified from *Phomopsis amygdali* cultures as starting material (Fig. 2A). Cortical neurons from E18 rat embryos were plated at low density in 96-well plates and compounds were added to the wells in duplicates at a single 10μM concentration in three independent experiments. The rho kinase inhibitor Y27632 was used as a positive control. After 24 hours of neurite outgrowth, the neurons were fixed and stained for beta III tubulin, a neuron-specific tubulin isoform that serves as a marker of cell morphology. Using a high-content screening system, images were acquired and analyzed for neurite outgrowth in a fully automated fashion, permitting the analysis of thousands of neurons in each condition. The resulting effects of the derivatives on neurite outgrowth were analyzed, revealing insight into SAR. Aglycone derivatives were the least potent. This is consistent with previous work which has shown that fusicoccane aglycones are less potent 14-3-3 PPI stabilizers(Anders et al., 2013). Four compounds with markedly higher potency than FC-A were identified (Fig. 2B). These compounds (F2, FC-7, F6 and F7) possess an amide substitution at C19. In fluorescence polarization binding assays, these derivatives are more than one order of magnitude more potent in stabilizing the binding of client peptides to 14-3-3 compared to FC-A (personal communication from Dr. Yusuke

Higuchi, Osaka University), suggesting that the 19-amide derivatives have stronger neurite outgrowth activity due to enhanced 14-3-3 PPI stabilization activity.

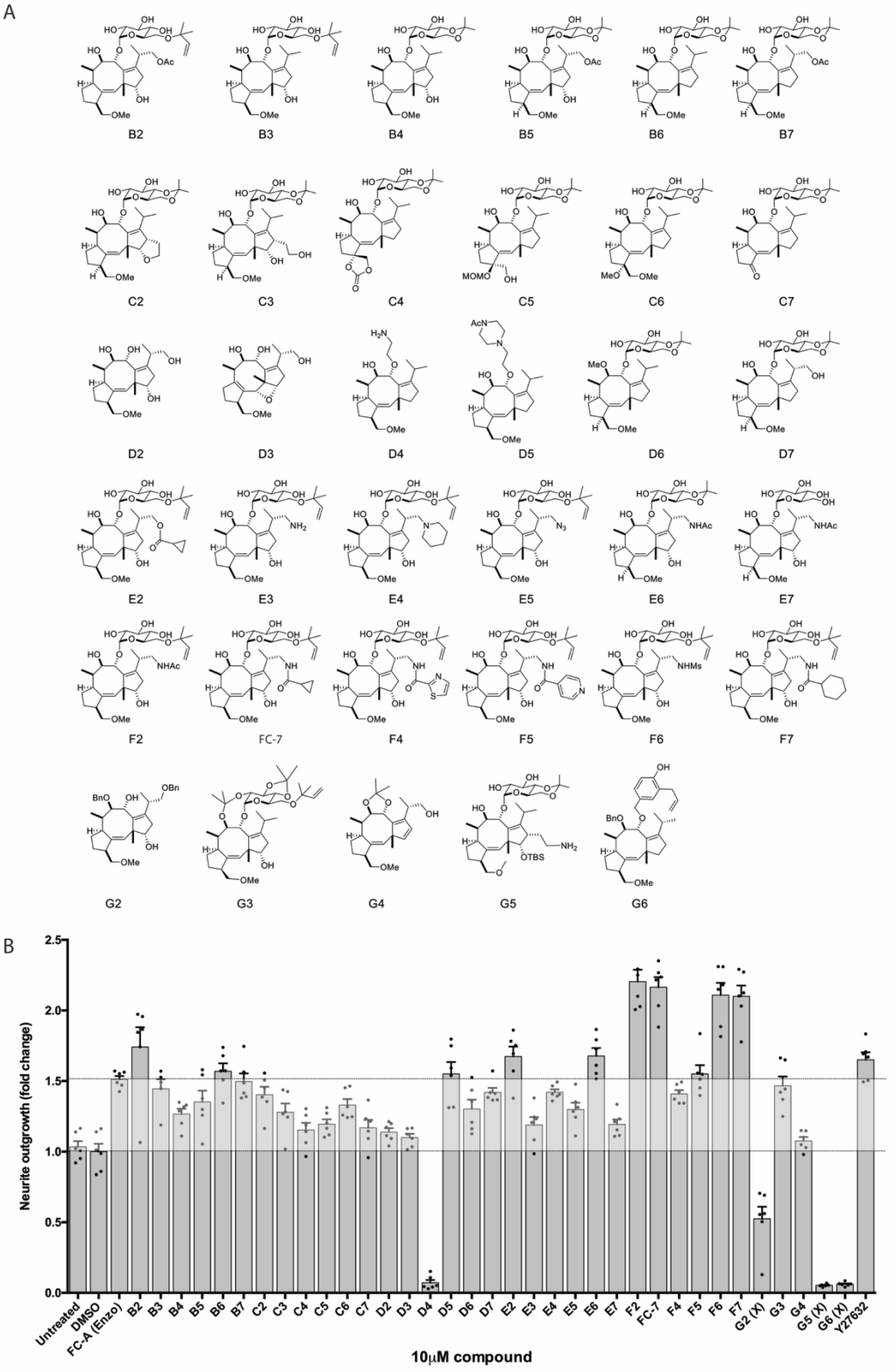
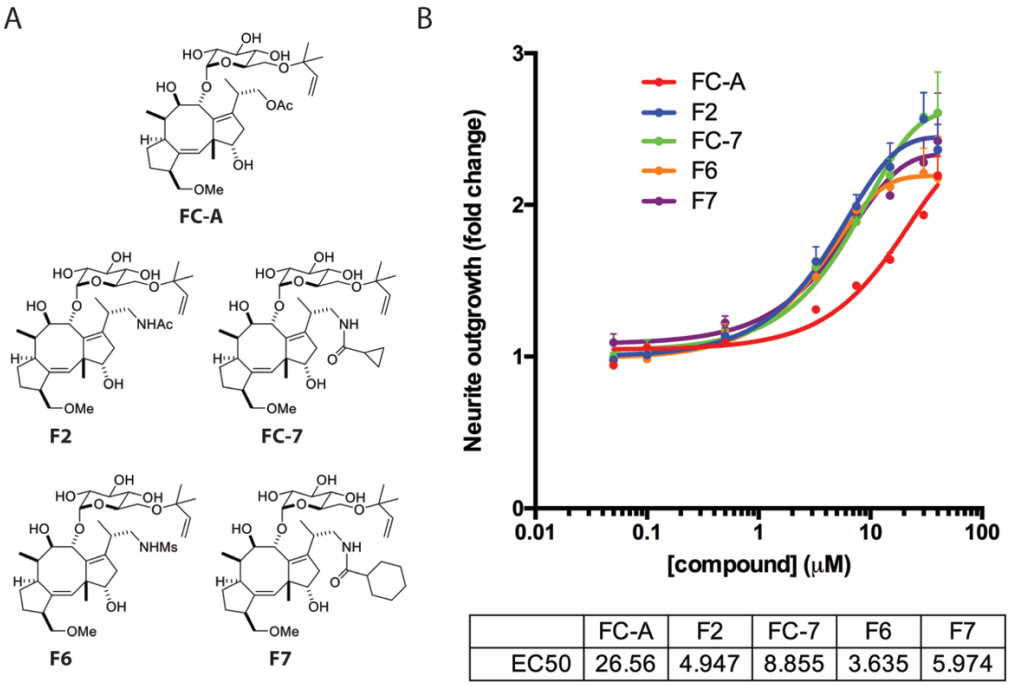


Figure 2. High-content neurite outgrowth screen of novel FC-A derivatives

A) Structures of 35 semisynthetic FC-A derivatives. B) E18 rat cortical neurons were cultured in 96-well plates and compounds were added to duplicate wells in three independent experiments at a single 10μM concentration for 24hr. Cultures were stained for beta III tubulin, a neuron specific tubulin isoform. Automated imaging and neurite outgrowth analysis were performed using the ImageXpress high-content screening system. Rho kinase inhibitor Y27632 and commercial FC-A (from Enzo) served as positive controls. Data points represent the fold-change in neurite outgrowth from individual wells relative to the DMSO control over all experiments. Bars indicate the mean +SEM. Compounds marked with (X) indicate that visible bacterial contamination from the compound stock was observed.

Compounds F2, FC-7, F6 and F7 were selected for further testing in dose curves to assess their neurite outgrowth activity. These analyses revealed a three to seven-fold decrease in EC50 (Fig. 3). Treatment of cortical neurons for 48 hours with these derivatives produces striking increases in neurite length, branching and neuritogenesis, drastically enhancing overall neuritic complexity (Fig. 4).



	FC-A	F2	FC-7	F6	F7
EC50	26.56	4.947	8.855	3.635	5.974

Figure 3. Dose curves of 19-amide derivatives identified from screen

A) Structures of 19-amide derivatives. B) Dose curves and EC50 values (μM) of compounds in neurite outgrowth assays with E18 rat cortical neurons. Data points represent the average fold change over the DMSO control (+SEM) of 3 independent experiments performed in duplicate wells.

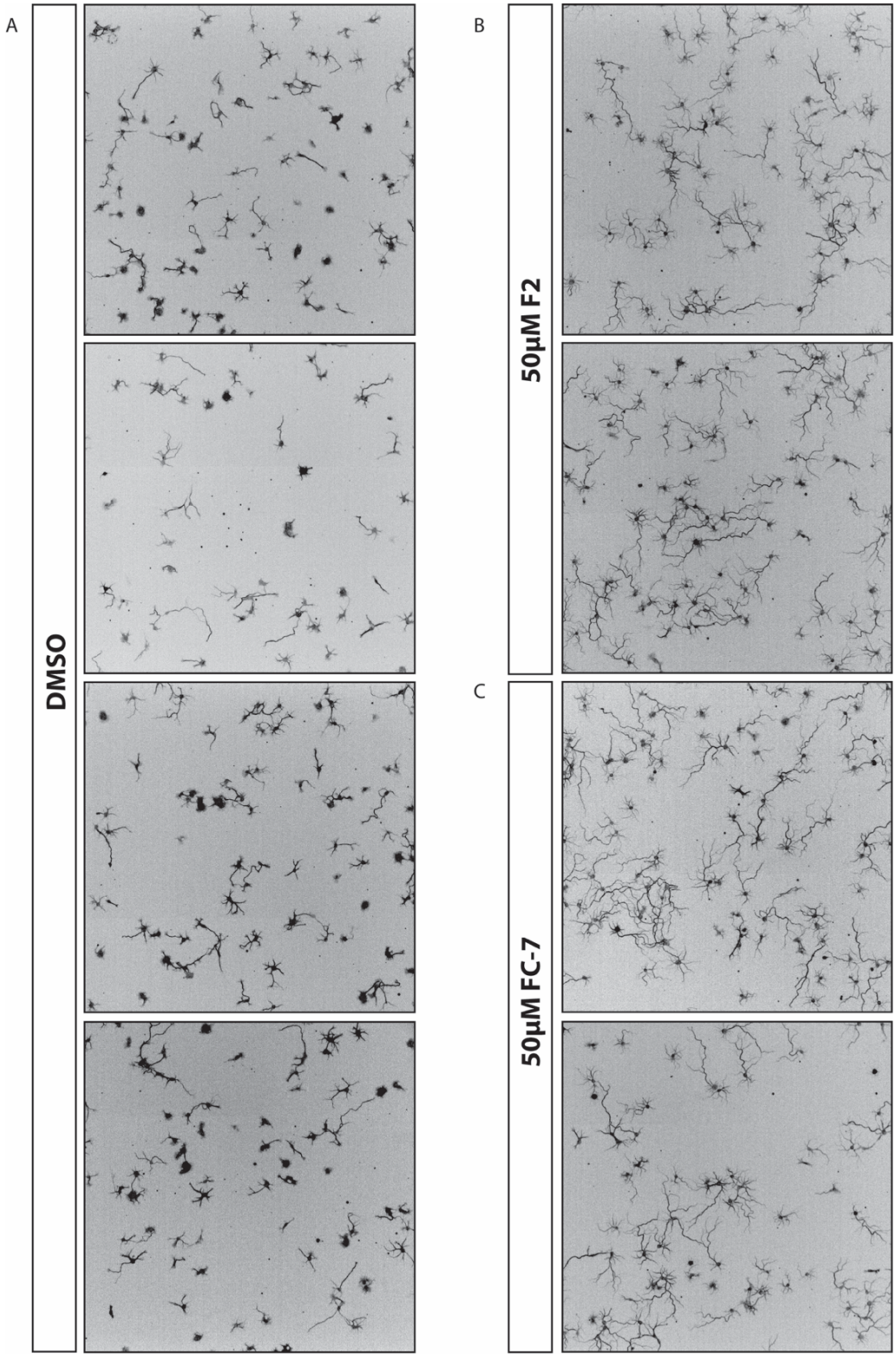


Figure 4. FC-A 19-amide derivatives induce striking enhancements in neurite growth

E18 rat cortical neurons were grown for 48hr in the presence of DMSO or 50μM F2 or FC-7. A) Four representative images of neurons treated with DMSO control. B) Two representative images of neurons

treated with B) **F2** or C) **FC-7**. Note the dramatic increase in neurite lengths, primary neurites, and branches.

4.4.2 SAR of 19-amide FC-A derivatives and FC-J derivatives

With the aim of selecting a lead compound for further testing in mouse models of axonal injury, we conducted a second round of SAR to further enhance the potency and improve the properties of the compounds for *in vivo* administration and potential blood-brain-barrier (BBB) permeability. FC-J, a natural biosynthetic intermediate that lacks 19-OAc was included in these analyses because of its smaller size and enhanced lipophilicity (Fig. 5A). The activity of FC-J in neurite outgrowth is comparable to that of FC-A (Fig. 5B). Conversion of the prenyl group on the glucose of FC-J to an acetonide group (**2**) mildly reduces the potency of the compound, while complete de-prenylation (**FC-4**) is inconsequential. In contrast, de-prenylation of **4** to yield **6**, results in a compound with drastically reduced potency. It is possible that the prenylation becomes critical for permitting the penetration of **4** across the plasma membrane because the amide makes **4** more hydrophilic compared to FC-J. The overall results of these analyses showed that compounds **4** and **FC-7** were the most potent of the compounds tested (Fig. 5). With the goal of administering these compounds in mice, we selected two compounds, **FC-4** and **FC-7**, for further testing. Although **FC-4** is mildly less potent than FC-A, it is significantly smaller in size (512.6Da) and it possesses an undecorated glucose, which could potentially facilitate its active transport across the BBB if recognized by glucose transporters. **FC-7** is the most lipophilic among the highest potency amide derivatives identified, potentially increasing the probability that it could cross the BBB.

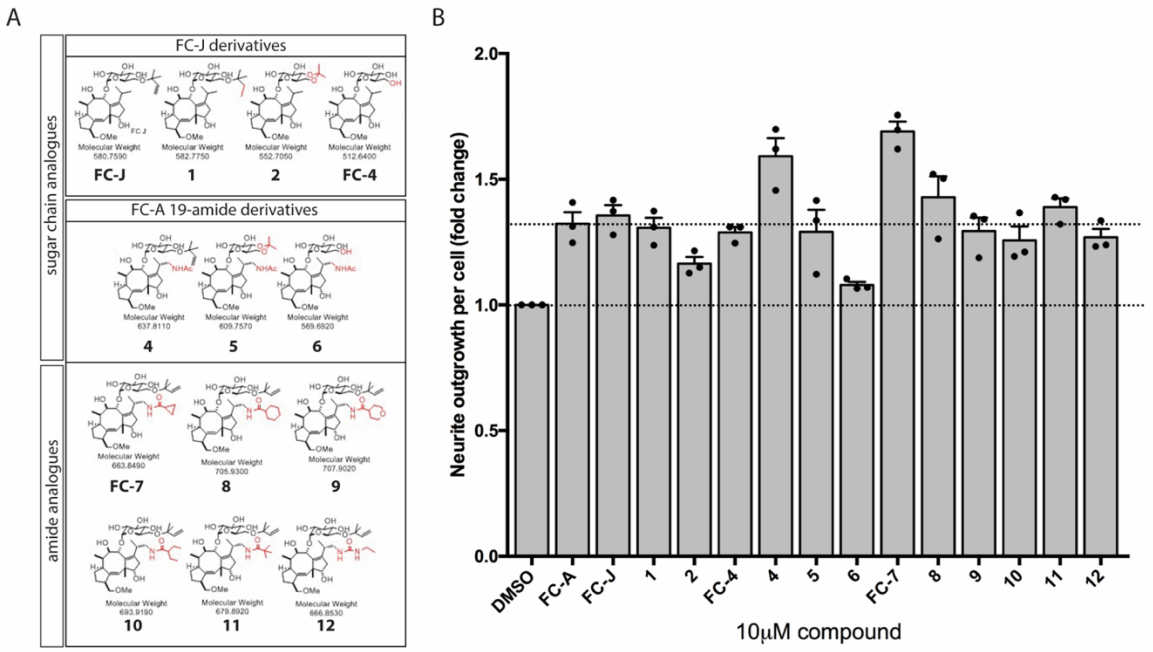


Figure 5. Structure activity relationship of FC-J and FC-A 19-amide derivatives

A) Structures of FC-J and FC-A 19-amide derivatives. B) E18 rat cortical neurons were grown in the presence of 10μM compound for 24hr and analyzed for neurite outgrowth. Data points represent the fold change over the DMSO control of 3 independent experiments performed in duplicate wells. Bars indicate the mean +SEM. n=3.

4.4.3 Effects of FC-4 and FC-7 on neuronal morphology

We next treated cortical neurons with a range of concentrations of FC-4, FC-7 and FC-A for 48 hours to examine effects on neuronal morphology. The compounds stimulate neurite extension (Fig. 6B), neuritogenesis (Fig. 6C) and branching (Fig. 6D), with FC-7 being the most potent and FC-4 the least. None of the compounds had any significant effect on the number of neurons identified in each field (Fig. 6E), suggesting no effect on basal cell survival. Intriguingly, the compounds significantly reduced the average area of the neuronal soma (Fig. 6F). This could result from an enhanced impetus to build and grow neurites, potentially diverting or exhausting cytoskeletal, lipid synthesis and membrane-addition pathways, hypothetically leading to a reduction in soma size. FC-4 stimulates the growth of long, straight, simple neurites. While FC-7 also induces this pattern of growth at lower concentrations, the appearance of supernumerary primary neurites and tortuous, highly branched neurites begins to predominate as the concentrations increase (Fig. 6G). This difference in growth pattern is likely a result of the difference in potency, but could also be due to differences in client-selectivity. It is

interesting to speculate that the chemical differences between these compounds could alter their selectivity for individual 14-3-3:client complexes.

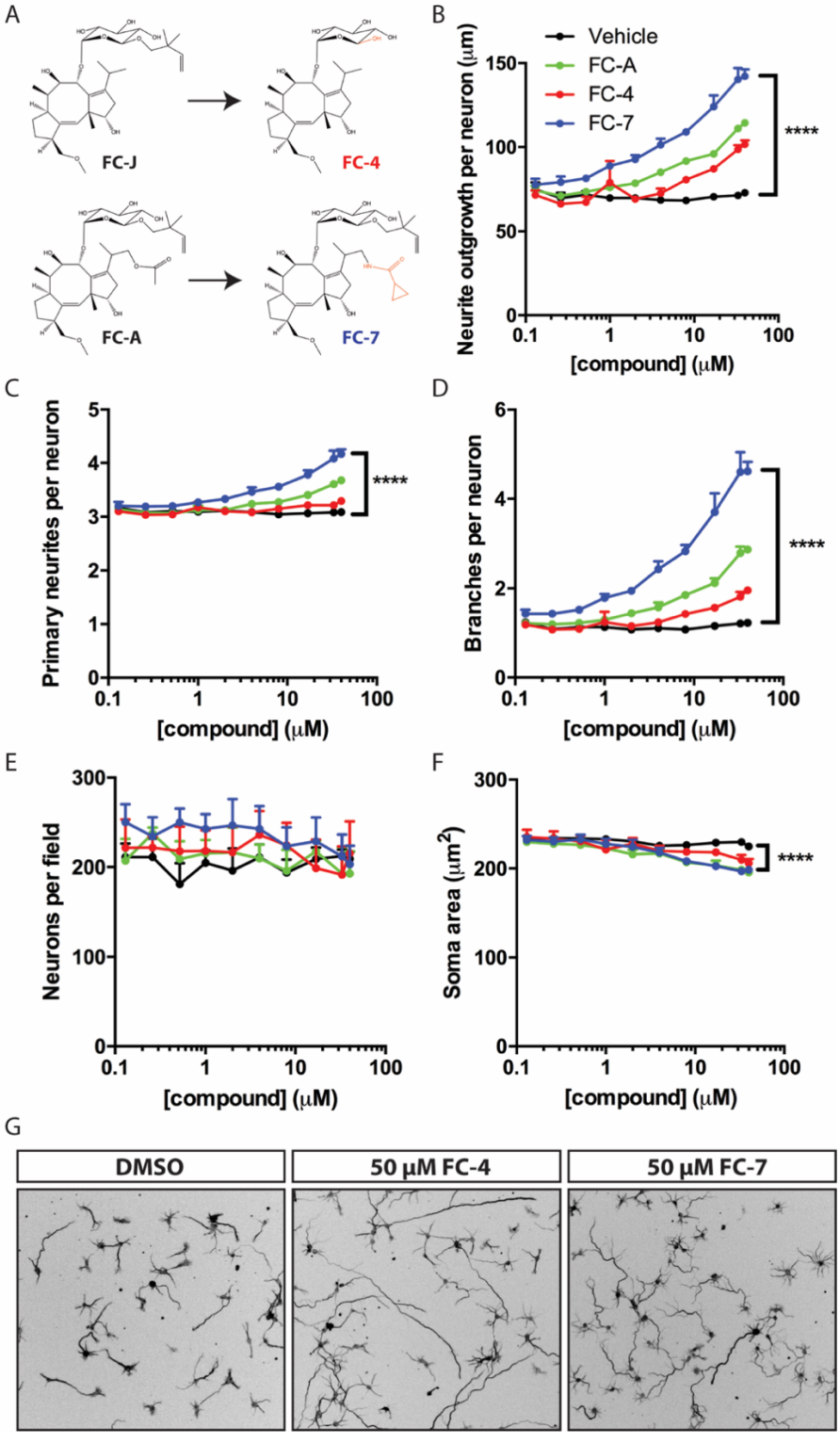


Figure 6. Examining the effect of FC-4 and FC-7 on neuronal morphology

A) Structures of **FC-4** and **FC-7**. E18 rat cortical neurons were grown for 48hr in the presence of DMSO, FC-A, **FC-4** or **FC-7** at a range of concentrations. Compounds significantly enhance B) neurite outgrowth, C) the number of primary neurites per neuron, and D) neurite branching. E) Compounds have no significant effect on the number of neurons. F) Compounds significantly reduce area of soma. Data points represent the means +SEM of raw values from 3 independent experiments performed in duplicate wells from thousands of neurons. ****p<0.0001, Two-way ANOVA, Tukey post-test, n=3.

We next established an *in vitro* model of axotomy to determine whether **FC-7** can stimulate regeneration after injury of mature axons. We cultured cortical neurons in microfluidic chambers for 11DIV, during which axons extend through 500μm micro-channels to a fluidically isolated compartment. Axotomy is then performed by vacuum aspiration of the axonal compartment, resulting in axonal breakage along the lower boundary of the channels. **FC-7** was then added to both the cell body compartment and the axonal compartment. Following a 48hr regeneration period, the cultures were fixed and stained with tuj1 and phalloidin (Fig. 7A). FC-7 treatment stimulated a significant increase in axon regeneration after injury (Fig. 7B-C). Tortuous axonal growth was prominent in **FC-7**-treated cultures, however, unlike the neurite outgrowth assays, an increase in axonal branching was not apparent. These results suggest both overlapping and distinct mechanisms underlying growth-stimulation during neuronal polarization early in culture and after injury of polarized neurons late in culture.

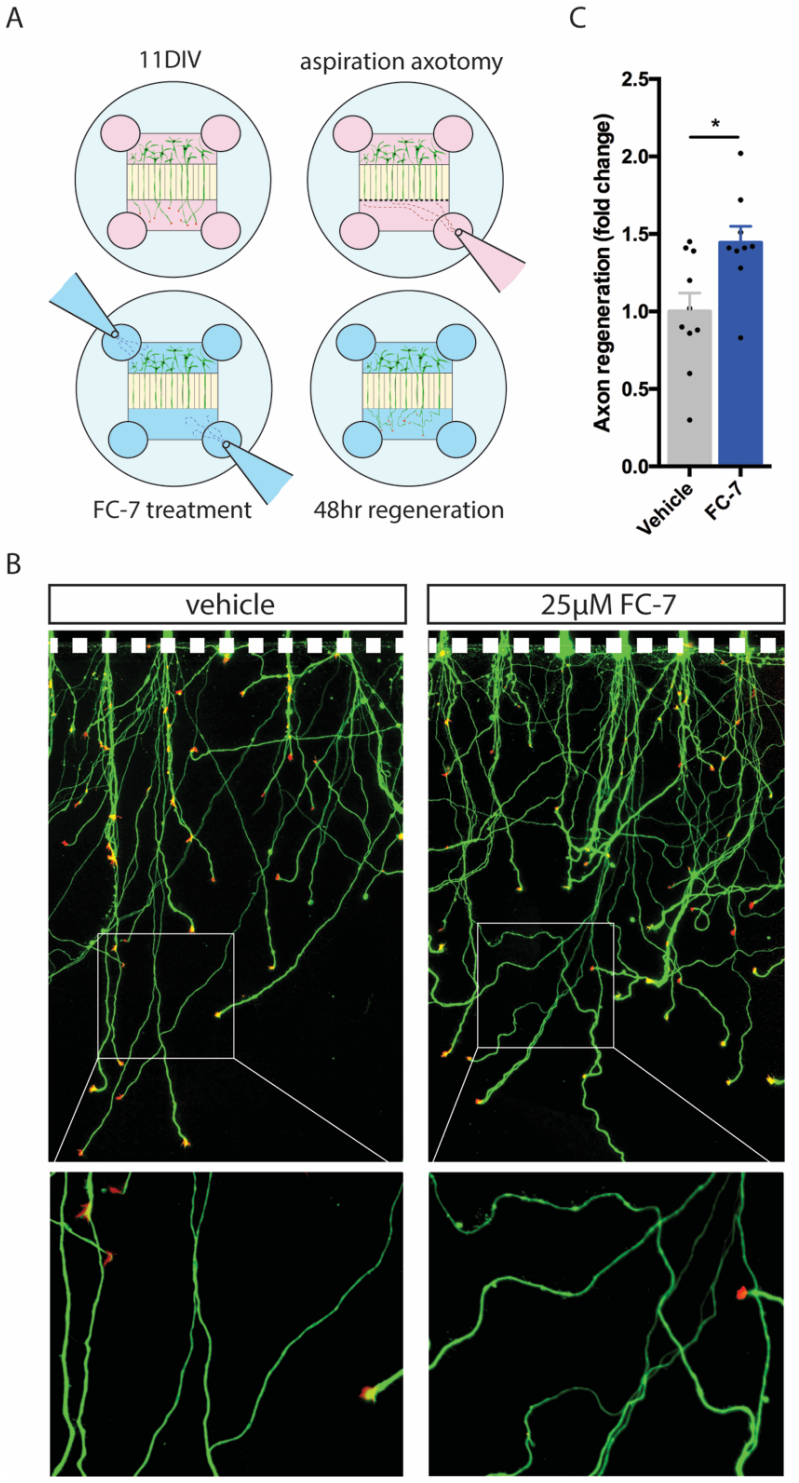


Figure 7. FC-7 stimulates axon regeneration in vitro

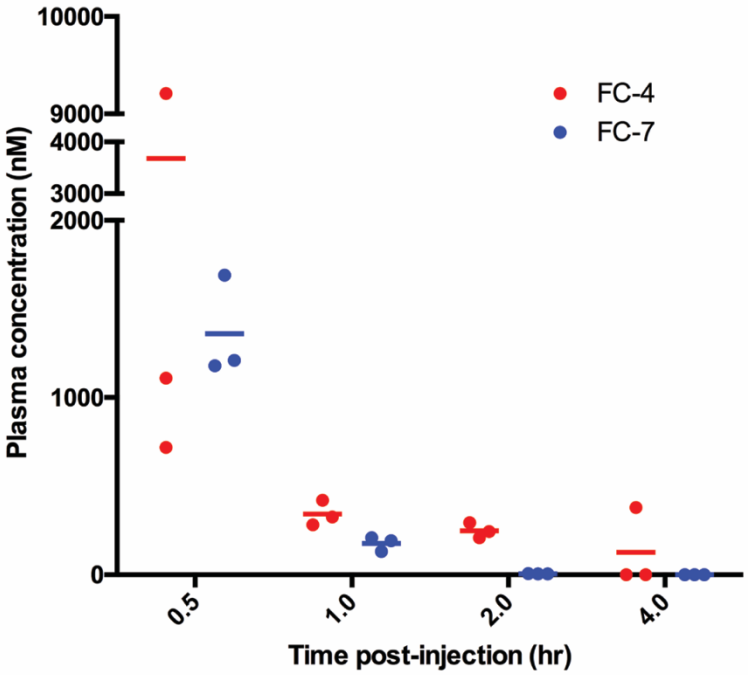
A) Schematic of *in vitro* axotomy model. E18 rat cortical neurons are grown in microfluidic chambers for 11DIV. Axons are cut by vacuum aspiration and then FC-7 is added to both the axonal and cell body compartments, followed by a 48hr regeneration period. B) Representative images of regenerating axons.

Note the tortuous growth pattern of FC-7-treated chambers. C) FC-7 stimulates axon regeneration (n=9-10 chambers, *p<0.05, unpaired t-test).

4.4.4 Pharmacokinetics of FC-4 and FC-7

Based on the neurite outgrowth activity and the chemical characteristics of FC-4 and FC-7, we next investigated the stability and distribution of these compounds after administration in mice. Mice received a single intraperitoneal (IP) injection at a 20mg/kg dose of either compound. Plasma and brain were extracted and analyzed at 0.5, 1, 2 and 4hr time-points. Both compounds were detected at low micromolar concentrations in plasma 0.5hr post-injection (Fig. 8A). FC-4 was not detectable in brain samples at any of the time-points tested. In contrast, FC-7 was detected at low nanomolar concentrations in the brain 0.5hr post-injection. The brain/plasma ratio ranged from 1.2-2%, suggesting that the compound can modestly cross the BBB and access the brain parenchyma (Fig. 8B). This suggests that FC-7 or structural analogues could be used in mouse models of nerve or spinal cord injury, in which uptake of the compound could be further enhanced by the local disruption of the BBB around the injury site.

A



B

Mouse #	Brain [nM]	Plasma [nM]	Brain/plasma ratio
FC-4			
1	<LLOQ	1110	NA
2	<LLOQ	718	NA
3	<LLOQ	9210	NA
FC-7			
1	24	1210	0.02
2	21.1	1690	0.012
3	15.5	1180	0.013

Figure 8. Pharmacokinetics of FC-4 and FC-7

Mice received a single intraperitoneal (IP) injection of FC-4 or FC-7 at a 20mg/kg dose. Plasma and brain samples were extracted and analyzed by LC-MS/MS at 0.5, 1, 2 and 4hr time-points post-injection. A) Plots of mouse plasma concentrations for each compound. Each data point indicates plasma concentration from 1 mouse. Bars represent the mean. B) Brain and plasma concentrations of compounds 0.5hr post-injection. Note that 1.2-2% of FC-7 plasma concentration is detected in the brain. <LLOQ = below lower limit of quantification.

4.5 Discussion

Drugs that can stimulate axon regeneration could be effective in restoring neurological function after spinal cord injury, nerve injury or in certain neurodegenerative conditions. Several small molecules that can stimulate axon growth have been identified. For instance, microtubule-stabilizing drugs, taxol and epothilone, have been shown to stimulate neurite outgrowth in cultured neurons and promote axon regeneration after spinal cord injury(Hellal et al., 2011; Ruschel and Bradke, 2017; Ruschel et al., 2015a). Recently, statins were shown to be highly potent stimulators of neurite outgrowth. Intravitreal administration of cerivastatin after optic nerve crush injury promoted axon regeneration after a single treatment(Li et al., 2016). The continued identification of compounds with neurite outgrowth activity will be important to the eventual development of a safe and effective drug to induce axon regeneration

We previously reported that fusicoccin-A (FC-A), a small molecule that stabilizes protein-protein-interactions (PPIs) between 14-3-3s and their client proteins, stimulates neurite outgrowth in cultured cortical neurons and improves axon regeneration when delivered by intravitreal injection after optic nerve crush injury(Kaplan et al., 2017b). Here we sought to improve the potency of FC-A in neurite outgrowth by exploring structure activity relationship of novel semisynthetic derivatives. We identified a series of derivatives with a 19-amide substitution that possess a three-to-seven-fold higher potency in neurite outgrowth compared to FC-A. These derivatives are also more than an order of magnitude more potent in stabilizing 14-3-3 PPIs (personal communication from Dr. Yusuke Higuchi, Osaka University). This indicates that these derivatives derive their enhanced biological potency from their improved 14-3-3 PPI stabilization activity.

We further examined the effects of an FC-A 19-amide derivative (**FC-7**) and an FC-J derivative (**FC-4**) and observed profound effects on neurite outgrowth, neuritogenesis and branching. Based on their activity and their chemical characteristics, we selected these two compounds for pharmacokinetic analyses after intraperitoneal injection in mice. We found that **FC-7**, but not **FC-4**, was readily detected at low nanomolar concentrations in the brain, representing 1.2-2% of the plasma concentration. This result suggests that **FC-7** can modestly penetrate the intact BBB. In the case of injury or disease, the BBB becomes locally compromised, allowing enhanced delivery of drugs to the injury site. After spinal cord contusion in rats, disruption of the blood-spinal cord barrier has been shown to persist for one to two months after injury, suggesting a long

window of time during which compounds with low BBB penetrance could undergo enhanced uptake around the injury site(Patel et al., 2009; Popovich et al., 1996).

Further optimization of FC-A 19-amide derivatives to enhance their metabolic stability and their ability to penetrate the BBB could result in compounds with efficacy in stimulating axon regeneration after systemic administration. FC-A derivatives have also been shown to have anti-tumor effects by sensitizing cancer cells to chemotherapeutic drugs. In studies where FC-A derivatives were delivered daily by systemic routes in mice with xenografted tumors, the compounds were efficacious in reducing tumor burden at doses that were well tolerated, without any apparent toxicity(Kawakami et al., 2012; Miyake et al., 2015). This suggests that these compounds may be safe and effective candidates for further development in CNS injury applications.

4.6 Materials and methods

4.6.1 Semisynthetic FC derivatives

FC-A and FC-J were purified from *Phomopsis amygdali* cultures as previously described and used as starting material for the synthesis of derivatives(Inoue et al., 2018).

4.6.2 Cortical neuron culture

All studies were approved by the McGill University Animal Care and Use Committee. For cortical neuron cultures, cortices were dissected from E18 Sprague Dawley rats in cold Leibovitz L15 medium (Thermo Fisher). Isolated cortices were incubated in 0.25% trypsin-EDTA for 30min at 37°C and dissociated into a single cell suspension by gentle trituration in DMEM supplemented with 10%FBS. Neurons were seeded in culture dishes coated with 100μg/mL poly-l-lysine (PLL) and washed 3 times with PBS. Culture medium consisted of Neurobasal medium supplemented with 2% B27, 1% N2, 1% penicillin/streptomycin and 2mM L-glutamine.

4.6.3 Neurite outgrowth assays

Cortical neurons were seeded in 96-well plates at a density of 7,000 cells per well. After 2hr, the medium was replaced with pre-diluted compounds prepared from 10mM DMSO stocks. Each treatment was performed in duplicate wells. After 24hr, the neurons were fixed with 4% paraformaldehyde/20% sucrose, permeabilized with 0.2% Triton-X, blocked with 5% BSA, and stained with anti-βIII tubulin (1:1000, BioLegend) for 1hr at

room temperature, Hoechst 33342 (1:10000, Sigma) and fluorescent secondary antibodies (1:1000, Thermo Fisher) for 1hr at room temperature. For analysis of neuron morphological parameters, neurons were cultured for 48hr.

4.6.4 High-content screening

Automated image acquisition of βIII tubulin and Hoechst stain was performed using the ImageXpress high-content imaging system (Molecular Devices). Automated neurite outgrowth analysis was performed using the neurite outgrowth module of the MetaXpress software (Molecular Devices), allowing the analysis of thousands of neurons per condition.

4.6.5 Microfluidic chambers and *in vitro* axotomy

E18 rat cortical neurons were cultured in microfluidic chambers with 500μm channels (Ananda Devices) for 11DIV. Medium was replenished every 2-3 days. On day 11, axons were aspirated with vacuum suction from the axonal compartment, resulting in axonal breakage along the lower boundary of the channels. 25μM FC-7 was added to both compartments and the chambers were maintained for an additional 48hr. The neurons were then fixed with 4% paraformaldehyde/20% sucrose, permeabilized with 0.2% Triton-X, blocked with 5% BSA, and stained with anti-βIII tubulin (1:1000, BioLegend) for 1hr at room temperature, Hoechst 33342 (1:10000, Sigma) and rhodamine-phalloidin (1:1000, Life Technologies) for 1hr at room temperature. Axon regeneration was quantified in ImageJ by skeletonizing images of βIII tubulin stain from the entire chamber, measuring the total skeleton length, and normalizing to the number of neurites.

4.6.6 Pharmacokinetics

Mice were administered a single intraperitoneal injection of FC-4 or FC-7 at a 20mg/kg dose. Plasma and brain samples were collected 0.5, 1, 2 and 4hr post-injection. Three mice per compound for each time-point were analyzed. After preparing calibration curves with the compounds, test samples were analyzed by LC-MS/MS to determine the concentration in each sample.

5 Chapter 4. Exploring the role of GCN1 in axon regeneration

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This is a manuscript in preparation for publication.

5.1 Preface

In this chapter, we follow up on our previous work implicating GCN1 as a potential target for stimulating axon regeneration. We profile the expression of GCN1 in the retina and conditionally delete GCN1 from retinal ganglion cells to examine effects on neurite outgrowth and axon regeneration. Results from these experiments will determine whether GCN1 could serve as a target for axon regeneration.

5.2 Abstract

Axons in the mature central nervous system (CNS) do not spontaneously regenerate and re-establish neural circuits after injury. This causes permanent deficits in motor and sensory function. Drugs that can stimulate axon regeneration could be effective in restoring function in spinal cord injury, diseases of the retina and optic nerve, and in neurodegenerative conditions. A better understanding of the molecular regulators of axon growth after injury will yield new targets for the development of therapies to stimulate axon regeneration. We identify the translational regulator GCN1 as a key regulator of neurite outgrowth and axon regeneration. In yeast, GCN1 is thought to mediate a reduction in global translation in response to cell stress by binding to the eIF2-alpha kinase GCN2. Using immunoprecipitation and mass spectrometry, we characterize the GCN1 interactome in rat cortical neurons, suggesting roles in intracellular trafficking and cytoskeletal regulation. These results provide rationale for targeting GCN1 to stimulate axon regeneration after CNS injury.

5.3 Introduction

Axonal injury causes persistent deficits in neurological function in spinal cord injury, nerve injuries and degenerative conditions in the central nervous system (CNS). Axons in the CNS have a poor capacity to regenerate and therapies that promote regeneration could be used to treat these devastating conditions. Intrinsic regulators of growth, including oncogenes and tumor suppressors can be genetically manipulated to induce axon regeneration in mouse models of CNS injury(He and Jin, 2016).

We previously identified GCN1, a regulator of translation in response to cell stress, as a potential intrinsic target for stimulation of axon regeneration(Kaplan et al., 2017b). The function of GCN1 is well characterized in yeast, but its role in mammals is unknown. Upon amino acid deprivation in yeast, GCN1 binds to the eIF2-alpha kinase GCN2. GTP-bound eIF2-alpha forms a complex with methionyl-tRNA to begin mRNA translation. When GCN2 phosphorylates eIF2-alpha on serine 51 (S51), this imparts a dominant negative function by enhancing its affinity for its GTP exchange factor (GEF), eIF2B, thereby reducing eIF2-alpha-GTP levels in the cell(Roffe et al., 2013). GCN1 is thought to promote the binding of uncharged tRNAs to GCN2, which is required for the activation of its kinase activity. In the absence of GCN1, GCN2 cannot phosphorylate eIF2-alpha. GCN2 is the only eIF2-alpha kinase in yeast. Mammals possess three other eIF2-alpha kinases, PERK, PKR, and HRI(Costa-Mattioli et al., 2009). Whether the role of GCN1 in this pathway is conserved in mammals is not well defined. The GCN1 sequence is characterized by a series of HEAT repeats, a domain that is thought to play a role in intracellular transport and scaffolding(Neuwald and Hirano, 2000), suggesting potential additional functions outside of the GCN2-eIF2-alpha pathway.

We recently reported that RNAi-mediated knockdown of GCN1 in cultured cortical neurons enhances neurite outgrowth(Kaplan et al., 2017b). Another study showed that a protein called IMPACT, which binds to GCN1 and antagonizes its interaction with GCN2, also promotes neurite outgrowth(Roffe et al., 2013). Here, we seek to determine whether removal of GCN1 can promote axon regeneration in the injured CNS. We used AAV-mediated delivery of Cre recombinase to remove GCN1 from retinal ganglion cells in neurite outgrowth assays in $GCN1^{fl/fl}$ mice. We further performed IP-mass spec and characterized the GCN1 interactome in neurons. We suggest that GCN1 could serve as a target to stimulate CNS axon regeneration.

5.4 Results

5.4.1 Expression profile of GCN1 in mouse retina

We first profiled the expression of GCN1 in the mouse cortex and retina. In western blots, we examined the expression of GCN1 in adult retina alongside cortex from a range of developmental stages. We found that GCN1 expression is highest during embryonic development in cortex and remains present in both adult cortex and retina (Fig. 1A). We also stained retinal cross sections from adult mice and observed strong staining restricted to the retinal ganglion cell (RGC) layer (Fig. 1B). RGCs were GCN1-positive, as revealed with anti-βIII tubulin (tuj1) co-labelling. The other cell population in this layer, displaced amacrine cells, also express GCN1.

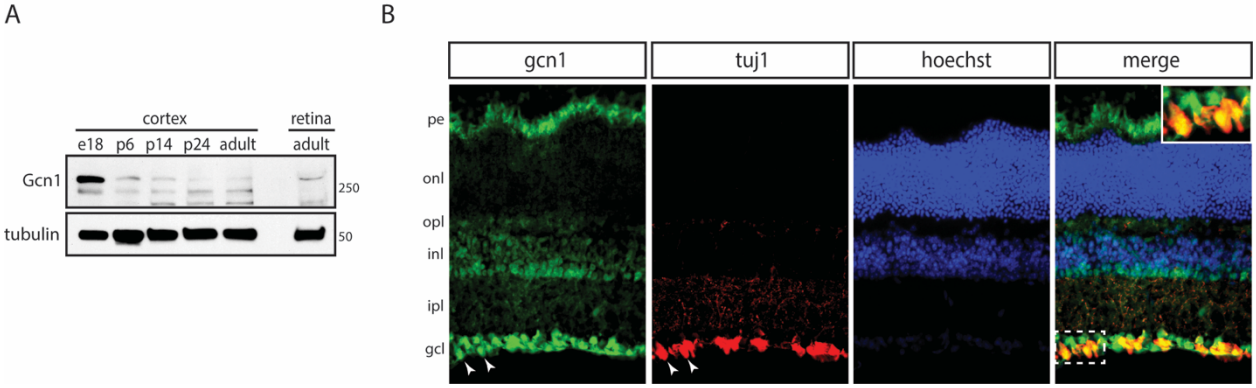

Figure 1. GCN1 expression profile in mouse cortex and retina

A) Western blots of cortex and retina lysates. Note the high expression of GCN1 during embryonic development in cortex. B) Immunohistochemistry of mouse retina cross-section. Note the high expression of GCN1 restricted to the retinal ganglion cell layer (gcl = ganglion cell layer, ipl = inner plexiform layer, inl = inner nuclear layer, opl = outer plexiform layer, onl = outer nuclear layer, pe = pigmented epithelium).

5.4.2 GCN1 knockout promotes neurite outgrowth

To assess the effect of GCN1 on neurite outgrowth, we used a line of mice that harbor loxP sites within the GCN1 locus (see Fig. S1 for genotyping), enabling the conditional deletion of GCN1 in the presence of Cre recombinase. We cultured retinal neurons from postnatal day 3 (P3) GCN1$^{fl/fl}$ mice and infected them with either AAV2-GFP or AAV2-Cre-GFP. On day 11 of culture, we fixed and stained the cultures for the RGC marker tuj1. GFP expression was observed throughout the cytoplasm in AAV2-GFP infected RGCs, and as expected, Cre-GFP fusion protein localized exclusively to the nucleus. RGCs

expressing Cre-GFP had significantly more neurite outgrowth compared to control RGCs, indicating that removal of GCN1 stimulates growth.

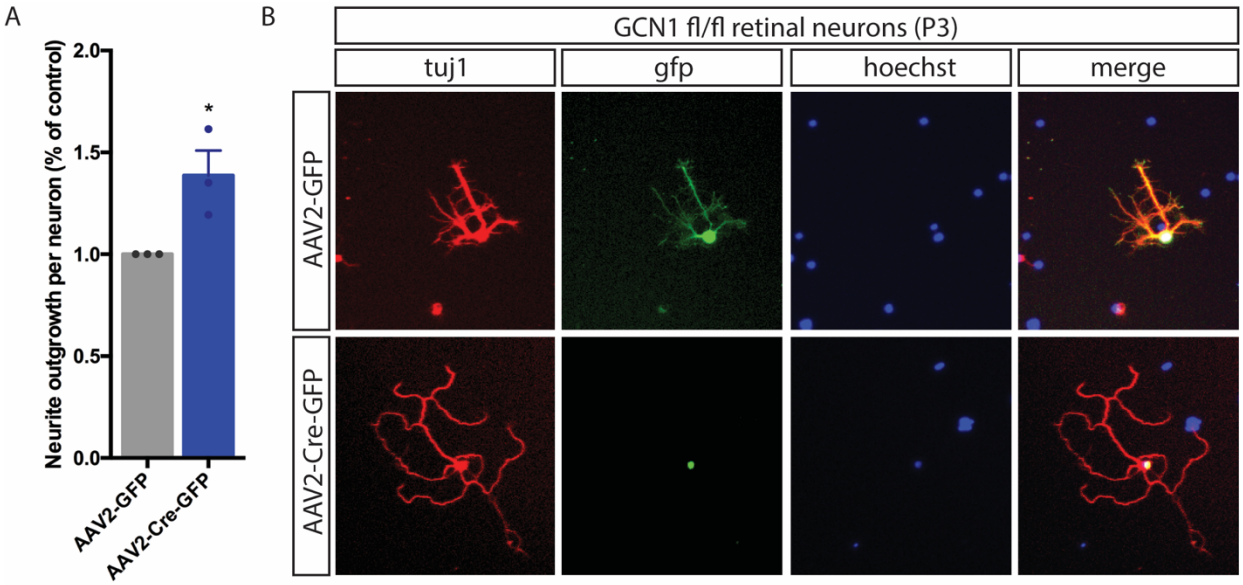

Figure 2. GCN1 knockout stimulates RGC neurite outgrowth

Retinal neurons from P3 mice were infected with AAV2-GFP or AAV2-Cre-GFP and fixed and stained for RGC marker tuj1 on day 11 of culture. A) Expression of AAV2-Cre-GFP significantly stimulates neurite outgrowth compared to control (*p<0.05, unpaired t-test, n=3). B) Images of cultured RGCs. Note that the expression of Cre-GFP fusion protein is restricted to the nucleus.

5.4.3 Characterization of neuronal GCN1 interactome

In order to gain insight into the mechanisms underlying GCN1-dependent regulation of neurite outgrowth, we next sought to characterize the repertoire of GCN1 interaction partners in cortical neurons. We immunoprecipitated GCN1 from cortical neuron lysates and subjected the samples to mass spectrometry, revealing proteins that co-precipitate with GCN1. The experiment was performed three independent times and the results were analyzed by 'significance analysis of interactome' (SAINT)(Choi et al., 2011). These analyses revealed twelve high-confidence interaction partners, defined by those having a SAINT score >0.8 (Table 1).

Protein		peptide counts control	anti-GCN1	SAINT score
GCN1, EIF2 Alpha Kinase Activator Homolog	GCN1L1	0|0|0	215|231|335	1
PTPRF Interacting Protein Alpha 3, Liprin alpha-3	PPFIA3	0|0|0	44|42|77	1
WD Repeat Domain 47, Nemitin	WDR47	0|0|0	12|16|42	1
WD Repeat Domain 37	WDR37	0|0|0	6|4|9	1
Centrosomal Protein 170	CEP170	0|2|2	15|9|37	1
Centrosomal Protein 170B	CEP170B	0|0|0	5|3|4	1
RAS P21 Protein Activator 1	RASA1	0|0|0	9|7|21	1
Lunapark, Limb and Neural Patterns	LNP	0|0|0	14|13|18	1
Heterogeneous Nuclear Ribonucleoprotein K	HNRNPK	1|3|5	14|17|23	1
Phosphofurin Acidic Cluster Sorting Protein 1	PACS1	0|0|0	10|3|16	1
Protein Phosphatase 2 Scaffold Subunit Alpha	PPP2R1A	0|0|0	2|3|9	0.98
Protein Phosphatase 2 Regulatory Subunit Gamma	PPP2R5C	0|0|0	2|2|7	0.96
Dynactin Subunit 2	DCTN2	1|0|1	3|4|7	0.8

Table 1. High-confidence GCN1 interaction partners

GCN1 was immunoprecipitated from 7DIV E18 rat cortical neuron lysate. Co-precipitating interactors were identified by mass spectrometry. Peptide counts correspond to the number of peptides identified in the IgG control or the GCN1 IP from 3 independent experiments. 'Significance analysis of interactome' (SAINT) scores are indicated. True interactors were considered those having SAINT score > 0.8.

5.5 Discussion

The identification of new molecular pathways that regulate axon growth could lead to the conception of new approaches to stimulate axon regeneration after injury. We previously found that the stress response protein GCN1 acts as an intrinsic 'brake' on neurite outgrowth in cultured cortical neurons(Kaplan et al., 2017b). Here we found that Cre-mediated removal of GCN1 from cultured $GCN1^{fl/fl}$ retinal ganglion cells also enhances neurite outgrowth. The molecular mechanisms underlying these effects are unknown.

In yeast, GCN1 plays a critical role in stimulating eIF2-alpha phosphorylation in response to cell stress by binding to GCN2 kinase(Sattlegger and Hinnebusch, 2000). However, it is unclear whether this pathway is conserved in mammalian cells. A protein called IMPACT has been shown to bind GCN1 and antagonize its interaction with

GCN2, resulting in enhanced neurite outgrowth in cultured neurons(Roffe et al., 2013). Upon actin depolymerization, IMPACT becomes sequestered by G-actin, resulting in GCN1 release and GCN2 activation(Sattlegger et al., 2011; Sattlegger et al., 2004; Silva et al., 2016). It is interesting to speculate that these mechanisms could play a role in the neuronal response to injury, whereby GCN1 could function as a sensor of cytoskeletal disruption that coordinates growth arrest through inhibition of protein synthesis.

We used an unbiased approach to characterize the GCN1 interactome in un-stressed cortical neurons. Using IP-mass spec, we identified twelve high-confidence interactors. Intriguingly, many of these proteins have established or putative functions as microtubule-associated proteins. CEP170 and CEP170B are components of the centrosome, which functions as the classical microtubule organizing center in eukaryotic cells(Kuijpers and Hoogenraad, 2011). WDR37 is also thought to be a centrosome-associated protein (Human Protein Atlas). WDR47, also known as Nemitin, localizes to the centrosome in non-neuronal cell lines, and its deletion in neurons impairs axon growth(Habib et al, Stanford Thesis, 2016). Recently-characterized WDR47 knockout mice were shown to have severe defects in brain development, including agenesis of the corpus callosum(Kannan et al., 2017). Moreover, ‘superior cervical ganglion 10’ (SCG10, also known as Stathmin-2) was identified as a WDR47 interaction partner. SCG10 is noted for its high expression in regenerating axons after nerve injury and is used as a marker of regenerating axons(Shin et al., 2014). WDR37 and WDR47 are characterized by WD40 repeats, which typically fold into beta-propeller structures(Xu and Min, 2011). Mutations in other microtubule-associated WDR proteins, including Lis1 and WDR62, cause lissencephaly or smooth-brain disease(Chen et al., 2014; Sapir et al., 1997). We also identified dynactin subunit 2 (DCTN2), a component of the dynactin complex which mediates dynein-dependent transport along microtubules. DCTN2 has also been found to localize to the centrosome(Uetake et al., 2004).

The over-representation of centrosome-associated proteins identified as GCN1 interactors suggests that GCN1 could be a novel centrosomal protein. Mutations in centrosomal proteins are known to underlie various neurodevelopmental and neurodegenerative diseases(Kuijpers and Hoogenraad, 2011). The centrosome plays a critical role in nucleating microtubules during formation of the mitotic spindle. In post-mitotic neurons, the centrosome has been shown to localize to the base of the axon

and associate with the golgi, where it plays a role in specifying the axon during neuronal polarization(de Anda et al., 2005). Disruption of microtubules in cultured hippocampal neurons is followed by re-nucleation primarily at the centrosome in newly polarized neurons, but not in mature neurons, suggesting a developmental loss in its function as a nucleation site for microtubules(Stiess et al., 2010). Based on these findings, it is interesting to speculate that GCN1 could function at the centrosome to regulate neuronal polarization and early outgrowth, but may lose its outgrowth activity in injured mature neurons. Assessment of axon regeneration after injury of GCN1-deleted mature neurons will shed light on this question. It is also important to consider that given the potential role of GCN1 in stress signaling, its interactome may change upon cell stress, where it may be recruited to GCN2 or other proteins involved in regulating stress-dependent inhibition of translation. Further study into the cellular and molecular functions of GCN1 and its associated proteins could reveal new mechanisms underlying neuronal development and regeneration.

5.6 Materials and methods

5.6.1 Retinal neuron culture

All studies were approved by the McGill University Animal Care and Use Committee. For retinal neuron cultures, retinae were dissected from P3 GCN1$^{fl/fl}$ mice in cold Leibovitz L15 medium (Thermo Fisher). Isolated retinae were dissociated using the Worthington Papain Dissociation System, according to manufacturer's protocol. Culture dishes were coated with 4μg/mL laminin (BD Biosciences) in 100μg/mL poly-l-lysine (PLL) for 2-4hr at room temperature. Culture medium consisted of Neurobasal medium supplemented with 200ng/mL CNTF (PeproTech), 2% B27, 1% N2, 1% penicillin/streptomycin and 2mM L-glutamine.

5.6.2 AAV infection and retinal neurite outgrowth assays

Neurons were seeded in 96-well plates at a density of 10,000 cells per well. On day 1 of culture, retinal neurons were infected with AAV2-GFP or AAV2-Cre-GFP at a multiplicity of infection (MOI) of 10,000. On day 11 of culture, the neurons were fixed with 4% paraformaldehyde/20% sucrose, permeabilized with 0.2% Triton-X, blocked with 5% BSA, and stained with anti-βIII tubulin (1:1000, BioLegend) for 2hr at room temperature, Hoechst 33342 (1:10000, Sigma) and fluorescent secondary antibodies (1:1000, Thermo Fisher) for 1hr at room temperature. Automated image acquisition of βIII tubulin and

Hoechst stain was performed using the ImageXpress high-content imaging system (Molecular Devices). Automated neurite outgrowth analysis was performed using the neurite outgrowth module of the MetaXpress software (Molecular Devices).

5.6.3 Immunoprecipitation

E18 rat cortical neurons were dissected and cultured as previously described(Kaplan et al., 2017b). On day 7 of culture, neurons were lysed in ice cold lysis buffer composed of 100mM NaCl, 50mM Tris-HCl, 5mM EDTA, 2mM $MgCl_2$, 10% glycerol and 0.5% NP-40 supplemented with protease inhibitors phosphatase inhibitors. 1.8mg of total cell lysate was precleared with protein A/G beads (Santa Cruz, sc-2003), and then GCN1 was immunoprecipitated with 2μg of anti-GCN1L1 (Abcam, ab86139) and 30μL A/G beads for 2hr at 4°C with rotation. The beads were subsequently washed 3 times with lysis buffer, twice more with lysis buffer lacking detergent, and then elution was performed with three 100μL applications of phosphoric acid, 10min each with light agitation on ice. Samples were stored at -80C until tryptic digestion and mass spectometry analysis.

5.7 Supplementary figures

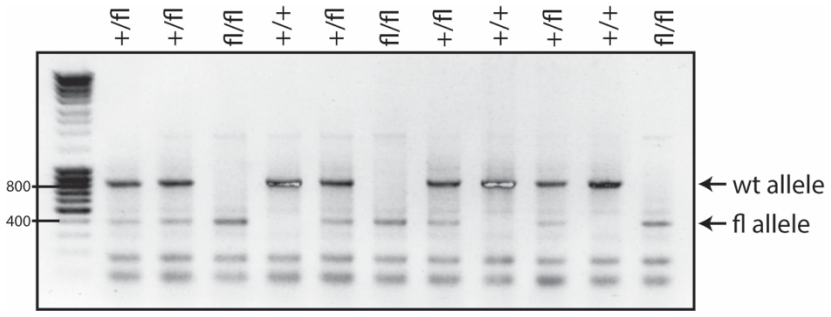

Figure S1. Genotyping from GCN1 +/fl crosses

Discussion and conclusion

6 Chapter 5. Discussion and conclusion

6.1 Barriers to CNS axon regeneration

The failure of axon regeneration in the CNS has increasingly been viewed as a neuron-intrinsic problem. In the same vein, the lines that were once drawn between 'extrinsic vs. intrinsic' have also given way to an appreciation that these two biologies are intertwined. Despite progress, the fundamental causes underlying failed axon regeneration are still poorly understood. Here we consider, in a broad perspective, some interesting hypotheses and put them in the context of our findings.

6.1.1 Fusicoccanes and neurite outgrowth

We have discovered that fusicoccanes (FCs) induce striking enhancements in neurite outgrowth. We found that these effects are also accompanied by a marked reduction in cell body area. It is interesting to speculate that these compounds may directly act on membrane-addition machinery at the growing tips of the neurites or may indirectly lead to exhaustion of these pathways, resulting in a cell body shrinkage. Membrane addition may be a critical limiting factor in the regeneration of severed axons in the mature CNS. The end-bulbs that form after injury become filled with vesicles and microtubules, which extend peripherally and loop back proximally toward the axon shaft (Bradke et al., 2012). This may indicate an attempt at protrusive growth which ends up 'clogging' the cut end of the axon. Why can't a new growth cone be re-established? When the shaft is transected, the appropriate machinery must conceivably be synthesized and transported to the lesion site. Perhaps this occurs to some extent, but certain key elements are missing (Fig. 1). Presumably, most of the cargo that would first arrive at the damaged tip would be destined to serve functions at the synapse. Some of these elements may overlap with typical growth cone machinery, some may be inconsequential, and some may be inhibitory to growth. Identifying the limiting factors may help devise strategies to enable growth cone formation. One interesting possibility is that there is an absence of membrane-addition machinery, limiting the requisite expansion of plasma membrane for growth cone formation and outgrowth. During development, membrane-addition plays an essential role in neuronal polarization and axonal outgrowth. For instance, the exocyst is an octameric protein complex that recruits and tethers exocytic vesicles to the plasma membrane before fusion. Depletion of exocyst proteins impairs polarization and neurite outgrowth(Dupraz et al., 2009; Lalli,

2009). The accumulation of free vesicles in the end-bulb suggests the absence of vesicle tethering or fusion machinery. The re-initiation of a motile growth cone will likely rely on a full complement of machinery, and identifying the elements that are missing from an end-bulb could help devise strategies to enable axon regeneration.

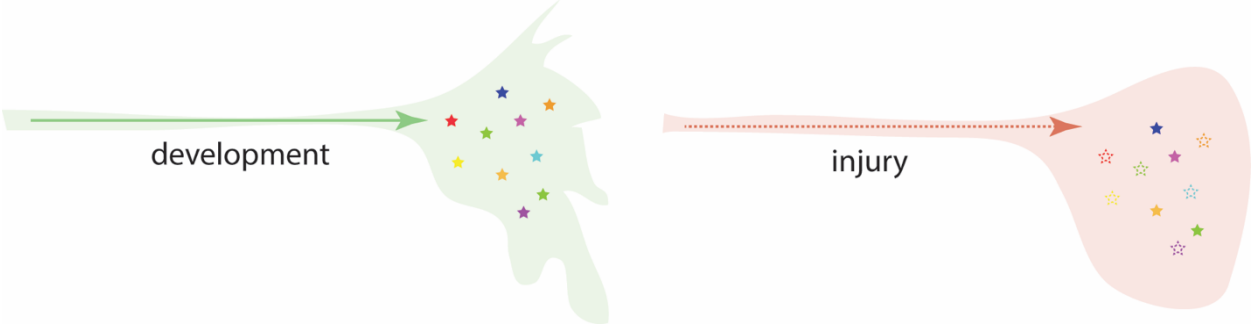

Figure 1. Growth machinery during development and after injury in the adult CNS

During development, a functionally diverse arsenal of machinery is transported to the growth cone to orchestrate the propulsion of the axon. After injury in the mature CNS, the cut end of the axon may only receive a partial installment of the 'ingredients' needed to re-initiate growth, resulting in the formation of a swollen end-bulb.

6.1.2 Extrinsic induction of synaptogenesis within the lesion

CNS injury is followed by the deposition of scar tissue, most notably by reactive astrocytes. CSPGs produced by reactive astrocytes are thought to be a major barrier to regeneration through the lesion. We examined the effect of FC-A on neurite outgrowth on aggrecan, a CSPG found in SCI lesions, and found that although it promoted outgrowth, there was still a persistent inhibition of growth relative to a neutral substrate. Interestingly, this is often the case with many interventions that are reported to enhance neurite outgrowth on CSPGs. For instance, rho kinase inhibition is frequently described to relieve the inhibitory action of CSPGs, however, the data that are often reported do not support this conclusion because these substrates persist in inhibiting growth relative to a neutral substrate (reviewed in Kaplan et al, 2015). Although reactive astrocytes have long been viewed as inhibitory to axon growth, recent work challenges this dogma. A study from the Sofroniew group shows that ablation of reactive astrocytes after SCI in mice worsens axonal die-back from the lesion and renders pro-regenerative interventions ineffective(Anderson et al., 2016). However, this study employs quite drastic methods which do not distinguish between the possible positive and negative contributions of reactive astrocytes, but rather suggests a net positive effect on axonal growth. Work from the Silver group has suggested that axons do not regenerate past the lesion because CSPGs produced by reactive glia may promote adhesion within the

lesion. They provide evidence that axons may form 'synapse-like' contacts with reactive glia, particularly NG2+ cells(Filous et al., 2014; Lang et al., 2015). These studies suggest that axons are 'stuck' in the lesion, rather than the more classical view that they are repelled by the lesion.

6.1.3 Intrinsic switch from growth to synaptogenesis

Axons must stop growing once they synapse with their appropriate targets during development. This switch is likely accompanied by global changes in intracellular signaling. Once a neuron becomes a unit within a circuit, it must adopt a 'receiver-transmitter' function. Upon injury, it is conceivable that the axon synapses within the lesion because the neuron is intrinsically programmed to form synapses. Strategies aimed at suppressing this disposition to form synapses could allow for the neuron to revert to a growth state. For instance, work from the Bradke group has shown that inhibition of the voltage-gated calcium channel *cacna2d2* with pregabalin impairs synaptic transmission and promotes axonal growth and regeneration after SCI. Treatment of embryos with pregabalin induces DRG axonal overgrowth in the spinal cord, supporting the idea that inhibiting activity leads to more exploratory growth(Tedeschi et al., 2016). Interestingly, FC-A has been shown to stabilize 14-3-3 binding to the TASK-3 potassium channel at a classical mode III motif, masking an ER-retention motif and promoting its trafficking to the plasma membrane(Anders et al., 2013). Although we did not detect TASK-3 in our analysis of FC-A binding proteins, it is possible that FC-A may act on this channel in neurons. This could conceivably hyperpolarize the membrane, which may be beneficial for axon regeneration. Whole-cell recordings upon treatment with FC-A could reveal potential changes in channel currents.

6.1.4 Stress response pathways

Although we are beginning to understand how to manipulate signaling pathways to favor regeneration after injury, little is known about injury-induced signaling and how the stress response could be targeted to promote growth. Proteomic analyses of RGCs and DRGs after injury of the optic and sciatic nerves suggests that in the CNS, a multitude of pro-growth molecules are downregulated, a response that does not occur in the PNS. Many of these proteins are regulated by the transcription factor c-Myc. Overexpression of c-Myc prior to, or 2-3 weeks after optic nerve crush promotes robust RGC axon regeneration(Belin et al., 2015). These data suggest that blocking the injury-induced loss of growth molecules or restoring the expression of these molecules after injury can

enable a regenerative response. It will be important to identify the mechanisms underlying the loss of growth molecules after CNS injury. We identified GCN1 as a regulator of neurite outgrowth. In yeast, GCN1 is required for stress-induced inhibition of global protein synthesis through activation of the eIF2-alpha kinase GCN2(Roffe et al., 2013). It is conceivable that the GCN1-GCN2-eIF2-alpha pathway could be induced upon axonal injury, leading to a reduction in protein synthesis (Fig. 2). Such a response could favor cell survival early after injury by inhibiting the synthesis of pro-apoptotic molecules. However, this could also become maladaptive, effectively thwarting a regenerative growth response. Our analyses of GCN1-interacting proteins in un-stressed cortical neurons suggest translation-independent functions at steady-state. We identified a number of microtubule-associated proteins, particularly those which have been found at the centrosome, suggesting that GCN1 may have functions in microtubule nucleation and dynamics. Further study is required to understand the functional significance of these interactions.

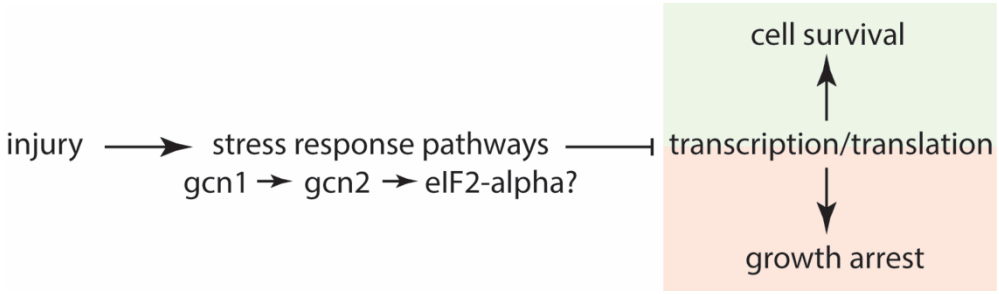

Figure 2. Double-edged sword of stress response after injury

Injury-induced stress may initiate adaptive responses that shut down protein synthesis and other pro-growth pathways, resulting in growth arrest. A possible role for GCN1-GCN2-eIF2-alpha is unknown.

6.1.5 14-3-3 phosphorylation

14-3-3s can be phosphorylated on multiple residues, most notably serine 58 (S58), a residue that is conserved among all isoforms except sigma. S58 is located within the dimer interface of 14-3-3. This residue can be phosphorylated by multiple kinases including Akt, MAPKAP2, and Ste20 stress response kinase 1 (SOK1)(Powell et al., 2002; Powell et al., 2003; Zhou et al., 2009). S58 phosphorylation abrogates 14-3-3 dimerization and impairs binding to client proteins, making this a critical regulatory switch to control 14-3-3 function(Powell et al., 2003). Intriguingly, we found that 14-3-3 S58 phosphorylation in the cerebral cortex increases dramatically during development. In cultured cortical neurons, overexpression of 14-3-3 promotes neurite outgrowth, but a phospho-mimetic mutant has no effect. 14-3-3s are very abundant proteins, particularly

in CNS neurons, however, we hypothesize that the pool of functional 14-3-3s is limited by phosphorylation. In the adult, the striking increase in phosphorylation suggests a global down-regulation of 14-3-3 function. It is interesting to speculate that this could be a factor in the developmental decline in neuron-intrinsic growth capacity. Although we do not have quantitative information about the levels of phosphorylated and un-phosphorylated 14-3-3, we propose that there is a pool of 'client-competent' 14-3-3 and a pool of 'readily functionalized' 14-3-3 (Fig. 3). In this manner, 14-3-3 functions as a 'molecular rheostat' to control cell signaling. This is tuned by multiple kinases and phosphatases whose expression levels and activity are dynamically regulated. How these kinases and phosphatases are regulated after injury is an important question. Moreover, the phosphatases that may act on 14-3-3 have yet to be identified. It is interesting to consider that spatiotemporal control of 14-3-3 phosphorylation within the cell could underlie axon guidance and growth events during development. Perhaps certain cues may locally activate kinases or phosphatases which act on 14-3-3s. Phosphorylated 14-3-3s could act as a sort of 'readily functionalized' reserve pool, waiting to be rapidly and reversibly functionalized as needed. This could be a mechanism to establish exquisite control of signaling events in the cell. In the adult CNS, the ratio of 'client-competent' 14-3-3 may be much lower than during embryogenesis when neurons are actively growing. 14-3-3s bind to hundreds of client proteins, many of which are highly abundant themselves, suggesting that the pool of 'client-competent' 14-3-3s may be limiting, particularly in the adult. This means that small changes in this ratio could have drastic effects on cell signaling and cell biology.

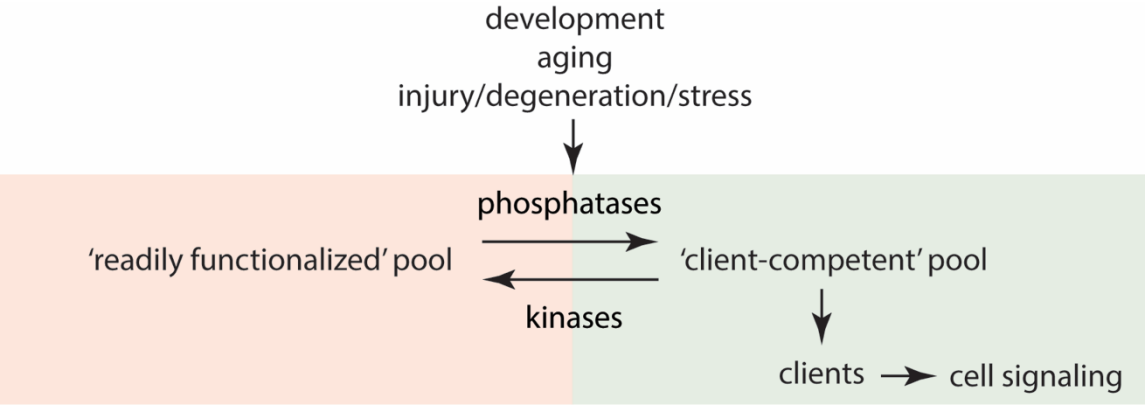

Figure 3. Model for rapid and reversible regulatory control of 14-3-3 function

Development, age and injury/stress may impinge on phosphatases and kinases that act on 14-3-3 'molecular rheostat' to establish spatiotemporal control over cell signaling events.

6.2 Drug discovery for axon regeneration

6.2.1 High-content screening

High-content image-based screening can be used to identify compounds with neurite outgrowth activity, an *in vitro* surrogate of axon regeneration. Neurite outgrowth assays are typically performed using embryonic or early postnatal neurons from various parts of the rodent CNS. It can be argued that the initial outgrowth of neurites in cultured neurons is 'regenerative growth' due to the injury that is inherent to dissociation of the neural tissue. However, the young age of the neurons used in these assays means that they possess a greater growth competency than adult neurons. Moreover, the initial outgrowth that occurs *in vitro* more closely resembles developmental growth, during which neuritogenesis and neuronal polarization occur. Growth-inhibitory substrates, including myelin and CSPGs, can be used to mimic the lesion environment, however, it is difficult to establish appropriate concentrations that are physiologically relevant. These are all important limitations that must be considered upon the identification of compounds with neurite outgrowth activity. Nonetheless, these assays are a great starting point for screening small-molecule libraries, and have led to the identification of compounds that have efficacy in *in vivo* injury models(Li et al., 2016; Usher et al., 2010). Recent advances in the generation of different populations of human iPSC-derived neurons will also provide cell culture models that may be superior in predicting activity in human patients.

6.2.2 Neurite outgrowth phenotypes

We used a high-content screening system to explore neurite outgrowth activity of an FC-A derivative small molecule library. Using this platform, we analyzed various morphological features of neurite outgrowth. These analyses revealed that FC-A compounds possess three primary activities: (1) neuritogenesis, (2) neurite extension and (3) neurite branching. Qualitatively, at doses below the EC50, these compounds primarily induce neurite extension, while doses above the EC50 also stimulate neuritogenesis and branching. At the maximal response, the effect on neuritogenesis becomes so striking that some neurons possess upwards of 10 primary neurites, many of which fail to polarize by 2DIV (Fig. 4B). Neurons treated with FC-7 and other higher potency amide derivatives also undergo remarkable increases in branching along the neurite shaft. These branches are sometimes thin and whispery protrusions emanating *en masse* from a single focus on the shaft (Fig. 4C). Solitary collateral branches and extensive 'arborization' at the terminus of the axon are also commonly observed (Fig.

4D-E). All of these phenotypes are highly unusual in cortical neurons that have only been cultured for 2 days. It is interesting to consider that some of these effects may also be due to inhibition of neurite retraction. During the initial stages of polarization and outgrowth, there is a dynamic interplay between protrusion and retraction. It is possible that in addition to promoting growth, these compounds may also interfere with neurite retraction, resulting in longer and more numerous neurites. It is also important to note that these cultures are heterogeneous, composed of neurons that normally adopt varied morphologies in different cortical layers *in situ*. Our analyses do not make a distinction between possible differences between populations, but rather assess neurite outgrowth as a universal biological process. Another striking feature of neurons treated with these compounds is the tortuous pattern in which the neurites grow out from the cell body (Fig. 4F). This is particularly evident in cultures treated with the amide derivatives. These effects appear to differ somewhat from those observed after injury of mature axons in microfluidic chambers. In those assays, tortuous growth was apparent, but without obvious effects on branching. It is also interesting to note that the effects on neuritogenesis, branching and tortuous growth are not as pronounced in neurons treated with FC-J derivatives (eg. FC-4), which do not possess a functional group at C19. *In silico* docking of the amide derivatives against 14-3-3:peptide crystal structures indicates the establishment of an additional hydrogen bond between the amide and an aspartic acid in the back wall of the 14-3-3 binding groove (personal communication from Dr. Yusuke Higuchi). This aspartic acid may be used by some clients which span the groove. It is therefore possible that these derivatives may, in some cases, function as competitive inhibitors of 14-3-3 PPIs. The phenotypic differences we observe between the amide derivatives and the FC-J derivatives could therefore be due to differences in client selectivity. However, it is also very likely that these effects are merely a function of potency and that enhancements in neuritogenesis and branching would be apparent at very high concentrations of FC-J compounds. Identification and comparison of client proteins bound by these compounds could answer these questions.

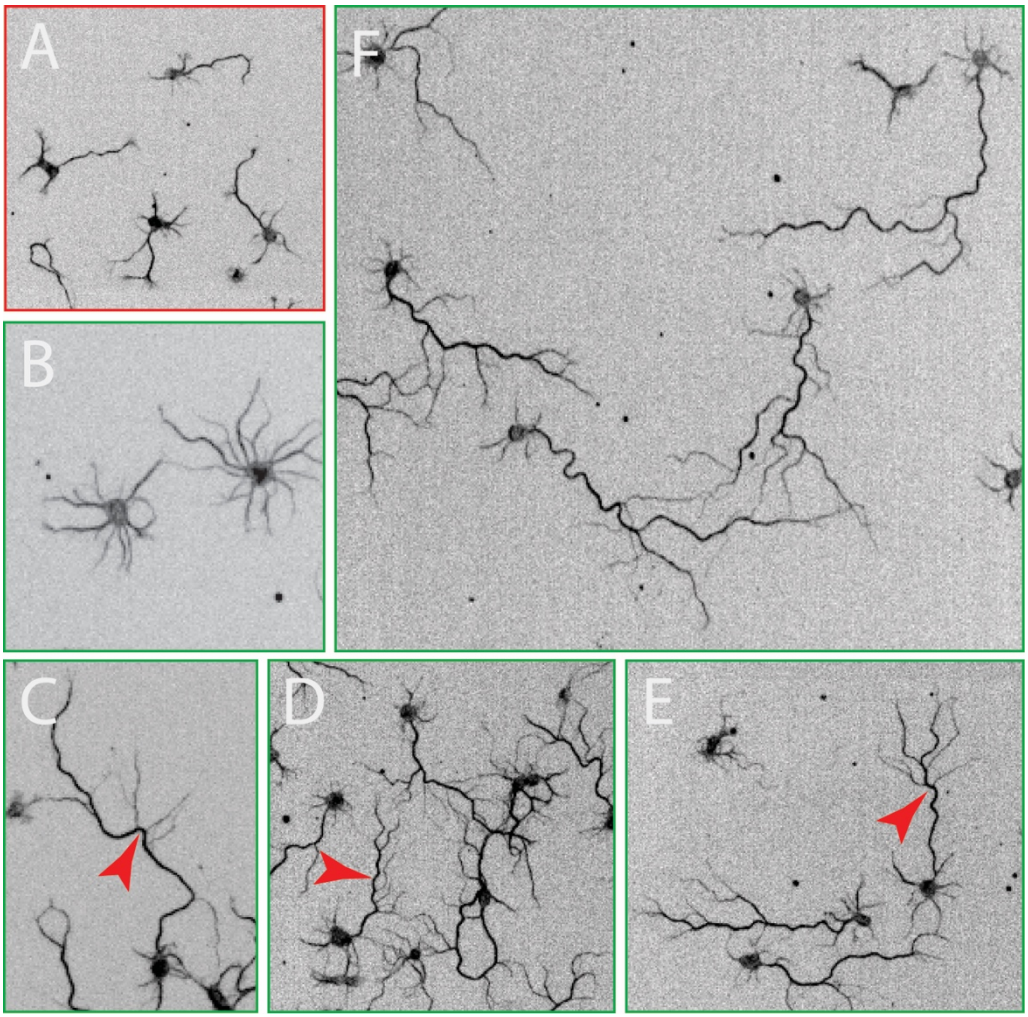

Figure 4. Morphological features of 2DIV E18 rat cortical neurons treated with 19-amide FC-A derivatives. A) DMSO vehicle control. B-F) Neurons treated with 19-amide derivative. B) Unpolarized neurons with supernumerary primary neurites. C) Extensive focal branching on axon shaft (indicated by arrowhead). D) Numerous solitary collateral braches spaced along axon shaft (indicated by arrowhead). E) Extensive arborization at axonal terminus (indicated by arrowhead). F) Axons exhibiting tortuous outgrowth pattern. (some images are reproduced from chapter 3)

6.2.3 *In vivo* models of CNS injury

Promising compounds that have activity in cell-based models can then be tested in rodent models of CNS injury. CST transection and optic nerve crush are two commonly used models to assess axon regeneration. Many of the cortical neurons that give rise to CST fibers survive indefinitely after axonal transection in the spinal cord(Wannier et al., 2005), whereas the vast majority of RGCs die after injury to the optic nerve. The Sanes and He groups have identified an RGC sub-type, the alpha-RGCs, as the most resilient after optic nerve crush(Duan et al., 2015). These cells survive and regenerate to some

extent after injury. Studying the molecular differences between resilient and vulnerable RGC populations could identify new targets for intervention. We found that intravitreal injection of FC-A promotes RGC axon regeneration, but does not affect injury-induced RGC death. We did not examine RGC sub-type specificity in our studies, but it is possible that FC-A acts on the resilient alpha-RGC population. This raises the question of whether FC-A can enable growth cone formation after injury, or if it merely stimulates the extension of axons from neurons that possess an intrinsic ability to survive and regenerate. In the spinal cord, we found that local application of FC-A on the cut ends of CST axons reduced axonal die-back from the lesion. This could indicate that FC-A stimulated some regrowth. Examination of different time-points after injury could answer this question. An important difference between these two *in vivo* experiments is the site of FC-A application. In the optic nerve crush model, the cell bodies had the highest exposure to the compound, whereas in the SCI model, the axons had the highest exposure. The sub-cellular site of action of FC-A currently remains unknown, but these results suggest that FC-A has activity in both domains. Compartmentalized culture systems can be used to differentially treat the cell bodies or the axons, potentially shedding light on this question.

6.3 14-3-3 PPI stabilization in drug development

We discovered that fusicoccanes stimulate neurite outgrowth and axon regeneration by stabilizing 14-3-3 PPIs. Here we consider some of the opportunities and challenges associated with developing these compounds as drugs for CNS and other indications.

6.3.1 Fusicoccanes

Total synthesis of the fusicoccane scaffold is chemically demanding and has not been achieved, however, FC-A and natural derivatives can be purified in high yield from *Phomopsis amygdali* cultures. The group of Kato has optimized sub-kilogram bench-scale production of these compounds(Inoue et al., 2018). Pilot-plant kilogram-scale production has also been achieved(Ballio et al., 1968). Using FC-A and natural analogues as starting material, novel semi-synthetic derivatives can be produced to explore SAR and improve potency and bioavailability. We performed a high-content neurite outgrowth screen with a library of semi-synthetic derivatives and discovered that substitution of the acetoxy group at C19 with an amide improves the potency in neurite outgrowth by a factor of three to seven-fold. This modification also enhances the potency

of the compound in stabilizing 14-3-3 PPIs (personal communication from Dr. Yusuke Higuchi), indicating that this is an 'on-target' 14-3-3-dependent effect. We examined the effects of these compounds on neurite outgrowth and observed dramatic increases in neuritogenesis, neurite extension, and branching. We selected two derivatives for pharmacokinetic analyses and identified a compound that modestly penetrates the intact BBB, providing a lead compound that can undergo further optimization to enhance metabolic stability.

Fusicoccanes are also being studied for applications in cancer. While FC-A and derivatives do not have potent anti-cancer effects on their own, they markedly sensitize various cancer cell lines to chemotherapeutic drugs(Inoue et al., 2018; Kawakami et al., 2012; Miyake et al., 2015; Molzan et al., 2013). This suggests that these compounds could be used in combination therapies to enhance the efficacy of chemotherapy. Many of these studies have focused on FC-A derivatives that have been modified to lack the C12 hydroxylation to mimic CN-A, which possesses more potent anti-cancer activity. The absence of the C12 hydroxylation likely changes the repertoire of 14-3-3 clients and perhaps increases the overall number of clients that are stabilized, due to a greater toleration for bulky side-chains that would otherwise cause steric hindrance downstream of the phosphorylation site. These compounds are non-toxic and well-tolerated with repeated dosing in rodents(Kawakami et al., 2012; Miyake et al., 2015). Together these studies support further development of FC-A-derived compounds for cancer and CNS indications.

6.3.2 Synthetic 14-3-3 PPI stabilizers

The discovery of synthetic compounds that stabilize 14-3-3 PPIs in an analogous fashion to FC-A suggests alternative routes toward the development of drugs with this unique mechanism of action. A high-throughput screen for compounds that stabilize 14-3-3 binding to a mode III motif led to the discovery of epibestatin and pyrrolidone 1. Both these compounds bind within the FC-A pocket and stabilize the complex(Richter et al., 2012; Rose et al., 2010). Interestingly, recent work from the Ottmann group has also identified druggable pockets on the 14-3-3 surface outside of the groove where client proteins make secondary contacts(Sijbesma et al., 2017). These sites could also be targeted with small-molecule stabilizers. Moreover, this could allow for greater client specificity due to the more variable nature of the 14-3-3:client interaction outside of the primary binding site in the groove. The goal of this field is to develop compounds that

are selective for a specific 14-3-3 PPI with one client protein. However, engagement of multiple targets could also be advantageous for indications where targeting a single protein is unlikely to be sufficient. In the case of axon regeneration, the problem is highly complex and likely involves many molecular pathways. A ‘dirty’ drug that targets many molecules may therefore be more effective in addressing multiple deficiencies in the neuron.

6.4 Conclusion

Axonal damage is a fundamental feature of SCI and other injuries and diseases of the CNS. Damaged axons do not spontaneously regenerate after injury, making this a central pathobiological process that could be targeted to restore neurological function in a wide variety of CNS indications. In this thesis, we sought to discover and develop pharmacological approaches to stimulate axon regeneration. We focused our efforts on 14-3-3 adaptor proteins, a family of proteins for which there is a wealth of high-quality structural data and small molecule tool compounds. We found that inhibiting or knocking down 14-3-3s impairs axon growth, whereas overexpression promotes growth. These results provided rationale for exploring strategies to stimulate 14-3-3 function as a means to induce axon regeneration. This led to our idea of using 14-3-3 PPI stabilizing compounds to harness the activity of 14-3-3s. We discovered that fusicoccin-A (FC-A) stimulates neurite outgrowth and axon regeneration. We further showed that the effects of FC-A on neurite outgrowth are ‘on-target’ and require 14-3-3s. We undertook a SAR campaign and improved the potency of the compound, suggesting routes for further development. We used an affinity chromatography and mass spectrometry approach to identify client proteins targeted by FC-A. Our analyses led to the discovery of GCN1, a regulator of protein translation, as a key target involved in FC-A-induced neurite outgrowth. We discovered that GCN1 acts as an intrinsic ‘brake’ on growth, suggesting a new pathway that could be targeted to promote axon regeneration. Our findings establish new avenues of research that could lead to the development of drugs to induce axon regeneration.

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