



RÉSEAU DE RECHERCHE
EN SANTÉ DE LA VISION

PRIX RÉSEAU VISION
PROGRAMME ÉTUDIANT
RÉSEAU DE RECHERCHE EN SANTÉ DE LA VISION
Concours 2017-2018

Objectif

Le Prix Réseau vision vise à récompenser l'excellence de la recherche en santé de la vision par des membres étudiants du RRSV. Les objectifs particuliers du programme sont:

- Promouvoir les carrières en recherche en santé de la vision au Québec.
- Stimuler l'implication des étudiants et stagiaires de recherche au sein du RRSV.
- Faire reconnaître l'excellence de leur travail au sein de la communauté scientifique du Québec et du Canada.

Prix

- Le prix est de 250 \$ par étudiant par publication (si plusieurs auteurs ont contribué au même titre à la recherche, le prix est partagé également entre eux).
- L'article primé et un résumé du parcours du/des boursier(s) sont publiés sur notre site Web.

Admissibilité

Voici les critères d'admissibilité propres au présent appel de demandes:

- Pertinence par rapport à la mission et aux domaines de recherche en santé de la vision du RRSV. Les demandes qui ne correspondent pas au mandat du RRSV ne seront pas évaluées.
- Avoir publié un article comme premier auteur entre le 1^{er} janvier et le 31 décembre de l'année courante. Les manuscrits sous presse ou soumis pour publication doivent être publiés avant le 31 décembre pour être admissibles.
- Ce concours est ouvert à tous les membres étudiants du Réseau vision. Vous devez être étudiant ou stagiaire dans une université du Québec, un hôpital universitaire, un centre de recherche ou dans un autre organisme de santé sous la supervision d'un membre du Réseau vision. (p. ex. : étudiants de premier cycle ou des cycles supérieurs, chercheurs postdoctoraux et résidents).
- Les étudiants de tous niveaux sont admissibles, et ce, jusqu'à 13 mois après la date de complétion de leur formation (c.-à-d.) dernier diplôme obtenu: diplômes de premier cycle, de cycle supérieur ou de résidence. Au moment de la date de tombée du concours, les chercheurs postdoctoraux doivent avoir complété leur stage postdoctoral depuis un maximum de 13 mois).
- Les Québécois et les étrangers qui font de la recherche au Québec sont admissibles.
- Les Québécois qui font de la recherche à l'étranger ne sont pas admissibles.
- Il n'est pas nécessaire d'être boursier du FRQS, ni du RRSV.
- Une seule demande par candidat sera acceptée.

Processus d'examen et critères d'évaluation

Pour attribuer un prix, le comité d'évaluation se fonde sur l'excellence de la recherche, son lien avec le mandat du RRSV, son orientation interdisciplinaire et ses avantages potentiels pour la santé des Québécois. Chaque demande sera évaluée et classée selon son mérite individuel et collectif, en se basant sur les critères suivants:

- Niveau de l'étudiant ou du stagiaire et stade de la carrière: qualité de la recherche en fonction de l'expérience du candidat.
- Contribution de l'étudiant ou du stagiaire: la contribution de la personne à la conception de l'étude ainsi qu'à l'acquisition, à l'analyse et à l'interprétation des données.
- Qualité de la publication: Évaluation de l'impact de la revue et/ou pertinence du sujet de la recherche. Innovation et contribution au domaine de recherche.
- Impact de la publication: impact potentiel de la recherche sur le public cible et au-delà du domaine particulier de recherche (la recherche a-t-elle le potentiel de stimuler de la recherche scientifique au-delà de la publication soumise ou d'être traduite en produits, services, politiques, matériel éducatif ou documents de pratique ?)

Dates importantes

Le comité scientifique du RRSV se réunit une fois par an pour évaluer toutes les demandes reçues dans le cadre de ce concours. Les dates importantes à retenir sont les suivantes:

- 1^{er} janvier 2017 au 31 décembre 2017: date de publication de l'article
- 1^{er} décembre 2017: date limite de soumission au RRSV
- 15 décembre 2017: annonce du (des) gagnant(s)
- 31 décembre 2017: soumission obligatoire de l'article ou des articles par le(s) gagnant(s) au concours national *Cerveau en tête* de l'Institut des neurosciences, de la santé mentale et des toxicomanies des IRSC: <http://www.cihr-irsc.gc.ca/f/9600.html>

Engagement

Tout étudiant récipiendaire du Prix Réseau vision s'engage à soumettre son article gagnant au concours national *Cerveau en tête* de l'Institut des neurosciences, de la santé mentale et des toxicomanies (INSMT) dont la date limite tombe seulement deux semaines après l'annonce des résultats du Prix Réseau vision (voir calendrier ci-haut). Il est donc fortement recommandé aux étudiants soumettant leur candidature au Prix réseau vision de lire attentivement les règlements du concours *Cerveau en tête* dès l'initiation de leurs démarches de soumission au concours du Prix Réseau vision: <http://www.cihr-irsc.gc.ca/f/9600.html>. Par ailleurs, l'étudiant récipiendaire du Prix Réseau vision s'engage à soumettre son article au concours du FRQ *Étudiants-chercheur étoile* s'il est admissible (voir règlements: <http://www.frq.gouv.qc.ca/etudiants-chercheurs-etoiles/regles>)

Important: Le prix Réseau vision ne sera remis aux gagnants que sur remise de la preuve de leur inscription au concours *Cerveau en tête* de l'INSMT.

Comment faire une demande

Les demandes qui répondent aux critères d'admissibilité peuvent être envoyées n'importe quand durant l'année. L'évaluation n'a lieu qu'une fois par an (voir calendrier ci-haut).

Documents à fournir par l'étudiant:

- formulaire ci-dessous dûment complété
- copie de la publication en format PDF
- photographie de l'étudiant

Date limite de soumission: 1^{er} décembre 2017

Nombre de prix octroyés: Tributaire de la disponibilité des fonds.

Prière de faire parvenir une copie électronique de votre demande au:

Réseau de recherche en santé de la vision

reseau.vision.hmr@ssss.gouv.qc.ca

Pour de plus amples informations, n'hésitez pas à communiquer avec:

Réseau de recherche en santé de la vision

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adriana.di.polo@umontreal.ca****Titre de l'article:** **Insulin signalling promotes dendrite and synapse regeneration and restores circuit function after axonal injury.****Citation complète de l'article (format Pubmed):**

[Insulin signalling promotes dendrite and synapse regeneration and restores circuit function after axonal injury.](#) **Agostinone** J, Alarcon-Martinez L, Gamlin C, Yu WQ, Wong ROL, Di Polo A. Brain. 2018 Jul 1;141(7):1963-1980. doi: 10.1093/brain/awy142.

Nom complet du journal: **Brain****Statut de la publication:** **Publié****Date de publication finale:** **01 juillet 2018****Type de recherche :** Clinique Fondamentale Épidémiologie-Santé publique autre, précisez :**Cet article est-il en lien avec la DMLA ?** oui non



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Description de votre contribution

- **Expliquez en quoi votre demande s'inscrit dans le cadre du mandat du RRSV.** Donnez un sommaire de votre recherche et sa pertinence par rapport au mandat du RRSV (Maximum 250 mots).

Les maladies neurodégénératives telles que le glaucome représentent un véritable challenge socio-économique dans le monde entier. Pourtant, il n'existe à ce jour aucun remède et les seuls traitements disponibles réussissent au mieux à ralentir la progression de la maladie.

Une raison pour laquelle la plupart des neuropathologies n'ont toujours pas de thérapie ou que celles-ci sont en général peu efficaces réside dans le fait que les approches sont axées sur la préservation des axones, qui permettent aux neurones de transmettre le message nerveux. Mais des études récentes ont démontré que des changements précoces au niveau des dendrites, ces longs prolongements qui permettent aux neurones de recevoir l'information nerveuse, joueraient un rôle prépondérant dans les maladies neurologiques. Il y a donc un réel besoin de trouver des stratégies permettant de régénérer les structures dendritiques mais aucune étude n'avait encore démontré qu'un neurone adulte est capable de régénérer ces structures. Notre étude est la première non seulement à démontrer que les neurones adultes peuvent faire repousser leurs dendrites et synapses alors qu'elles sont déjà rétractées mais aussi à identifier les voies moléculaires impliquées. En effet, une administration quotidienne d'insuline, par voie systémique (intrapéritonéale) ou topique (gouttes oculaires) quelques jours après une lésion axonale aiguë permet de restaurer les arbres dendritiques des cellules ganglionnaires de la rétine (CGRs) grâce à l'activation de la voie mTOR (*mammalian Target of Rapamycin*). De plus, l'insuline a rétabli les connectivités synaptiques des CGRs ainsi que la fonctionnalité de la rétine et favorisé la survie neuronale.

- **Décrivez votre contribution** à la conception de l'étude et à l'acquisition, l'analyse et l'interprétation des données (Maximum 250 mots).

Dans le cadre de mon doctorat, j'ai participé à l'élaboration de l'hypothèse principale de cette étude en collaboration avec ma directrice de recherche. J'ai également joué un rôle primordial dans la conception et la réalisation du projet de recherche, des protocoles expérimentaux, de toutes les figures ainsi que dans l'écriture de l'article scientifique.

J'ai pratiqué l'intégralité des expérimentations animales comprenant les microchirurgies (axotomie du nerf optique chez la souris) et les traitements par injections intrapéritonéales et intravitréales ou par gouttes ophtalmiques. J'ai aussi procédé à l'ensemble des analyses apparaissant dans les figures (exceptées les figures 5-A à H) et les figures supplémentaires : modélisations tridimensionnelles (dendrites et synapses), analyses immunohistochimiques et par immunobuvardages. Dû à la grande fragilité des marqueurs synaptiques et à l'absence de protocole efficace et reproductible pour leur visualisation endogène,

j'ai également créé un protocole original pour l'analyse des synapses sur cryosections à partir de tissus frais. Le second auteur a réalisé, avec mon assistance, les enregistrements électrophysiologiques ainsi qu' à leur analyse pour l'étude fonctionnelle de la rétine (figure 5-A à H). Des collaborateurs de l'Université de Washington m'ont enseigné la technique du gene gun (transfection biolistique) et nous ont fourni les plasmides requis. J'ai ensuite réalisé moi-même les transfections qui m'ont permis de visualiser et de quantifier les synapses en fonction de différents sous-types de cellules ganglionnaires de la rétine (figure 4G à U).

- **Décrivez l'impact de votre publication.** Expliquez la pertinence et l'importance de votre article dans le domaine de la recherche en santé de la vision et au-delà de ce domaine particulier (Maximum de 250 mots).

En plus de la charge financière que représentent les maladies neurodégénératives telles que le glaucome ou encore la maladie d'Alzheimer, elles ont un impact considérable sur la vie des personnes atteintes ainsi que sur celle de leur entourage. Malheureusement, les seuls traitements disponibles ne permettent généralement pas la guérison et ils sont souvent lourds et invasifs.

Dans le cas du glaucome, les traitements consistent d'une part en l'inoculation de gouttes ophtalmiques plusieurs fois par jour et d'autre part en une chirurgie invasive dont les risques d'aggraver la maladie sont élevés. L'insuline a une faible toxicité chez l'humain, même en administration topique, et comme il s'agit d'un médicament déjà approuvé par Santé Canada, le traitement proposé pourrait faire l'objet d'un protocole de recherche chez l'homme plus rapidement que dans d'autres programmes de recherche. Notre étude pourrait donc avoir des implications majeures dans la prévention des déficits visuels liés au glaucome en proposant une stratégie thérapeutique non invasive, facile à mettre en œuvre et peu coûteuse. D'autre part, ces avancées pourraient s'appliquer à plusieurs neuropathologies autres que le glaucome, comme la maladie d'Alzheimer. En effet, des études récentes tendent à démontrer que la résistance à l'insuline jouerait un rôle prépondérant dans la pathogénèse. Ainsi, la réactivation de la voie mTOR pourrait permettre de stopper la progression de la maladie et de régénérer les structures cérébrales atteintes afin d'améliorer les symptômes.

- **Rédigez une courte biographie** décrivant votre formation en cours et passée, ainsi que votre expérience en recherche. **(En anglais et en français)**

Lors de mon cursus universitaire, j'ai d'abord effectué une maîtrise en Neurosciences dans le laboratoire du Dr Di Polo. J'ai ensuite eu l'opportunité de réaliser un passage accéléré au doctorat. L'acquisition d'une expertise poussée en vision ainsi qu'en microchirurgies chez la souris m'ont permis d'être invitée à un stage de recherche dans un laboratoire de renommée aux USA ainsi qu'à participer à plusieurs collaborations.

During my university curriculum, I first carried out a Master's degree in Neurosciences in the laboratory of Dr Adriana Di Polo. I then had the opportunity to do a fast-track master's to Ph.D. program. The acquisition of an extensive expertise in vision and microsurgery in mice allowed me to be invited to do a research stay in a renowned laboratory in the US and to participate in several collaborations.

Insulin signalling promotes dendrite and synapse regeneration and restores circuit function after axonal injury

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See Peterson and Benowitz (doi:10.1093/brain/awy165) for a scientific commentary on this article.

Dendrite pathology and synapse disassembly are critical features of chronic neurodegenerative diseases. In spite of this, the capacity of injured neurons to regenerate dendrites has been largely ignored. Here, we show that, upon axonal injury, retinal ganglion cells undergo rapid dendritic retraction and massive synapse loss that preceded neuronal death. Human recombinant insulin, administered as eye drops or systemically after dendritic arbour shrinkage and prior to cell loss, promoted robust regeneration of dendrites and successful reconnection with presynaptic targets. Insulin-mediated regeneration of excitatory postsynaptic sites on retinal ganglion cell dendritic processes increased neuronal survival and rescued light-triggered retinal responses. Further, we show that axotomy-induced dendrite retraction triggered substantial loss of the mammalian target of rapamycin (mTOR) activity exclusively in retinal ganglion cells, and that insulin fully reversed this response. Targeted loss-of-function experiments revealed that insulin-dependent activation of mTOR complex 1 (mTORC1) is required for new dendritic branching to restore arbour complexity, while complex 2 (mTORC2) drives dendritic process extension thus re-establishing field area. Our findings demonstrate that neurons in the mammalian central nervous system have the intrinsic capacity to regenerate dendrites and synapses after injury, and provide a strong rationale for the use of insulin and/or its analogues as pro-regenerative therapeutics for intractable neurodegenerative diseases including glaucoma.

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Keywords: insulin; dendrite regeneration; retinal ganglion cell; mammalian target of rapamycin; optic nerve

Abbreviations: ERG = electroretinogram; mTOR = mammalian target of rapamycin; PhNR = photopic negative response; pSTR = positive scotopic threshold response; RGC = retinal ganglion cell

Introduction

Dendrites are exquisitely specialized processes that determine how neurons receive and integrate information within neuronal circuits. Dendrite retraction and synapse disassembly are early signs of pathology in a number of psychiatric and neurodegenerative disorders (Stephens *et al.*, 2005; Grutzendler *et al.*, 2007; Cochran *et al.*, 2014; Agostinone and Di Polo, 2015; Hong *et al.*, 2016; Yan *et al.*, 2016; Kweon *et al.*, 2017). Dendritic pathology occurs prior to soma or axon loss and correlates with substantial functional deficits (Cochran *et al.*, 2014). A critical question is whether dendrites from adult injured neurons can regenerate and reconnect with presynaptic targets once they have retracted. In mammals, CNS neurons have an extremely limited capacity to regenerate after injury (Aguayo *et al.*, 1990). While a large number of studies have focused on axonal regeneration (He and Jin, 2016; Benowitz *et al.*, 2017), the ability of mammalian neurons to regrow dendrites and re-establish functional synapses has been largely ignored. This is a critical issue because pathological disconnection from presynaptic targets leads to persistent functional impairment and accrued neuronal death.

Aberrant or insufficient insulin signalling, even in the absence of diabetes, has been associated with neurodegeneration in diseases characterized by dendritic pathology, notably Alzheimer's and Parkinson's disease, as well as glaucoma (Athauda and Foltynie, 2016; Song *et al.*, 2016; Bloom *et al.*, 2017). Traditionally viewed solely as a peripherally acting hormone, insulin crosses the blood–brain barrier and can influence a number of physiological brain processes including neuronal survival, neurotransmission, and cognitive performance (Ghasemi *et al.*, 2013). It has been suggested that insulin signalling may be potentially targeted for disease modification (Bedse *et al.*, 2015); however, its role in the response of vulnerable neurons is poorly understood. Along these lines, intranasal insulin administration has been reported to improve memory and attention in patients with Alzheimer's disease (Freiherr *et al.*, 2013), but the mechanism underlying this effect is currently unknown. Here, we asked whether insulin signalling promotes dendrite regeneration and the re-establishment of functional connections and, if so, which intracellular pathways regulate this response.

To address this, we focused on retinal ganglion cells (RGCs), a population of long-projecting CNS neurons that convey visual information from the retina to the brain via their axons in the optic nerve. The selective death of RGCs is a crucial element in the pathophysiology of glaucoma, the leading cause of irreversible blindness worldwide (Tham *et al.*, 2014). The rapid retraction of RGC dendrites is one of the earliest pathological changes in glaucoma (Agostinone and Di Polo, 2015). Insulin receptors are abundantly expressed by adult RGCs (Bu *et al.*, 2013), and impairment of insulin signalling in these

neurons inhibits neurite outgrowth (Song *et al.*, 2015). Insulin binding to its receptor activates phosphoinositide-3' kinase (PI3K) and its target Akt leading to potent activation of the mammalian target of rapamycin (mTOR) complexes 1 and 2 (mTORC1 and mTORC2) (Saxton and Sabatini, 2017). The mTORC1 multiprotein complex includes the core components mTOR, Raptor (regulatory protein associated with mTOR, encoded by *RPTOR*), and mLST8 (mammalian lethal with Sec13 protein 8) (Hara *et al.*, 2002; Kim *et al.*, 2003). Instead of Raptor, mTORC2 contains Rictor (rapamycin insensitive companion of mTOR, encoded by *RICTOR*), a completely unrelated protein that serves an analogous function (Dos *et al.*, 2004; Jacinto *et al.*, 2004).

Our data demonstrate that insulin, administered at a time when there is substantial dendritic arbour retraction, promotes remarkable dendrite regeneration. We show that activation of both mTORC1 and mTORC2 is essential for successful insulin-dependent dendritic regeneration. Furthermore, insulin treatment leads to striking regeneration of pre- and postsynaptic components, with marked increase in excitatory postsynaptic sites in ON-sustained, OFF-sustained, and OFF-transient RGCs. Importantly, insulin promoted robust neuronal survival and rescued light-triggered retinal responses. Together, our findings demonstrate that mammalian CNS neurons have the capacity to regenerate dendrites and synapses after injury, and identify the insulin-mTORC1/2 signalling pathway as a critical component for dendritic arbour repair and restoration of circuit function.

Materials and methods

Experimental design and animals

All animal procedures were approved by the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) Animal Care Committee, and followed the ARRIVE and the Canadian Council on Animal Care guidelines. Surgical procedures were carried out in B6.Cg.Tg[Thy1-YFPH]2Jrs/J mice (Jackson Laboratory) or wild-type littermate controls (3–4 months of age) maintained under 12-h light/12-h dark cyclic light conditions with an average in-cage illumination level of 10 lx. All experiments were performed under general anaesthesia using 2% isoflurane (0.8 l/min), except for electroretinogram (ERG) recordings (see below). The number of animals used in each experiment is indicated in the tables and in the figure legends. Data analysis was always carried out blinded by third party concealment of treatment using uniquely coded samples.

Optic nerve axotomy

Axonal injury was induced by complete transection (axotomy) of the optic nerve to trigger rapid and stereotypical loss of RGCs (Alarcón-Martínez *et al.*, 2010; Galindo-Romero *et al.*, 2011). Briefly, an incision in the skin over the superior orbital rim was made to gain access to the back of the eye. The

dural sheath was longitudinally opened to expose the optic nerve, which was then cleanly transected at 0.5–1 mm from the optic nerve head. Care was taken not to damage the central retinal artery, and fundus examination was routinely performed before and after the procedure to verify the integrity of the retinal circulation. Animals showing compromised blood supply were excluded from the study. Axotomy can induce changes in the contralateral eye (Ramírez *et al.*, 2015) therefore, with the exception of ERG recordings (see below), all control eyes in this study were from naïve, non-injured animals.

Retinal immunohistochemistry

Animals were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde (PFA). The eyes were immediately collected and the retinas were dissected out. For flat-mount preparations, the retinas were free-floated for 3 days in 2% Triton™ X-100 and 0.5% dimethyl sulphoxide (DMSO) in phosphate-buffered saline (PBS), followed by incubation for 2 h in blocking solution [10% normal goat serum (NGS), 2% Triton™ X-100, 0.5% DMSO]. The following primary antibodies were applied and incubated overnight at 4°C: SMI-32 (NF-H, 10 µg/ml, Sternberger Monoclonals) or GFP (4 µg/ml, Sigma-Aldrich). Retinas were washed and incubated with secondary antibodies: anti-mouse Alexa Fluor® 594 (2 µg/ml, Molecular Probes) or anti-rabbit Alexa Fluor® 488 (2 µg/ml, Molecular Probes). For retinal cross sections, eyes were embedded in optimal cutting temperature (O.C.T.) compound and cryosections (12 µm) were prepared (Lebrun-Julien *et al.*, 2009). Sections were incubated overnight at 4°C in the following primary antibodies: phospho-S6 (Ser^{240/244}, 1:200, Cell Signaling Technology) and RNA binding protein with multiple splicing (RBPMS, 1:1000, Phosphosolutions), followed by secondary anti-guinea pig or anti-rabbit antibodies (Alexa 594 or 488, 2 µg/ml, Molecular Probes). Three retinal cross sections per eye were analysed at two areas (central and peripheral) for a total of six output measures per mouse. Samples were mounted and visualized with a Zeiss AxioSkop 2 Plus microscope (Carl Zeiss).

Dendritic arbour analysis

Dendritic arbour reconstruction and measurements were performed blinded to manipulations. High-resolution images of yellow fluorescent protein (YFP)-labelled RGC dendrites were acquired using a confocal microscope (Leica Microsystems Inc.). Scans were taken at 0.5 µm intervals (1024 × 1024 pixels) with an average of three to five images per focal plane. Reconstruction of dendritic trees was carried out using the computer-aided filament tracing function of Imaris (Bitplane). The following parameters were measured: (i) total dendritic length: the sum of the lengths of all dendrites per neuron; (ii) total dendritic field area: the area within the contour of the arbour created by drawing a line connecting the outermost tips of the distal dendrites; (iii) total number of branches: the sum of all dendritic branches per neuron; and (iv) Sholl analysis: the number of dendrites that cross concentric circles at increasing distances (10-µm interval) from the soma. RGCs located in all retinal quadrants and eccentricities were included in our analysis.

Insulin and drug administration

Human recombinant insulin diluted in sterile, endotoxin free PBS (15–30 U/kg/day, Sigma-Aldrich; or Humulin-R U100, Eli Lilly) was administered by daily intraperitoneal (i.p.) injections or eye drops (5 µl drop) as per the regimen outlined in Fig. 1F.

For topical delivery, the permeation enhancer polyoxyethylene-20-oleyl ether was added to the insulin solution (0.5%, Brij93, Sigma-Aldrich). Control animals received daily intraperitoneal or eye drops of vehicle. No allergic response, inflammation, or side-effects were detected, as previously reported in humans (Bartlett *et al.*, 1994). Recombinant FITC-tagged insulin (Sigma-Aldrich) was used to examine insulin access to retinal tissue. The dual mTORC1/2 inhibitor KU0063794 (8 mg/kg/day, i.p., Tocris Bioscience) (Zhang *et al.*, 2013), which crosses the blood–retinal barrier, was administered alone or in combination with insulin. Rapamycin (6 mg/kg, LC Laboratories) was administered by intraperitoneal injection.

Short interfering RNA sequences and intravitreal delivery

The following short interfering RNA (siRNA) sequences against *RPTOR*/Raptor and *RICTOR* were purchased from Dharmacon (ON-TARGET plus, Smartpool GE Dharmacon) (sense strands). *RPTOR*/Raptor: (i) 5'-UAG AGGUAGCU GCGAUUAA-3'; (ii) 5'-AUACUGACCGGGAGACGAA-3'; (iii) 5'-AGAAUGAAGGAUCGGAU GA-3'; and (iv) 5'-CUG AGGAACACUCGAGUCA-3'; *RICTOR*: (i) 5'-CUUAGAAG AUCUCGUGAAA-3'; (ii) 5'-AUGUAGAAUUAGAGCGAAU-3'; (iii) 5'-CAUUAUUUGCGGUUGGAAA-3'; and (iv) 5'-CGAUUUUG GCCAUAGUGAA-3'. A non-targeting siRNA was used as control (siCtl, ON-TARGET plus, Smartpool, GE Dharmacon). In some experiments a non-targeting Cy3-tagged control siRNA (siCtl-Cy3) was used to visualize siRNA uptake by retinal cells (Thermo Fisher Scientific). Each siRNA pool (7 µg/µl, total volume: 2 µl) was injected into the vitreous chamber of the eye using a custom-made glass microneedle (Wiretrol II capillary, Drummond Scientific Co). Under general anaesthesia, the sclera was exposed and the tip of the needle inserted into the superior ocular quadrant at a 45° angle through the sclera and retina into the vitreous space. This route of administration avoided injury to the iris or lens, which can promote RGC survival (Mansour-Robaey *et al.*, 1994; Leon *et al.*, 2000).

Western blot analysis

Whole retinas were rapidly isolated and homogenized in ice-cold lysis buffer: 150 mM NaCl, 20 mM Tris, pH 8.0, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 1 mM EDTA, supplemented with 2 mM NaVO₃, and protease and phosphatase inhibitors. Protein samples were resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Life Science). Blots were incubated with each of the following antibodies: phospho-Akt (Ser⁴⁷³, 1:2000, Cell Signaling Technology), total Akt (1:2000, Cell Signaling Technology), Rictor (1:2000, Thermo Fisher Scientific), Raptor (0.96 µg/ml, Abcam), or β-actin (0.5 µg/ml, Sigma-Aldrich), followed by anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (0.5 µg/ml, GE Healthcare). Densitometric

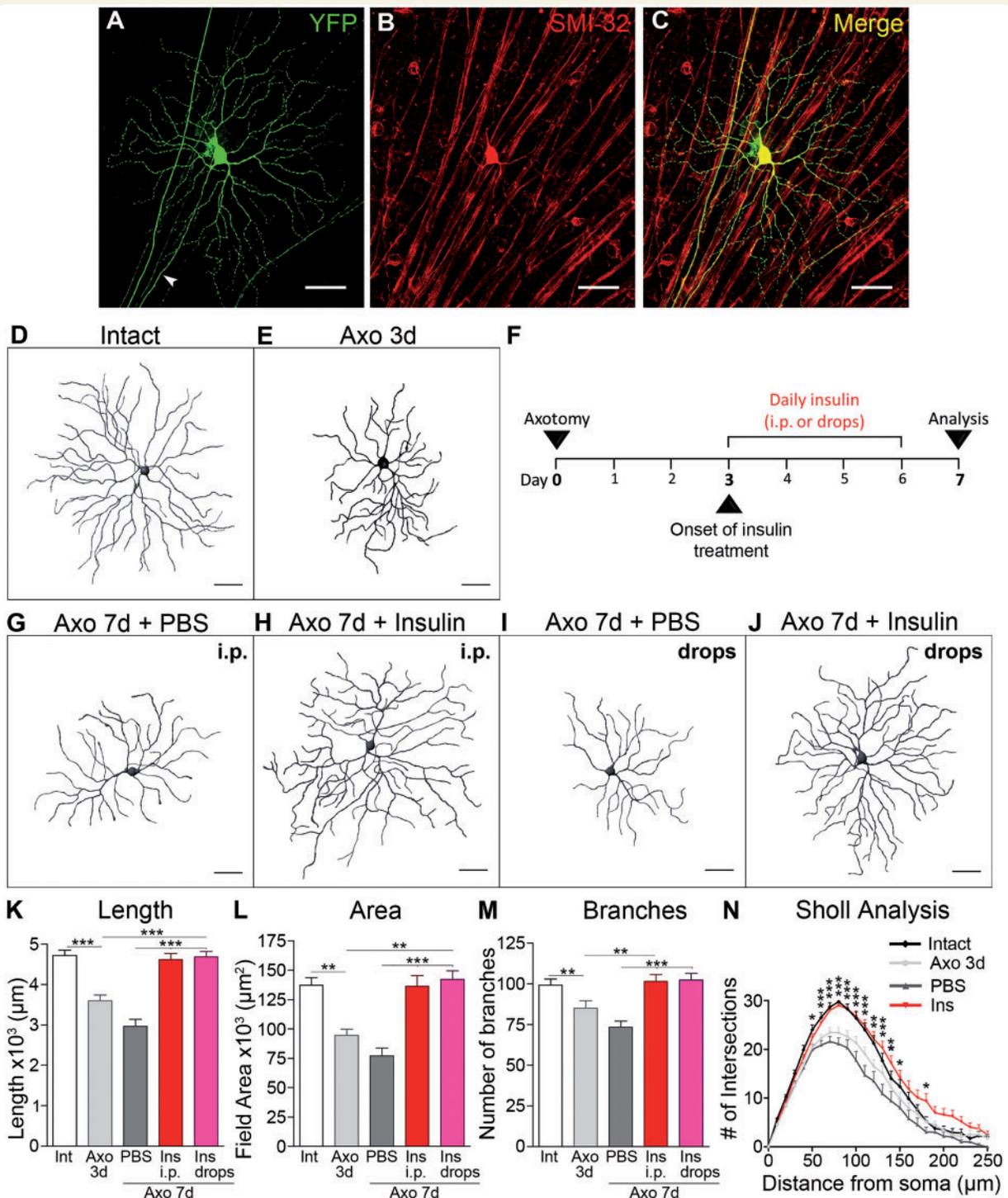


Figure 1 Insulin promotes dendritic regeneration in adult RGCs after axonal injury. (A–C) RGCs co-expressing YFP and SMI-32 (NF-H) with a clearly identifiable axon (arrowhead) were selected for dendritic arbour imaging and 3D reconstruction. (D and E) Three days after axotomy, RGCs had visibly smaller and simpler dendritic arbours relative to intact, non-injured neurons. (F) Human recombinant insulin or vehicle (PBS) were administered daily for four consecutive days by intraperitoneal injection (i.p.) or topically (eye drops) starting at 3 days post-axotomy, a time when there is already substantial dendrite retraction. RGC dendritic arbour analysis was carried out 7 days after injury. (G–J) Representative examples of dendritic arbours from axotomized retinas treated with vehicle or insulin, following the regimen described in (F), visualized at 7 days post-lesion (4 days of insulin treatment). (K–N) Quantitative analysis of dendritic parameters revealed that insulin-treated neurons had longer dendrites and markedly larger and more complex arbours than vehicle-treated controls (insulin i.p.: red, insulin eye drops: pink, PBS: dark grey). Values are expressed as the mean \pm SEM. (ANOVA, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $n = 28$ –46 RGCs/group, Table 1). The number of cells analysed in each group is indicated in Table 1. Scale bars: A–C = 25 μm , E–J = 50 μm .

analysis was performed using ImageJ (<http://imagej.nih.gov/ij/>) on scanned autoradiographic films obtained from five independent western blots, each carried out using retinal samples from different experimental groups.

Analysis of synaptic markers on retinal cross-sections

Mice were sacrificed by decapitation under deep anaesthesia (5% isoflurane), and the eyes were immediately collected. The cornea was carefully pierced with a 30-gauge needle and the eye was incubated in ice-cold 4% carbodiimide (Thermo Fisher Scientific) for 30 min. Retinal cryosections (16 μm) were generated and incubated with each of the following primary antibodies overnight at 4°C: VGLUT1 (1:800, Synaptic System) and PSD95 (2 $\mu\text{g}/\text{ml}$, Abcam). Sections were washed and incubated with secondary antibodies: anti-guinea pig and anti-mouse (Alexa 594 or 488, 2 $\mu\text{g}/\text{ml}$, Molecular Probes). Three retinal cross sections per eye were analysed at two areas (central and peripheral) for a total of six output measures per mouse. Fluorescent labelling was visualized with a Leica SP5 confocal microscope (Leica Microsystems Inc.), and 7.5- μm thick z -stacks were sequentially obtained at 0.13- μm intervals (1024 \times 1024 pixels) with an average of three images per focal plane. Quantitative analysis of voxels, which measured the 3D volume occupied by pre- and postsynaptic markers, was carried out using Imaris (ImarisColoc, Bitplane). VGLUT1 and PSD95 did not co-localize in the same structure, but some overlap was detected due to light diffraction-limited resolution.

Biolistic gene delivery and analysis of excitatory postsynaptic site density

Mice were deeply anaesthetized, killed by cervical dislocation, and the eyes were immediately collected in oxygenated mouse artificial CSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl_2 , 2.5 mM CaCl_2 , 1 mM NaH_2PO_4 , 11 mM glucose, 20 mM HEPES, pH 7.4). Retinas were quickly dissected out and flat-mounted onto nitrocellulose filter paper (Millipore Corporation). Gold particles (12.5 mg, 1.6 μm , Bio-Rad) were coated with CMV:tdTomato (20 μg) and CMV:PSD95-YFP (7 μg) DNA plasmids. A Helios gene gun (Bio-Rad) was used to biolistically deliver the DNA-coated gold particles to whole-mounted retinas, which were then transferred to an oxygenated humidified chamber and maintained for 24 h at 32°C (Della Santina *et al.*, 2013; Ou *et al.*, 2016). Samples were fixed in 4% PFA, mounted, and images were acquired using a Leica SP5 confocal microscope (0.120 \times 0.120 \times 0.294 μm). RGCs were classified according to their dendritic arbour morphology and stratification level in the inner plexiform layer, defined as the distance between the RGC soma and the plane of dendrite ramification. Dendritic arbours and PSD95 puncta were 3D reconstructed and analysed using the Imaris filament tracing and dot finding functions (Bitplane). Candidate puncta present in only one confocal plane or <0.25 μm in diameter were not included in our analysis (Della Santina *et al.*, 2013; Ou *et al.*, 2016).

Electroretinography

Animals were dark adapted overnight prior to ERG recordings and all manipulations were carried out under dim red light. Mice were anaesthetized by intraperitoneal injection of ketamine (20 mg/kg, Bimeda), xylazine (2 mg/kg, Bayer), and acepromazine (0.4 mg/kg, Vétquinol). Bilateral pupil dilation was induced by applying tropicamide on the cornea (1%, Mydracyl[®], Alcon). The intensity of the stimuli was calibrated using a dual-biosignal generator device adapted for ERG responses. The recording system used Burian-Allen bipolar electrodes adapted for use in mice. The active electrode, which has a corneal contact shape, was placed on the cornea following application of a drop of hydroxypropyl methylcellulose (Isopto[®] Tears, 0.5%, Alcon). The reference electrode was placed behind the ears, and the ground electrode in the tail. Electrical signals generated in the retina were amplified (1000 \times) and filtered (band-pass filter: 1–1000 Hz) using a commercial amplifier (Power Lab, ADInstruments). The recorded signals were digitized (Power Lab, ADInstruments) and displayed on a computer. Bilateral ERG recordings were performed simultaneously from both eyes. Measurements from non-injured naïve eyes (pre-injury) served as baseline, and contralateral eyes were used for data normalization. The ERG responses were recorded by stimulating the retina with light intensities ranging between 10^{-6} and 10^{-4} cd s/m² for the scotopic threshold response (STR), and 10^2 cd s/m² for the photopic negative response (PhNR). For each light intensity, a series of responses per flash were averaged (50 recordings). The interval between light flashes was adjusted to allow for response recovery. A calibration protocol was established to ensure homogenous stimulation and recording parameters and was performed immediately prior to each experiment.

Quantification of neuronal survival

Mice were subjected to transcardial perfusion with 4% paraformaldehyde and retinas were dissected out and fixed for an additional 15 min. Free-floating retinas were blocked overnight at 4°C in 10% normal goat serum, 2% bovine serum albumin, 0.5% Triton[™] X-100 in PBS, and incubated with the RGC-specific marker RBPMS (1:1000, PhosphoSolutions) for 5 days at 4°C. Retinas were then incubated with Alexa 488-coupled secondary antibody (2 $\mu\text{g}/\text{ml}$, Molecular Probes) for 4 h at room temperature, mounted with the nerve fibre layer side up, and visualized with a Zeiss Axio Observer (Carl Zeiss). RBPMS-labelled RGCs were counted within three square areas at distances of 0.25, 0.625 and 1 mm from the optic nerve disc in each of the four retinal quadrants for a total of 12 retinal areas.

Statistical analysis

Data analysis and statistics were performed using GraphPad Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA) followed by a Bonferroni or Tukey *post hoc* tests, or by a Student's *t*-test as indicated in the legends.

Table 1 Dendritic parameters in experimental and control groups (data shown in Figs 1–3)

Condition	Total dendritic length, μm Mean \pm SEM	Dendritic field area, μm^2 Mean \pm SEM	Dendritic branches, n Mean \pm SEM	Animals, n	RGCs, n
Intact	4718 \pm 130 (100%)	137 284 \pm 6451 (100%)	99 \pm 4 (100%)	5	43
Axo 3 days	3598 \pm 142	94 632 \pm 5140	85 \pm 5	4	28
Axo 7 days + PBS	2961 \pm 180	77025 \pm 6637	73 \pm 4	5	32
Axo 7 days + Ins (i.p.)	4612 \pm 156 (98%)	136 343 \pm 9021 (99%)	101 \pm 4 (102%)	6	46
Axo 7 days + PBS (drops)	2781 \pm 80	80 673 \pm 3736	64 \pm 3	4	31
Axo 7 days + Ins (drops)	4677 \pm 135 (99%)	142 063 \pm 7520 (103%)	102 \pm 4 (103%)	5	44
Axo 7 days + Ins + siCtl	4585 \pm 155	145 340 \pm 9600	100 \pm 3	4	39
Axo 7 days + Ins + siRaptor	4115 \pm 138	136 243 \pm 6847	71 \pm 2	5	43
Axo 7 days + Ins + siRictor	3138 \pm 144	79 132 \pm 5394	98 \pm 5	5	43
Axo 7 days + Ins + KU	2649 \pm 108	66 964 \pm 3692	65 \pm 3	4	38
Intact + KU	4758 \pm 145	135 612 \pm 6716	99 \pm 4.3	4	41
Intact + siRaptor	4625 \pm 131	138 960 \pm 5784	97 \pm 4.22	3	31
Intact + siRictor	4753 \pm 182	150 757 \pm 12 470	98 \pm 5.13	3	25

Axo = axonal injury; KU = KU0063794; siCtl = control siRNA; siRaptor = siRNA against Raptor.

Results

Insulin promotes robust dendrite regeneration in adult RGCs after axonal injury

To investigate whether adult neurons have the capacity to regenerate dendrites, we used a well-characterized model of acute optic nerve transection to selectively damage RGC axons in transgenic mice expressing YFP under control of the *Thy1* promoter (Thy1-YFPH) (Feng *et al.*, 2000). Anatomical and functional studies have identified 15 to 32 RGC subtypes (Sun *et al.*, 2002; Coombs *et al.*, 2006; Volgyi *et al.*, 2009; Baden *et al.*, 2016), many of which are present in the Thy1-YFPH retina. The antibody SMI-32, which recognizes non-phosphorylated neurofilament heavy chain, was used to identify alpha RGCs characterized by strongly labelled somata and large dendritic arbours (Bleckert *et al.*, 2014; Baden *et al.*, 2016). YFP-positive RGCs that co-labelled with SMI-32 and had a clearly identifiable axon were selected for dendritic arbour imaging and 3D reconstruction (Fig. 1A–C). At 3 days after axotomy, dendrites had visibly retracted relative to non-injured, intact neurons (Fig. 1D and E). Analysis of total dendritic length and total dendritic area demonstrated a reduction of 23% and 31%, respectively, compared to intact SMI-32-positive RGCs (ANOVA $P < 0.001$, Table 1). Dendritic shrinkage occurred prior to RGC soma or axon loss, which starts at 5 days post-lesion in this model (Morquette *et al.*, 2015).

Based on the marked injury-induced dendritic retraction observed at 3 days, we initiated administration of human recombinant insulin or vehicle (PBS) at this time point and analysed dendritic length, area, and complexity 4 days later (7 days post-lesion). The insulin administration regimen

shown in Fig. 1F, consisting of a daily dose of insulin over the course of 4 days starting at Day 3 after axotomy, was used for all the experiments in this study. Insulin was delivered by intraperitoneal injection, which led to mild and transient reduction in blood glucose levels followed by a quick recovery, or topically as eye drops with no detectable changes in glycaemia (Table 2). Dendritic arbour reconstruction and measurements were performed in RGCs co-labelled with YFP and SMI-32, and were always carried out blinded to treatments. At 7 days after axotomy, vehicle-treated RGCs displayed shrunken dendritic arbours with considerably fewer branches (Fig. 1G and I). In contrast, insulin administration, independently of delivery route, promoted robust RGC dendrite regeneration and restored process length, arbour area and complexity (Fig. 1H and J). For example, quantitative analysis of dendritic parameters following insulin treatment demonstrated a 36% increase in process length and 43% larger arbour area relative to vehicle-treated neurons (Fig. 1K and L, ANOVA $P < 0.001$ and $P < 0.01$, respectively, Table 1). Insulin also increased the number of dendritic branches relative to control neurons (Fig. 1M) and promoted a global surge in the number of branch intersections at all distances from the soma, as evidenced by Sholl analysis (Fig. 1N), suggesting enhanced arbour complexity. Overall, insulin restored all dendritic parameters to values similar to those in naïve uninjured retinas. Relative to intact retinas (100%), systemic insulin increased dendritic length, area, and branches to 98%, 99%, and 102%, respectively (Table 1). Similar dendritic regeneration was observed with topical insulin administration (Table 1). The observation that insulin delivered by two different routes, causing very small variations in blood glucose levels and yielding identical regenerative outcomes (Table 2 and Fig. 1H and J), rules out an effect of glycaemic changes on dendrite morphology. Administration of FITC-tagged recombinant insulin confirmed that insulin

Table 2 Blood glucose levels after insulin delivery

	Delivery route	Time after insulin administration (min)								
		0	60	120	180	240	300	360	420	480
Glycaemia, mmol/l	Systemic (i.p.) n = 5	6 ± 0.3	2.4 ± 0.3 (P < 0.001)	1.9 ± 0.1 (P < 0.001)	2.1 ± 0.1 (P < 0.001)	3.6 ± 0.2 (P < 0.001)	4.7 ± 0.2 (P < 0.01)	5.3 ± 0.4 (n.s.)	5.8 ± 0.1 (n.s.)	5.8 ± 0.3 (n.s.)
	Topical (drops) n = 5	5.6 ± 0.2	6.5 ± 0.5 (n.s.)	5.9 ± 0.6 (n.s.)	5.6 ± 0.5 (n.s.)	6.0 ± 0.8 (n.s.)	5.6 ± 0.5 (n.s.)	5.7 ± 0.5 (n.s.)	5.2 ± 0.3 (n.s.)	5.6 ± 0.3 (n.s.)

Data are shown as mean ± SEM. ANOVA, Dunnett's *post hoc* test, n = 5 mice/group, n.s. = not significant (P > 0.05).

effectively reached retinal cells (Supplementary Fig. 1). Collectively, these findings demonstrate that insulin, provided after dendrites have substantially retracted, promotes striking process regeneration and suggest that adult CNS neurons have the capacity to regrow dendrites after injury.

mTORC1 is required for insulin-mediated dendritic branching and restores arbour complexity

Insulin controls many aspects of cell growth and metabolism through activation of the mTOR pathway (Saxton and Sabatini, 2017). Recent evidence indicates that mTORC1 regulates dendritic arbour morphology (Morquette *et al.*, 2015; Skalecka *et al.*, 2016). To determine whether insulin-mediated RGC dendrite regeneration occurred through mTORC1 activation, we first examined whether dendritic retraction correlated with changes in endogenous mTORC1 activity in these neurons. mTORC1 activates the p70 ribosomal S6 kinase leading to phosphorylation of the ribosomal protein S6 at Ser240/244 residues (pS6^{Ser240/244}) thus stimulating protein translation (Jefferies *et al.*, 1997). Antibodies that recognize pS6^{Ser240/244} are widely accepted functional readouts of mTORC1 function (Ikenoue *et al.*, 2009). Immunolabelling of non-injured retinas with pS6^{Ser240/244} revealed two retinal cell populations with robust constitutive mTORC1 activity: one located in the ganglion cell layer and another in the inner nuclear layer (Fig. 2A). Co-labelling of pS6^{Ser240/244} with RBPMS (RNA-binding protein with multiple splicing), a selective marker of RGCs (Rodriguez *et al.*, 2014), revealed robust mTORC1 activity in these neurons in the ganglion cell layer (Fig. 2B–D). A marked decrease of pS6^{Ser240/244} in RGCs was detected at 4 days after axonal injury coinciding with early dendritic retraction, prior to the onset of neuronal death (Fig. 2E and H). Insulin treatment fully restored mTORC1 activity in injured RGCs, consistent with its ability to stimulate this signalling pathway (Fig. 2F and H). In the inner nuclear layer, pS6^{Ser240/244} co-localized with the calcium-binding protein calbindin, a marker of horizontal cells (Supplementary Fig. 2A–C). In contrast to RGCs, pS6^{Ser240/244} expression in horizontal cells remained unchanged after axotomy (Supplementary Fig. 2D–G), indicating RGC-specific mTORC1 downregulation in response to injury. The mTORC1 inhibitor rapamycin completely

eliminated retinal pS6 labelling, thus supporting that pS6 is a true readout of mTOR function in this system (Supplementary Fig. 2H–K).

To determine whether insulin-induced activation of mTORC1 played a role in RGC dendrite regeneration, we sought to selectively reduce mTORC1 function. Rapamycin is an effective mTORC1 inhibitor (Chung *et al.*, 1992), but disrupts mTORC2 function and can induce insulin resistance (Sarbasov *et al.*, 2006; Lamming *et al.*, 2012). To avoid off-target effects of rapamycin, we selectively inhibited mTORC1 with a siRNA against Raptor, a specific and essential component of mTORC1 function (Schalm *et al.*, 2003). First, we examined whether siRNA delivered intraocularly was taken up by mouse RGCs. A single intravitreal injection of non-targeting Cy3-tagged control siRNA (siCtl-Cy3) resulted in RGC labelling as early as 3 h after administration (Supplementary Fig. 1L–O). The mechanism for this rapid preferential uptake is unknown, but it might relate to the ganglion cell layer being directly exposed to the vitreous chamber allowing rapid siRNA diffusion into RGCs.

Next, we assessed the ability of the siRNA against Raptor (siRaptor) to knockdown retinal Raptor protein expression and inhibit mTORC1 function. Retinal immunohistochemistry demonstrated that siRaptor decreased by ~50% the number of pS6-positive axotomized RGCs, visualized with RBPMS, following insulin treatment (ANOVA, P < 0.001, Fig. 2G and H). Western blot analysis confirmed that, in the presence of insulin, retinas that received siRaptor showed a significant reduction of Raptor protein, while non-targeting control siRNA (siCtl) had no effect (Fig. 2I and J). Importantly, siRaptor did not reduce the levels of Rictor, the analogous component in mTORC2 (Sarbasov *et al.*, 2004), validating the specificity of this siRNA (Fig. 2I and K).

We then asked whether siRaptor-mediated knockdown of mTORC1 function had an effect on insulin-induced RGC dendrite regeneration. For this purpose, siRaptor was intravitreally injected concomitantly with the onset of insulin administration (i.p.) at 3 days after optic nerve axotomy, and the neuronal morphology of RGCs co-labelled with YFP and SMI-32 was analysed 4 days later (7 days post-lesion, Fig. 1F). Mice received a single siRaptor intravitreal injection at the onset of daily insulin treatment. Our data show that insulin-mediated regeneration was partially

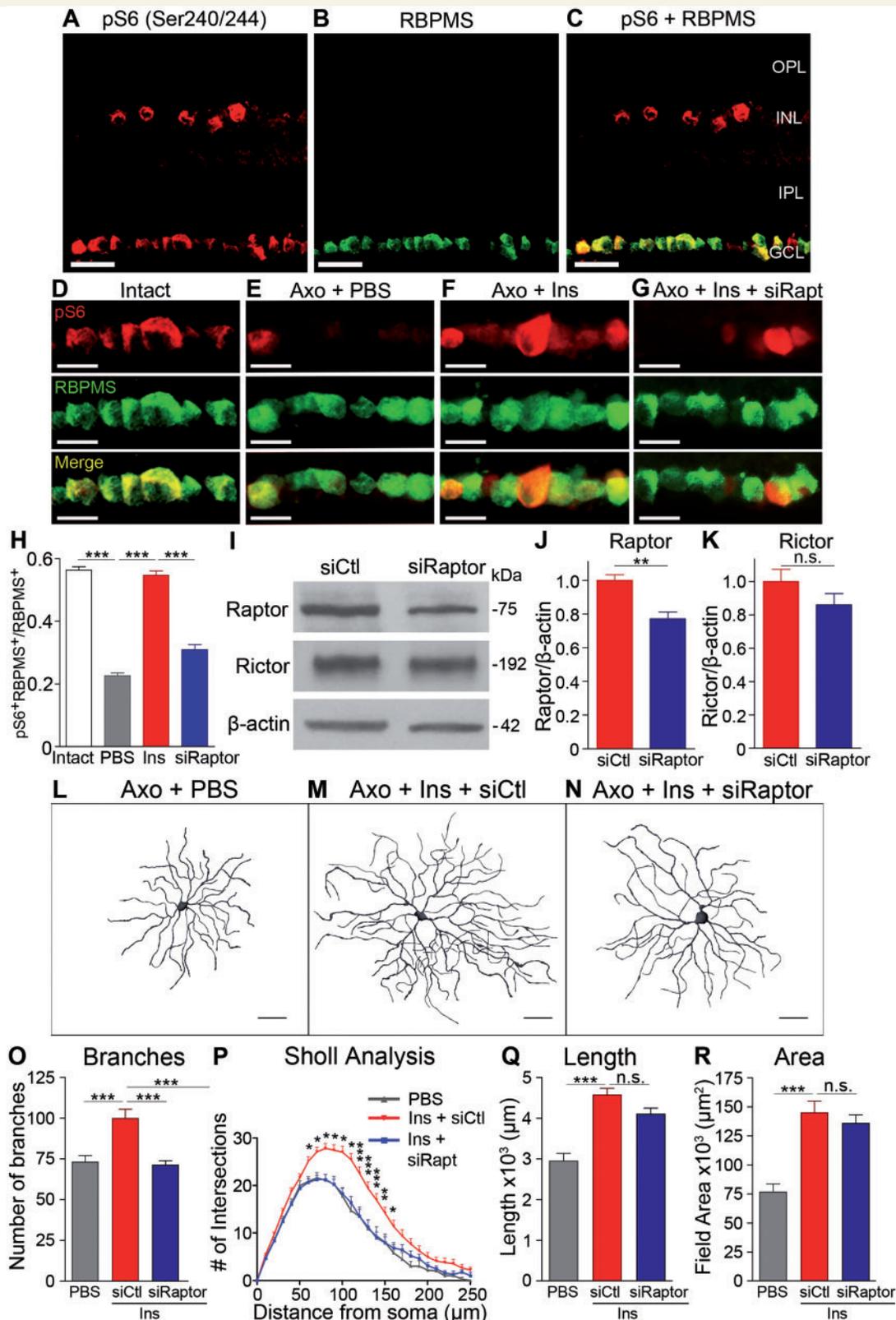


Figure 2 mTORC1 activity is required for insulin-mediated dendritic branching. (A–D) Immunohistochemical analysis of retinal cross sections with an antibody against pS6^{Ser240/244}, a readout of mTORC1 function, and RBPMS, a selective marker of RGCs, revealed robust mTORC1 activity in these neurons. (E and F) Axonal injury induced a marked decrease of pS6^{Ser240/244} labelling in RGCs, suggesting loss of mTORC1 activity, which was restored by insulin treatment. (G) Co-administration of insulin with siRNA against Raptor (siRaptor), an essential component of mTORC1 function, blocked the effect of insulin on RGC-specific pS6^{Ser240/244} levels. (H) Quantification of the number of cells expressing both pS6^{Ser240/244} and RBPMS relative to all RBPMS-positive cells confirmed that insulin fully restored mTORC1 activity in injured

(continued)

blocked by siRaptor (Fig. 2L–N). A 30% reduction in the number of branches was observed with siRaptor, resulting in less complex arbours relative to non-targeting siCtl (Fig. 2O, red versus blue bars, ANOVA, $P < 0.001$, Table 1). Sholl analysis further confirmed a marked decrease in dendritic complexity in retinas treated with siRaptor (Fig. 2P). In contrast, the total dendritic length and arbour area were not affected by siRaptor-mediated blockade of mTORC1 function (Fig. 2Q and R and Table 1). Administration of siRaptor alone in non-injured retinas was not toxic and did not elicit changes in dendritic parameters (Table 1). Our data show that selective mTORC1 knockdown blocks the ability of insulin to increase the number of regenerating branches, suggesting that mTORC1 contributes to the restoration of arbour complexity.

mTORC2 regulates process extension and length in regenerating dendritic arbours

The partial inhibition of insulin-induced dendritic arbour regeneration observed in conditions of lower mTORC1 activity, prompted us to ask whether other signalling components participate in this response. In addition to mTORC1, insulin activates mTORC2 (Saxton and Sabatini, 2017). A critical role of mTORC2 is the phosphorylation of Akt, a key effector of insulin/PI3K signalling (Sarbasov *et al.*, 2005). mTORC2 phosphorylates Akt on Ser473 residues (pAkt^{Ser473}) in a Rictor- and mTOR-dependent manner (Sarbasov *et al.*, 2005), therefore we used antibodies against pAkt^{Ser473} as a readout of mTORC2 activity. Western blot analysis of retinal homogenates demonstrated a substantial reduction of pAkt^{Ser473} in axotomized retinas treated with vehicle, while insulin administration rescued pAkt^{Ser473} levels (Fig. 3A and B), suggesting restoration of mTORC2 function. To establish the role of mTORC2 in insulin-mediated dendrite regeneration, we used an siRNA against Rictor (siRictor), a specific protein component essential for mTORC2 activity (Sarbasov *et al.*, 2004). In the presence of insulin, intraocular injection of siRictor resulted in effective depletion of retinal Rictor relative to non-targeting siCtl-treated eyes (Fig. 3C and D). Rictor knockdown resulted in pAkt^{Ser473} downregulation

indicative of mTORC2 function loss (Fig. 3C and E). Importantly, siRictor did not alter the levels of Raptor, thus validating its specificity (Fig. 3C and F).

To investigate whether mTORC2 played a role in insulin-mediated dendrite regeneration, siRictor and insulin were co-administered at 3 days after axotomy and dendritic arbours from YFP- and SMI32-positive RGCs were characterized 4 days later. Animals received a single siRictor intravitreal injection at the onset of daily insulin treatment (Fig. 1F). Loss of mTORC2 function impaired insulin-induced dendrite extension resulting in shorter dendrites and, consequently, considerably smaller arbour areas (Fig. 3G and H). Quantitative analysis of dendritic parameters confirmed 32% decrease in total dendritic length resulting in reduced surface coverage in eyes that received siRictor relative to non-targeting siCtl-treated controls (Fig. 3J and K, red versus black bars, ANOVA, $P < 0.001$, Table 1). Intriguingly, and contrary to what was observed with loss of mTORC1 function, decreased mTORC2 activity did not affect the number of branches or arbour complexity (Fig. 3H and L). Sholl analysis confirmed no significant changes in arbour complexity between retinas treated with insulin and siRictor or non-targeting siCtl (Fig. 3M).

Next, we asked whether mTORC1 and mTORC2 exerted additive roles in insulin-mediated dendritic regrowth. For this purpose, we co-administered insulin with KU0063794, a well-characterized cell permeable and selective dual inhibitor of mTORC1 and mTORC2 that does not affect other kinases (García-Martínez *et al.*, 2009), using the same regimen as above. Inhibition of both mTOR complexes completely abrogated insulin-mediated dendrite regeneration resulting in RGCs with rudimentary and shrivelled dendritic arbours, with shorter and considerably fewer processes, compared to control RGCs (Fig. 3I and J–L). Quantitative analysis confirmed that KU0063794 led to a dramatic reduction in total dendritic length, arbour area, and number of branches indicative of lower complexity (Fig. 3J–L, green bar and Table 1). Intraocular injection of KU0063794 alone in non-injured retinas did not induce cell toxicity or altered dendritic parameters (Table 1). Collectively, these data suggest that mTORC2 activity contributes to process extension and the re-establishment of arbour area, and that mTORC1 and mTORC2 play additive roles during insulin-induced dendrite regeneration.

Figure 2 Continued

RGCs relative to vehicle- or siRaptor-treated retinas (ANOVA, $***P < 0.001$, $n = 5$ mice/group). (I–K) Western blot and densitometry analysis confirmed that intravitreal delivery of siRaptor reduced retinal Raptor protein while a non-targeting control siRNA (siCtl) had no effect (Student's *t*-test, $**P < 0.05$, n.s. = not significant, $n = 6–7$ mice/group). siRaptor did not alter the levels of Rictor, confirming the specificity of the siRNA. The lower panel is the same blot as in the upper panel but probed with an antibody that recognizes β -actin to ensure equal protein loading. (L–N) Co-administration of insulin and siRaptor resulted in a marked loss of dendritic branches and simpler arbours relative to insulin alone or insulin combined with siCtl at 7 days post-injury. (O–R) Quantitative analysis of dendritic parameters, including Sholl analysis (P), confirmed that selective mTORC1 knockdown blocked the ability of insulin to increase the number of regenerating branches, while no significant changes in process length or arbour area were observed (Q and R) (ANOVA, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, n.s. = not significant; $n = 4–5$ mice/group, $n = 32–43$ RGCs/group, Table 1). Values are expressed as the mean \pm SEM. Scale bars: A–C and L–N = 50 μ m, D–G = 25 μ m. GCL = ganglion cell layer; INL = inner nuclear layer; IPL = inner plexiform layer; OPL = outer plexiform layer.

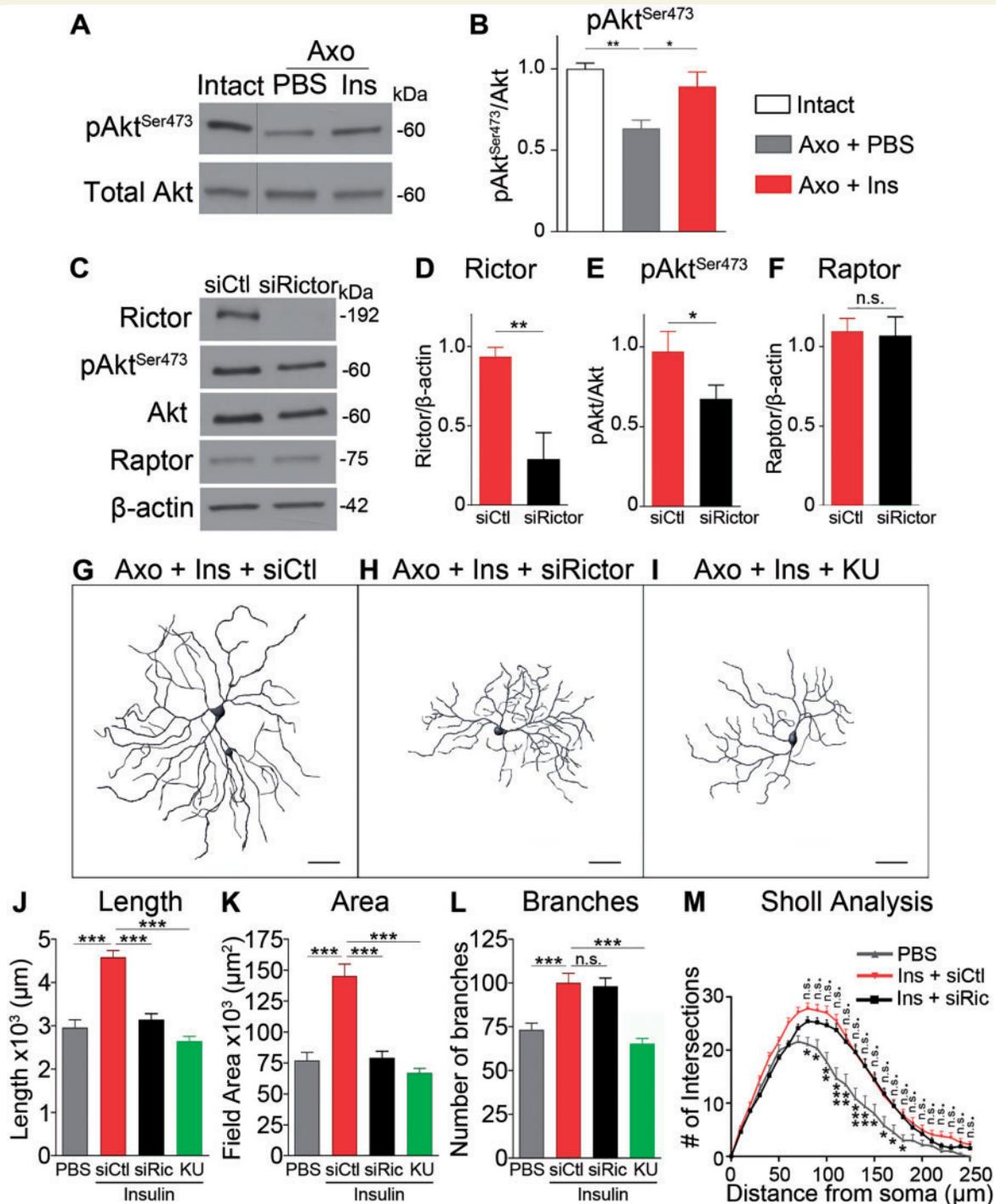


Figure 3 mTORC2 regulates process extension and length in regenerating dendritic arbours. (A and B) Western blot analysis of retinal homogenates demonstrated a substantial reduction of pAkt^{Ser473}, a readout of mTORC2 activity, in axotomized retinas treated with vehicle, while insulin administration rescued pAkt^{Ser473} levels (ANOVA, $**P < 0.01$, $*P < 0.05$, $n = 5$ mice/group). The lower panel is the same blot probed with an antibody against total Akt for normalization. (C–F) Western blot and densitometry analyses demonstrated that intravitreal injection of siRNA against Rictor (siRictor) led to reduction of retinal Rictor protein while a non-targeting control siRNA (siCtl) had no effect. Rictor knockdown also resulted in pAkt^{Ser473} downregulation, indicative of mTORC2 function loss, but did not alter the levels of Raptor, thus validating its specificity (Student's *t*-test, $**P < 0.01$, $*P < 0.05$, n.s. = not significant, $n = 6$ –7 mice/group). The lowest panel represents the same blot probed with an antibody against β -actin to ensure equal protein loading. (G and H) Co-administration of insulin and siRictor resulted in a dramatic reduction in dendritic length and arbour area at 7 days post-injury relative to control retinas. (I) Administration of insulin and KU0063794 (KU), dual mTORC1 and mTORC2 inhibitor, resulted in overt dendrite degeneration characterized by a dramatic loss of branches and much shorter processes. Scale bars = 50 μm . (J–M) Quantitative analysis of dendritic parameters, including Sholl analysis (M), confirmed that siRictor-mediated mTORC2 inhibition resulted in a substantial loss of regenerating branches, while no significant changes in process length or arbour area were observed (ANOVA, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, n.s. = not significant, $n = 4$ –5 mice/group, $n = 38$ –43 RGCs/group, Table 1). Values are expressed as the mean \pm SEM.

Insulin restores glutamatergic post-synaptic sites in injured neurons

Excitatory inputs from bipolar cells onto RGCs occur at the ribbon synapse, a specialized structure that enables fast and sustained neurotransmission required for vision (Heidelberger *et al.*, 2005). We asked whether insulin restored glutamatergic synapses on regenerated dendritic processes. For this purpose, we first examined changes in endogenous vesicular glutamate transporter 1 (VGLUT1), a presynaptic protein expressed at bipolar ribbon synapses (Johnson *et al.*, 2003), and postsynaptic density protein 95 (PSD95) in the inner plexiform layer, where RGC dendrites are located. A pronounced decrease in the expression of both VGLUT1 and PSD95 was observed after axonal injury in vehicle-treated eyes relative to naïve controls (Fig. 4A and B). Insulin, administered after synapse disassembly according to the regimen shown in Fig. 1F, promoted marked rescue of VGLUT1 and PSD95 expression in the inner plexiform layer (Fig. 4C). Quantitative analysis of pre- and postsynaptic voxels, which measures the 3D volume occupied by VGLUT1 and PSD95 in the inner plexiform layer, confirmed that insulin promoted robust synaptic regeneration compared to vehicle-treated retinas (Fig. 4D–F, $n = 4–6$ mice/group).

To characterize the effect of insulin on the distribution of excitatory postsynaptic sites further, we used biolistic transfection of CMV:PSD95-YFP and CMV:tdTomato plasmids onto injured or control retinal whole mounts followed by analysis of PSD95 puncta on individual YFP-positive RGC dendrites (Fig. 4G). This technique has been previously validated for the analysis of excitatory postsynaptic site density in RGC dendritic branches (see ‘Materials and methods’ section) (Morgan *et al.*, 2008). Axotomized neurons had many varicosities along dendrites, often accompanied by large retraction bulbs at their tips (Fig. 4G), as well as larger PSD95 puncta size (Fig. 4H and I). A recent study suggested that OFF-transient RGCs are more prone to lose synapses in a mouse model of ocular hypertension glaucoma (Della Santina *et al.*, 2013), thus we analysed excitatory postsynaptic site density in ON-sustained OFF-sustained, and OFF-transient cells. RGC subtypes were classified according to their morphology and dendritic stratification level in the inner plexiform layer (Pang *et al.*, 2003; Huberman *et al.*, 2008; Van Wyk *et al.*, 2009) (Supplementary Fig. 3). Substantial dendritic alterations as well as marked loss of PSD95 puncta were observed in all alpha RGC subtypes following axotomy (Fig. 4J–U). Quantitative analysis of PSD95-YFP confirmed extensive synapse disassembly and loss of PSD95 puncta density in injured RGCs treated with vehicle, independently of subtype (Fig. 4K, O and S). Interestingly, although all alpha RGCs were affected, ON-sustained and OFF-transient RGCs displayed the highest vulnerability to axonal injury with a loss of ~65% of synapses when compared to non-injured RGCs (Fig. 4J, K, M, R, S and U; ANOVA,

$P < 0.001$, $n = 3–6$ mice/group). Insulin promoted striking rescue of excitatory postsynaptic sites on RGC dendrites, again independently of subtype, returning PSD95 puncta to 76%, 93%, and 93% of control PSD95 densities on ON-sustained, OFF-sustained, and OFF-transient RGCs, respectively (Fig. 4L, M, P, Q, T and U, ANOVA, $P < 0.001$). We conclude that insulin restores postsynaptic site density on regenerating RGC dendrites.

Insulin rescues retinal function and increases neuronal survival

To assess the impact of insulin-mediated dendrite and synapse regeneration on RGC function, we measured two components of the full-field ERG: the pSTR and the PhNR. The pSTR and PhNR derive predominantly from RGC activity in the rodent retina, and are reduced after optic nerve injury and in glaucoma patients (Bui and Fortune, 2004; Alarcón-Martínez *et al.*, 2010; Chrysostomou and Crowston, 2013; Smith *et al.*, 2014; Wilsey and Fortune, 2016). Because of the non-invasive nature of the ERG, we longitudinally followed light responses in the same mice prior to axotomy, and after injury and administration of vehicle or insulin using the regimen outlined in Fig. 1F. Figure 5 shows representative pSTR and PhNR recordings from naïve retinas, prior to axotomy (Fig. 5A and D), and after lesion in mice treated with vehicle (Fig. 5B and E, PBS, blue trace) or insulin (Fig. 5C and F, red trace) relative to the contralateral non-injured eye (grey traces). Both the pSTR and PhNR responses were markedly reduced after optic nerve injury in vehicle-treated mice, with a 48% and 60% decrease in amplitude, respectively, relative to naïve eyes (Fig. 5G and H, ANOVA $P < 0.001$, $P < 0.05$, respectively). In contrast, insulin fully restored both the pSTR and PhNR amplitudes in injured retinas at 7 days post-axotomy (Fig. 5G and H, red bars, $n = 4–10$ mice/group) suggesting recovery of RGC function upon light stimulation. To rule out confounding secondary changes in the contralateral eye (Ramírez *et al.*, 2015), the contralateral control and experimental responses were compared. Our data show no significant differences between these responses (Supplementary Fig. 4) indicating that the contralateral eye was not affected in these conditions.

To investigate whether the pro-regenerative effect of insulin reflected on the ability of RGCs to survive after injury, we followed the same treatment regimen as above and analysed neuronal density on flat-mounted retinas at 1, 2, 4, and 6 weeks after axotomy. Retinas from eyes treated with insulin consistently showed higher densities of RBPMS-labelled RGCs than those treated with vehicle (Fig. 5I–L). Quantitative analysis demonstrated that insulin promoted substantial RGC survival relative to PBS-treated eyes at 1 week after optic nerve injury (insulin: 70% survival, vehicle: 46% survival, $n = 5–6$ mice/group, ANOVA $P < 0.001$) (Fig. 5M). Remarkably, insulin administration

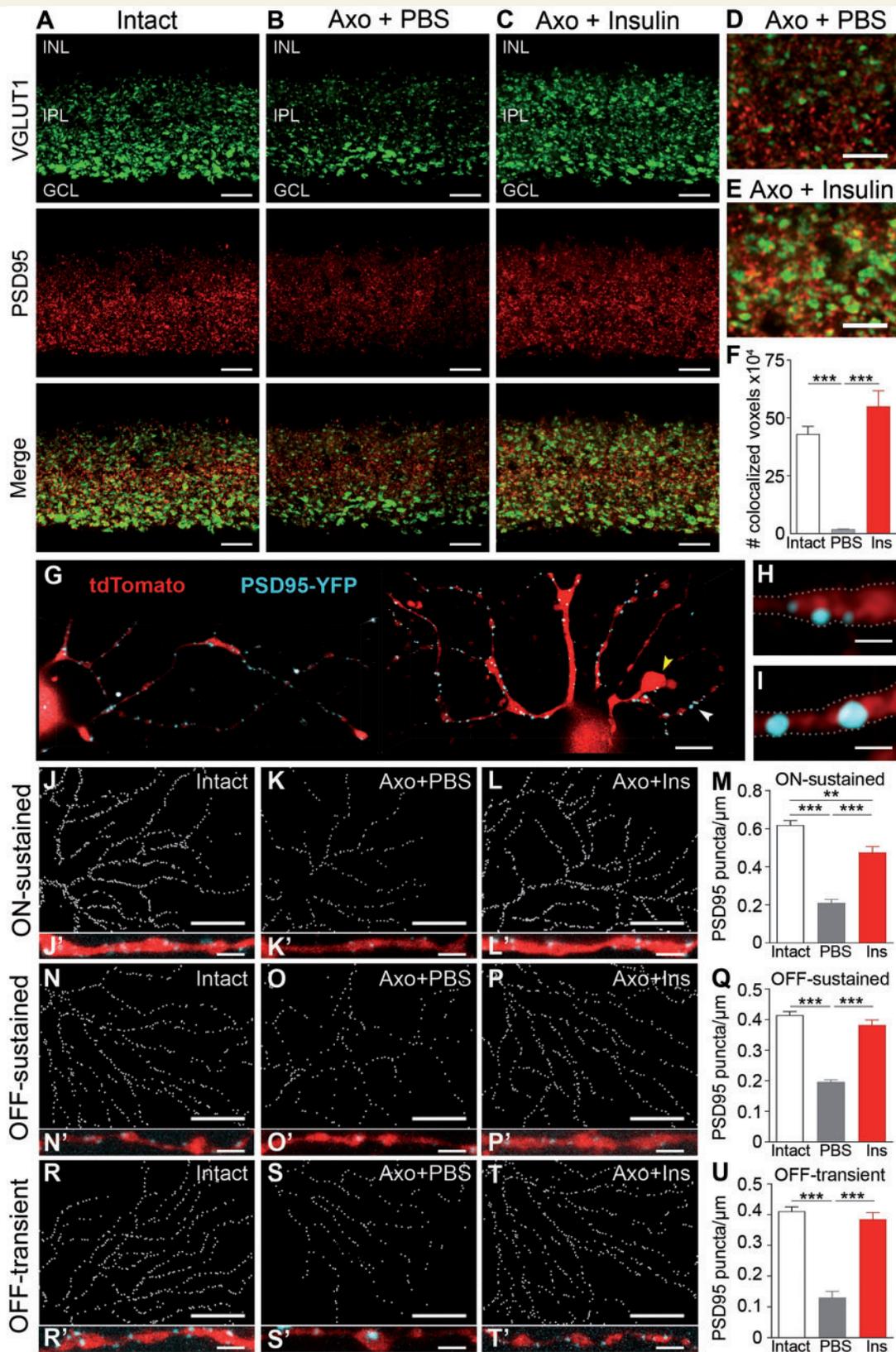


Figure 4 Insulin restores glutamatergic postsynaptic sites in injured neurons. (A) Glutamatergic synapses visualized in the inner plexiform layer (IPL) on retinal cross-sections using immunolabelling against VGLUT1 (green) and PSD95 (red), a pre- and a postsynaptic marker, respectively. (B and C) Axonal injury induced a pronounced loss of both VGLUT1 and PSD95 expression in the inner plexiform layer, which was completely restored by insulin treatment (analysis at 7 days post-axotomy). (D and E) High magnification of VGLUT1 and PSD95 puncta in the inner plexiform layer at the level of the OFF sublamina in retinas treated with insulin or vehicle. (F) Quantitative analysis of pre- and postsynaptic

(continued)

resulted in sustained neuroprotection promoting the survival of 60% of RGCs at 2 weeks after injury, when only 14% of RBPMs-positive neurons remained in vehicle-treated eyes ($n = 5-6$ mice/group, ANOVA $P < 0.001$) (Fig. 5M). Furthermore, 25% of RGCs survived at 4 and 6 weeks, respectively, relative to 8% observed in vehicle treated controls (Fig. 5M, $n = 5$ mice/group, ANOVA $P < 0.05$ and $P < 0.01$, respectively). Next, we asked whether insulin-induced mTORC1 and mTORC2 activation played a role in neuronal survival. For this purpose, insulin was co-administered with KU0063794 and neuronal density was quantified at 1 and 2 weeks after axotomy. KU0063794 fully inhibited insulin-mediated neuroprotection, reducing the number of RGCs to levels similar to those found in vehicle-treated retinas (Fig. 5L and M). Administration of KU0063794 alone in intact, non-injured retinas did not alter RGC numbers, ruling out a toxic effect of this drug. Thus, insulin rescues RGC function and extends the survival of these neurons after axonal injury through mTOR signalling.

Discussion

Dendrites are extremely dynamic during development, elaborating and retracting rapidly in response to intrinsic and environmental cues (Wong and Wong, 2000; Cline, 2001), but they become stable in adulthood and undergo few structural changes thereafter (Koleske, 2013). The stability of dendrites is severely compromised following axonal injury or during neurodegeneration. Shrinkage of RGC dendritic arbours has been observed in primate, cat and rodent models of glaucoma (Weber *et al.*, 1998; Shou *et al.*, 2003; Morgan *et al.*, 2006; Li *et al.*, 2011; Della Santina *et al.*, 2013; Feng *et al.*, 2013; Williams *et al.*, 2013; Morquette *et al.*, 2015) as well as in human glaucomatous retinas (Pavlidis *et al.*, 2003; Fard *et al.*, 2016). Consistent with this, we show that selective injury to RGC axons triggers rapid dendritic shrinkage and loss of arbour complexity, prior to overt neuronal loss. Remarkably, insulin administration, after dendrite retraction had already occurred, promoted robust process regrowth and restoration of arbour area and complexity. Because our study

focused on classic alpha RGCs expressing SMI-32, which are characterized by large somata and expansive dendritic fields with a typical branching pattern (Peichl, 1991), there is no risk of confounding retraction or lack of regeneration for neurons with more compact dendritic trees. Notably, spontaneous dendrite regeneration has been described in invertebrate systems. Peripheral nervous system sensory neurons in *Caenorhabditis elegans* and *Drosophila* display a robust ability to regrow the stereotypical pattern of dendritic branches after laser-induced dendrite ablation (Stone *et al.*, 2014; Thompson-Peer *et al.*, 2016; Oren-Suissa *et al.*, 2017). Recent work demonstrated the use of two-photon nanosurgery in the mouse brain to ablate single dendrites that resulted in rapid fragmentation of the distal end and formation of a retraction bulb (Zhao *et al.*, 2017). Using a prick-injury model in the cerebral cortex, another study showed dendritic regrowth by pyramidal cells after local injection of heparin-binding growth-associated molecule, an effect that was attributed to modification of the glial scar (Paveliev *et al.*, 2016). In our optic nerve injury model, RGC dendrites never regenerated unless they were stimulated with insulin, thus providing the first evidence of successful dendritic regeneration in retinal neurons. Failure to spontaneously regrow dendrites is reminiscent of the limited capacity for axonal regeneration in mammals relative to lower order vertebrate and invertebrate organisms (Nawabi *et al.*, 2012). Our data support the finding that injured CNS neurons are endowed with an intrinsic ability to regrow dendrites and can readily re-establish functional dendritic arbours upon insulin signalling.

Our data show that insulin-dependent activation of both mTORC1 and mTORC2 is required for dendrite regeneration, and these complexes cooperate in an additive manner to ensure successful restoration of dendritic length and arbour complexity. Intriguingly, our siRNA-mediated loss-of-function experiments revealed that mTORC1 and mTORC2 do not control the same aspects of dendrite regeneration. mTORC1 was required for branching and the restoration of arbour complexity, while mTORC2 regulated process extension and the re-establishment of arbour area. Of interest, partial raptor knockdown had a striking effect on dendritic branching confirming the importance of raptor within the mTORC1 complex. Indeed, raptor forms

Figure 4 Continued

co-localized voxels, which measured the 3D volume occupied by both VGLUT1 and PSD95 in the inner plexiform layer, confirmed that insulin promoted synaptic marker regeneration (insulin: red bar, vehicle: grey bar, ANOVA, $***P < 0.001$, $n = 4-6$ mice/group). (G) Representative examples of biolistically labelled RGCs with plasmids encoding tdTomato (red, RGC dendrites) and PSD95-YFP (cyan, PSD95 puncta) at 7 days after axotomy. In the absence of treatment, many retraction bulbs and varicosities (yellow arrowheads) as well as abnormally large PSD95 puncta (white arrowheads) were observed. (H and I) Higher magnification of dendritic branch segments show abnormally large PSD95 clusters in vehicle-treated retinas relative to intact RGCs. (J–U) Analysis of 3D-reconstructed PSD95 puncta density along RGC dendrites demonstrated striking insulin-mediated regeneration of excitatory postsynaptic sites in ON-sustained, OFF-sustained, and OFF-transient alpha RGCs relative to vehicle-treated controls. (J'–T') Higher magnification images of individual segments are provided to show PSD95-YFP puncta (blue) along dendrites (red) for each condition. Values are expressed as the mean \pm SEM. (ANOVA, $***P < 0.001$, $*P < 0.01$, $n = 5-6$ mice/group, $n = 3-6$ RGCs/group). Scale bars: A–C and G = 10 μ m, D and E = 5 μ m, H and I = 1 μ m, J–S = 30 μ m, J'–T' = 2.5 μ m. GCL = ganglion cell layer; INL = inner nuclear layer.

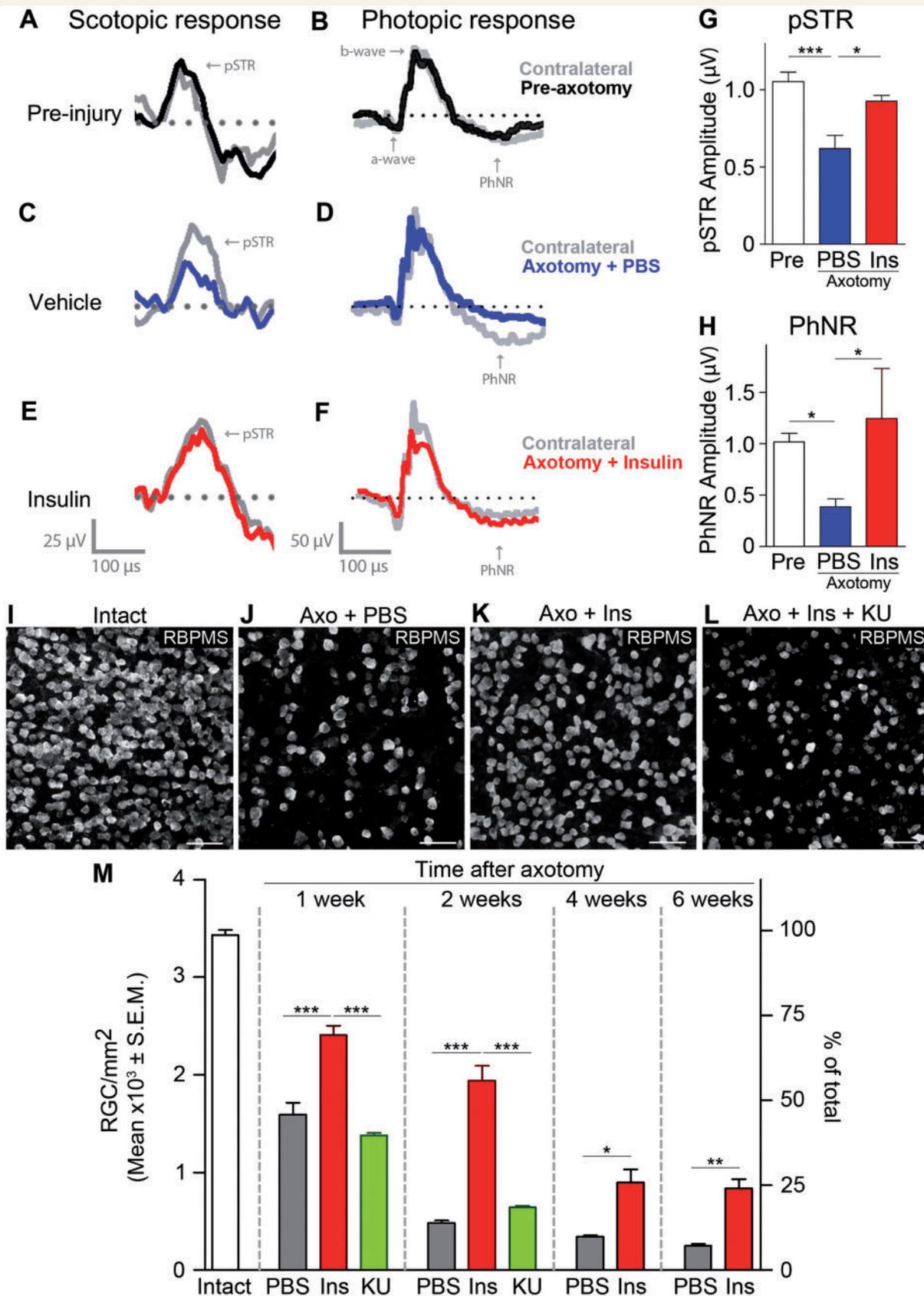


Figure 5 Insulin rescues retinal function and increases neuronal survival. (A–F) Representative examples of ERG recordings elicited by dim scotopic (A, C and E) or photopic (B, D and F) light stimulation prior to axotomy (pre-injury, black trace), or after axotomy and treatment with PBS (blue trace) or insulin (red trace). Pre- and post-axotomy recordings were normalized relative to the contralateral, non-injured eye (grey traces).

(continued)

a stoichiometric complex with mTOR tightly regulating its kinase activity (Kim *et al.*, 2002), thus a small change in raptor function is expected to have a large impact on mTORC1-mediated dendritic branching. Our finding that mTORC1 mediates insulin-induced branching in RGCs is consistent with developing hippocampal neurons in which mTORC1 inhibition reduces the number of dendritic branches and arbour complexity (Jaworski *et al.*, 2005). This response was mimicked by p70 ribosomal S6 kinase knockdown (Jaworski *et al.*, 2005), thus it is tempting to speculate that mTORC1 controls the addition of new branches during insulin-induced regeneration through activation of protein translation. Consistent with our observation that mTORC2 mediates dendrite extension, Rictor-deficient Purkinje cells lacking mTORC2 have shorter dendrites and decreased arbour area (Thomanetz *et al.*, 2013). In neurons, mTORC2 controls actin polymerization for neuronal shape modification and synaptic plasticity required for the consolidation of long-term memory (Angliker and Rüegg, 2013; Huang *et al.*, 2013; Thomanetz *et al.*, 2013). Therefore, mTORC2 might promote dendrite regenerative growth through regulation of cytoskeleton dynamics.

Deletion of phosphatase and tensin homolog (PTEN), the negative regulator of PI3K upstream of mTOR, enhances axonal regeneration in rodent RGCs and corticospinal neurons, as well as sensory neurons in flies (Park *et al.*, 2008; Liu *et al.*, 2010; Song *et al.*, 2012). Mutations in PTEN also stimulate dendrite regrowth after dendrotomy in *Drosophila* sensory neurons (Song *et al.*, 2012). These findings, together with the data presented here, support a key role of mTOR signalling in both axon and dendrite regeneration after injury. However, our study also highlights important differences in the intrinsic mechanisms used for dendrite versus axonal regeneration in adult RGCs. First, successful dendrite regeneration depends on both mTORC1 and mTORC2 activation (our data), while axonal regeneration requires primarily mTORC1 (Park *et al.*, 2010; Duan *et al.*, 2015), with mTORC2 being growth inhibitory (Miao *et al.*, 2016). Second, we show that insulin-mediated mTORC1/2 activation is sufficient for dendritic regrowth and reconnection to presynaptic targets, whereas mTOR enhancement alone is not enough for RGC axons to reach their brain targets and re-establish functional synapses (Sun *et al.*, 2011; de Lima *et al.*, 2012; Lim *et al.*,

2016). For example, mTOR activation must be combined with approaches that increase neuronal activity, such as high-contrast visual stimulation, to achieve long-distance RGC axon regeneration and target-specific connections (Lim *et al.*, 2016). It is likely that RGC dendrites being in the more growth-permissive retinal environment, relative to the optic nerve, and in closer proximity to their targets require regeneration over shorter distances facilitating the restoration of functional connections. A better understanding of the molecular interplay between dendrite and axon regeneration will be important to design strategies that result in complete circuit restoration.

We show that axonal injury leads to dendrite degeneration and rapid synapse disassembly. Axotomized RGC dendrites display varicosities, retraction bulbs, and larger PSD95 clusters, similar to inactive synapses observed during retinal development (Okawa *et al.*, 2014). Recent studies suggest that RGCs with dendritic stratification in the OFF sublamina are more vulnerable to synaptic alterations in mouse models of ocular hypertension glaucoma (Della Santina *et al.*, 2013; El-Danaf and Huberman, 2015; Ou *et al.*, 2016). However, our finding that OFF and ON alpha RGCs are similarly affected after axotomy suggests that subtype vulnerability depends on injury modality. Remarkably, insulin treatment rescued excitatory postsynaptic sites on RGC dendrites, independently of subtype. In addition to rescuing synaptic inputs, insulin restored the ability of regenerating RGCs to respond to light stimulation restoring circuit function after axonal damage.

Our observation that insulin leads to mTORC1/2-dependent RGC survival after axotomy suggests that increased connectivity with presynaptic targets increases neuronal activity, which might subsequently enhance cell viability. The transient insulin regimen used here, initiated after dendrite retraction and synapse disassembly, promoted ~60% RGC survival at 2 weeks post-axotomy, a time when only 10% of these neurons remained alive in vehicle-treated animals. Insulin-mediated survival was higher than that observed with single or multiple injections of brain-derived neurotrophic factor (BDNF) or insulin growth factor (IGF1), which promoted 38–45% or 20–28% survival, respectively, at 2 weeks after optic nerve injury (Kermer *et al.*, 2000; Cheng *et al.*, 2002; Galindo-Romero *et al.*, 2013; Duan *et al.*, 2015). Analysis of neuronal survival at longer time points after

Figure 5 Continued

(G and H) Quantitative analysis of the pSTR or PhNR amplitudes demonstrated restoration of RGC function in insulin-treated eyes relative to controls that received vehicle at 7 days post-axotomy (ANOVA, *** $P < 0.01$, * $P < 0.05$, $n = 4–6$ mice/group). (I–K) Flat-mounted retinas from eyes treated with insulin displayed higher densities of RBPMS-positive RGCs compared to control retinas treated with vehicle at 1 week after optic nerve axotomy. (L) Co-administration of insulin with KU0063794 completely abrogated the pro-survival effect of insulin, strongly suggesting a role for both mTORC1 and mTORC2 in insulin-mediated RGC survival. Scale bars = 50 μm . (M) Quantitative analysis of RBPMS-positive cells confirmed that insulin (red) promoted significant RGC soma survival at 1, 2, 4, and 6 weeks after optic nerve lesion compared to control eyes treated with vehicle (grey), or a combination of insulin and KU0063794 (green). The densities of RGC soma in intact, non-injured animals is shown as reference (white, 100% survival). Systemic administration of KU0063794 alone did not induce RGC death in intact retinas ruling out any toxic effect of this compound. Values are expressed as the mean \pm SEM. (ANOVA, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, $n = 5–6$ mice/group).

axotomy demonstrated that 25% of RGCs survived at 4 and 6 weeks, respectively, relative to only ~8% in vehicle-treated controls. Although still significant, the gradual decrease in RGC survival after 2 weeks is likely to reflect the transient nature of the insulin treatment used here. It should be of future interest to examine the neuroprotective effect of chronic insulin administration on RGC viability after axonal injury. Among RGC subtypes, alpha cells appear to have a greater capacity to survive after axotomy (Duan *et al.*, 2015). However, the high survival rate observed here suggests that other RGC subtypes are likely to be insulin responders. Previous studies have reported subtype-specific alterations in dendritic morphology after optic nerve injury (Thanos, 1988; Watanabe *et al.*, 1995; Leung *et al.*, 2011; Liu *et al.*, 2014; Pérez de Sevilla Müller *et al.*, 2014; Ou *et al.*, 2016). For example, a recent study showed reduction of dendritic branching and length in α RGCs and melanopsin-positive RGCs after induction of ocular hypertension, while ON direction-selective RGCs showed no changes (El-Danaf and Huberman, 2015), suggesting differential responses among subtypes. A detailed characterization of dendritic alterations in distinct RGC types relative to their ability to regenerate and/or survive should expand our understanding of injury-induced responses in the retina.

In summary, this study reveals that adult retinal neurons are endowed with the ability to effectively regenerate dendrites and synapses after injury. We identify insulin, through mTORC1 and mTORC2 signalling, as a powerful strategy to restore dendritic morphology thus enhancing the function and survival of injured neurons. The observation that insulin eye drops exert a potent pro-regenerative effect is reinforced by findings that insulin applied at doses as high as 100 U/ml, several-fold higher than those tested here, were innocuous and produced no detectable clinical toxicity when applied topically in humans (Bartlett *et al.*, 1994). In this regard, it will be of interest to assess whether there is a correlation between glaucoma progression and insulin intake in such patients affected by diabetes. Collectively, these data support the rationale for using insulin and its analogues as pro-regenerative therapeutic targets that are potentially applicable to glaucoma and a host of other intractable neurodegenerative diseases.

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Supplementary material

Supplementary material is available at *Brain* online.

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Mammalian dendritic regrowth: a new perspective on neural repair

This scientific commentary refers to ‘Insulin signalling promotes dendrite and synapse regeneration and restores circuit function after axonal injury’, by Agostinone *et al.* (doi:10.1093/brain/awy142).

Neuroscientists studying injuries and diseases of the CNS have largely focused on mechanisms of neuronal dysfunction or death and of axon degeneration; and reciprocally, on developing strategies to maintain cellular function and to promote axon regeneration (Fig. 1). We now know the pathways that lead to cell death or dysfunction in multiple models, and promising treatment strategies for neuroprotection and/or cellular replacement are under development. Because communication within the CNS occurs largely through axodendritic synapses, it is also essential to protect or restore the axons and dendrites of surviving neurons. Axons are commonly compromised in CNS injuries and diseases, and therapies that address physical or chemical barriers to growth, as well as neuron-intrinsic,

glial, and inflammatory regulators of axon growth, have emerged in animal studies, with a few potential treatments having entered clinical trials. Dendrites are likewise affected by CNS injury and disease, and several CNS diseases are known to directly affect synaptic structures. Importantly, however, the structural degeneration of dendrites has been little studied, and their regeneration relatively neglected. In this issue of *Brain*, Agostinone and co-workers help to fill this gap by identifying an intracellular signalling pathway that underlies injury-induced dendritic retraction and growth in the mammalian visual system, and by demonstrating a relatively straightforward strategy to restore dendritic structure and circuitry (Agostinone *et al.*, 2018).

Using an *in vivo* model of traumatic nerve injury, this article provides evidence that the dendritic and somatic atrophy that occur in retinal ganglion cells (RGCs) after injury to the optic nerve can be reversed with insulin acting through the mTOR pathway.

The rodent optic nerve injury model closely mimics clinical optic nerve trauma and is a useful model for clinical glaucoma. This model has also helped generate strategies to address other types of CNS injury. The visual system is particularly well suited to investigating neural responses to injury, with the dendrites, somata, and axons of RGCs occupying distinct zones in the retina (inner plexiform layer, retina ganglion cell layer, and nerve fibre layer) and optic nerve, which are relatively accessible CNS structures. Current consensus from optic nerve injury, other glaucoma models, and clinical data is that dendritic retraction is an early event in glaucomatous pathology, beginning before somatic loss, axon pathology, and overt functional changes. In agreement with this literature, Agostinone *et al.* demonstrate that RGC dendritic complexity is reduced by 3 days after optic nerve transection, as determined using four common histological measures of genetically-labelled dendritic trees: total dendritic length, dendritic field

Glossary

Dendritic complexity/morphology: In adult mammals, the shape and location of dendritic arbors can be used to identify neuronal subclasses (including RGCs). Dendritic morphology is commonly assessed using measurements of dendrite length, area, branching, and Sholl analysis, which involves counting the number of intersections between dendrites and concentric rings centred at the soma. Dendritic arbours shrink and lose complexity, or degenerate, in injury and disease. Along with dendritic morphology, circuit function requires the structural and functional integrity of synaptic connections.

area, number of dendrite branches, and Sholl analysis.

To date, most studies of endogenous and treatment-induced dendritic regrowth have used invertebrate peripheral sensory neuron injury models. Recent experiments in *Caenorhabditis elegans* described spontaneous dendrite regeneration (Oren-Suissa *et al.*, 2017), while experiments in *Drosophila* confirmed and extended these findings and have begun to elucidate the underlying mechanism, which appears to involve the PTEN/PI3K/Akt/mTOR pathway and bantam microRNA (Song *et al.*, 2012) and to be activity-dependent

(Thompson-Peer *et al.*, 2016). Two recent, intriguing papers make use of *in vivo* two-photon microscopy to show dendritic growth in the brains of adult rodent models. Zhao *et al.* (2017) provide 3 h of time-lapse images of a single, normal, mostly morphologically stable dendrite in the mouse motor cortex. They contrast this to images of a single dendrite after nanosurgical ablation, showing a dynamic spontaneous morphological response, although the ultimate result is increased distance between the proximal and distal ends of the severed dendrite. These data hint at an exciting potential for

regrowth while remaining in line with other literature suggesting that the summation of endogenous signals present during injury and disease results in dendritic arbour retraction in mammals. Paveliev *et al.* (2016) present images and quantification of spontaneous and treatment-induced dendritic growth in mouse somatosensory cortex after brain prick injury. However, because therapy with pleiotrophin began immediately after injury, it is difficult to distinguish protective from regenerative effects.

The experiments by Agostinone *et al.* address regeneration by showing that daily insulin treatment starting 3

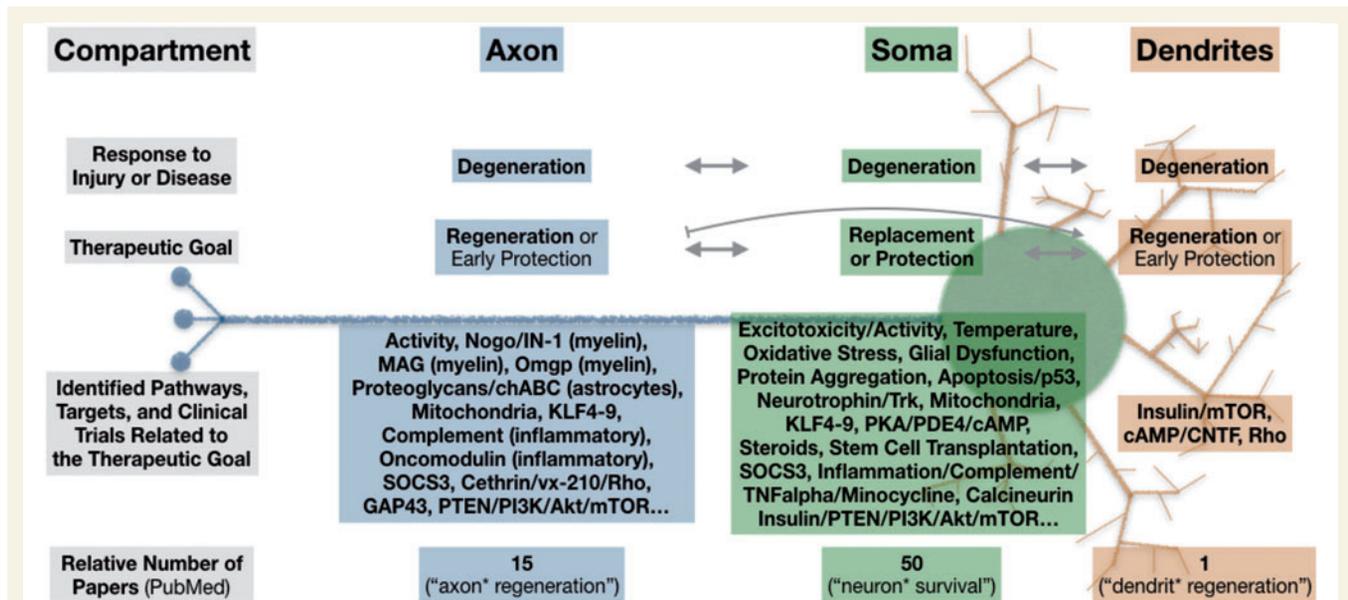


Figure 1 Little is known about adult mammalian dendrite regeneration. Along with somata and axons, dendrites degenerate in CNS injuries (including optic nerve transection) and diseases (including glaucoma). Despite the obvious importance of dendrites to CNS function, and the interdependence (arrows) between neuronal compartments for both degeneration and regeneration/protection/replacement, much more research has been directed toward axon regeneration and soma replacement/protection. A simplified measure of this disparity is the relative number of publications found on PubMed (search terms in parentheses, asterisk represents wildcard), which reveals that for every one dendrite regeneration paper, there are ~15 publications on axon regeneration and 50 for neuron survival. In this issue of *Brain*, Agostinone *et al.* identify a role for insulin/mTOR signalling in dendrite regeneration. Note common targets, including mTOR, as well as unique targets, and possible antagonism of dendrite growth on axon growth (bar-headed line). This simplified diagram does not include data from non-mammalian or developmental models and is not exhaustive. Axon and dendrite protection strategies likely require very early clinical intervention and are therefore noted but not emphasized here.

days post-injury successfully reverses the loss of dendritic complexity, with multiple dendritic measures returning to normal. Importantly, the timing of insulin intervention to several days post-axotomy—after dendrite retraction has begun—allows the authors to conclude that insulin promotes regrowth of dendrites, as opposed to promoting dendritic stabilization or preventing dendritic loss after optic nerve transection. Given the characterization of dendritic pathology as very early events that occur before clinical presentation in many CNS injuries and diseases, and the resulting timing of intervention, dendritic regrowth is a more relevant therapeutic target than dendritic protection. Another rodent visual system study with similarly delayed treatment identified other dendritic growth strategies [blocking Rho GTPase; elevating ciliary neurotrophic factor (CNTF) plus cAMP], albeit in the presence of a peripheral nervous system graft, and found that while the agents used had effects, the resulting dendritic morphology was abnormal (Drummond *et al.*, 2014). Interestingly, the outcome of the present study appears to be a restoration of normal morphology. This study's elegant, simple design allows for a conceptual distinction between preservation and restoration, with consequences for treatment identification, characterization, and clinical administration, and should be considered for future studies on mammalian dendrite repair.

Agostinone and colleagues further show, using siRNA-mediated knock-down techniques, that the various insulin-mediated dendritic effects require two separate mTOR signalling complexes, mTORC1/Raptor and mTORC2/Rictor, and that pharmacological inhibition of all mTOR signalling fully blocks the dendritic growth effects of insulin. Yet while this paper appears to be alone in describing a partial mechanism for regrowth of dendrites in the mammalian CNS, the key proteins and pathway identified are not new to us. Insulin or insulin-like growth factor-1 (IGF1) have been reported to be

neuroprotective in this and other rodent CNS injury models. Agostinone *et al.* confirm previous reports with data showing insulin-, and specifically, mTOR-mediated neuroprotection of RGCs at subacute time points (7 and 14 days post-injury), and extend the published time course for this model by reporting significant neuroprotection with reduced effect sizes at more chronic time points (28 and 42 days). These more chronic data are key to any treatment aimed at clinical translation, especially given that most neuroprotective effects in this model have been disappointingly transient, including those of mTOR as shown here, making these data impressive but imperfect. This reinforces the need for neuroprotective treatments (or combinations of treatments) with long-term effects, and the development of improved slow-release or repeated administration strategies.

The PTEN/PI3K/Akt/mTOR pathway mechanism, shown here to be required (mTOR) for insulin-induced dendritic regrowth, is shared not only with that for dendritic development, invertebrate dendritic regeneration, and neuroprotection, but also with a well-described mechanism for CNS axon regeneration (Park *et al.*, 2008). The suggestion of a common pathway for soma protection, axon regeneration, and dendrite regeneration in multiple CNS injury models may have notable implications for clinical therapeutic manipulations of this pathway. The larger literature strongly suggests some interdependence of neuronal compartments for maintenance, degeneration and regrowth. Because intact somata, axons, and dendrites are all essential for neuronal communication and CNS function, a shared pathway is an exciting prospect and should be highlighted as a general target, meriting even more research in this area.

In contrast, other evidence points to an antagonism between dendrite growth and innervation versus axon growth or regeneration (Cull, 1974; Goldberg *et al.*, 2002; Francis and Freeman, 2016). Such antagonism

may or may not play a role in the current work by Agostinone *et al.*, but axonal regeneration requires a different injury model and was not investigated here. However, this issue has clear implications for therapeutic development for any disease or injury with axonal pathology, including glaucoma. The present study provides limited evidence of insulin-mediated synapse restoration and improvement in retinal circuit function at 7 days post-injury, which may be indicative of local recovery, but the study did not address perception or behaviour, which would require regeneration of injured axons and which are critical issues for clinical therapies. Future studies will be required to determine the full effect on the larger system, perhaps using a glaucoma model in which, during a therapeutic window that needs to be defined, retinal axons are compromised but still present. In addition, if the potential inhibitory action of dendrites on axons is confirmed in future studies, careful attention to the timing of intervention may improve outcomes.

Although much remains to be explored, the translational potential of insulin treatment for glaucoma is exciting. This potential may even reach beyond glaucoma to other CNS injuries and diseases. Enthusiasm is based on the strong, partially sustained neuroprotection and neuro-restoration after injury, the current clinical use of insulin, the post-injury timing of administration, and the eye drop route of administration, which all seem favourable for translation. Moreover, prying open the figurative window to the vastly under-explored but crucial research topic of dendrite regeneration, and demonstrating the potential for treatment-induced dendrite regrowth, are important steps forward.

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A broader view of dementia: multiple co-pathologies are the norm

This scientific commentary refers to ‘Neurodegenerative disease concomitant proteinopathies are prevalent, age-related and APOE4-associated’, by Robinson *et al.* (doi:10.1093/brain/awy146).

Alzheimer’s disease and other dementias are defined by hallmark protein abnormalities found in brain tissue post-mortem. Despite increasingly accurate diagnosis of primary pathology in life, treatments targeting the underlying protein abnormalities in Alzheimer’s disease have so far failed to work. Why is dementia proving so hard to treat? One argument is that treatments are given too late in the course of illness—by the time of diagnosis, disease has progressed for a decade or more, has initiated self-perpetuating secondary processes and is no longer modifiable. A related, but distinct argument is presented in this issue of *Brain* by Robinson and co-workers, who demonstrate concurrence of multiple different abnormal proteins in dementias such as

Alzheimer’s disease, hinting at the likelihood that treatment might require a multi-pronged approach (Robinson *et al.*, 2018).

Accuracy of clinical diagnosis

Brains from 766 people who had died with dementia, and age-matched controls, were classified using standard diagnostic criteria as having either a neurodegenerative disease or minimal pathology. As in previous studies, the clinical and neuropathological diagnoses did not always agree. For example, high levels of Alzheimer’s disease pathology at post-mortem examination had only a 79% sensitivity and 59% specificity for a clinical diagnosis of Alzheimer’s disease. The figures are in keeping with those of other large clinicopathological studies (e.g. Beach *et al.*, 2012). Fourteen per cent of brains with high levels of Alzheimer’s disease pathology came from patients who had been diagnosed clinically as having

frontotemporal dementia. This highlights the complexity and imprecision of clinical diagnosis, even in the best centres, and the importance of moving to molecular markers of disease processes that cause dementia to ensure the right patients enter the right trials.

Ubiquity of tau

A striking finding was that neuronal accumulation of tau protein was almost universal, even in the minimal pathology group, within which neurofibrillary tangles were found in 93% of brains. Neurofibrillary tangles were present in at least 88% of brains with all other primary pathologies, and some of these—frontotemporal dementia and Lewy body diseases in particular—had tau co-pathology in 100% of cases. The prevalence and extent of neuronal tau pathology increased with age and incipient Alzheimer’s disease. Robinson *et al.* did not address the relevance of astroglial tau, which also increases with age. One argument is that neuronal