Regeneration of retinal ganglion cell dendrites: the role of insulin signaling to stimulate connections and restore vision in glaucoma.

Adriana Di Polo[1], Luis Alarcon-Martinez[1], Jessica Agostinone[1]

[1]Department of Neurosciences, University of Montreal Hospital Research Center (CR-CHUM), Quebec, Canada

INTRODUCTION: Loss of vision in glaucoma results from the irreversible death of retinal ganglion cells (RGCs). The dendrites of RGCs are the substrate for receiving synaptic inputs. The structural stability of dendritic arbors is, therefore, essential for the normal function of RGCs and their ability to transmit visual information. The rapid retraction of RGC dendrites and loss of synapses is one of the earliest pathological features of ocular hypertension damage. A crucial step towards circuit repair in glaucoma is to promote damaged RGCs to regenerate not only axons, but also dendrites to successfully reconnect with their synaptic partners. Paradoxically, although much is known about axonal regeneration, the capacity of injured RGCs to regenerate dendrites has been large ignored.

HYPOTHESIS: Here, we tested the hypothesis that insulin will stimulate dendrite regeneration and the re-establishment of synaptic connections thus improving survival and function in injured RGCs.

SPECIFIC AIMS

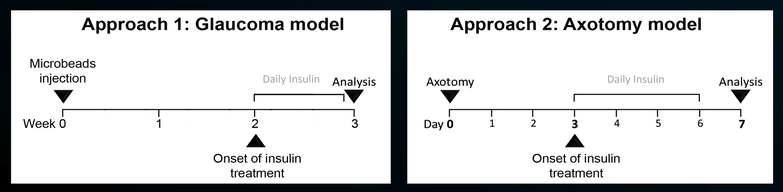
Aim 1: Characterize the role of insulin on RGC dendrite regeneration and survival after axonal injury.

Aim 2: Determine the efficacy of insulin to regenerate RGC synapses and promote functional recovery.

METHODS

Glaucoma model and axotomy: Ocular hypertension was induced by intracameral injection of magnetic microbeads in Thy1-YFP mice followed by intraocular pressure measurements as described by us. Axotomy was performed by exposing the optic nerve (ON), which was cleanly transected at 0.5-1 mm from the ON 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.

Insulin administration: Human recombinant insulin diluted in sterile, endotoxin free PBS (15-30U/Kg/day) was administered by daily intraperitoneal (i.p.) injections or eye drops (5 µl drop) as per the regimens outlined here.



Dendritic arbor analysis: High-resolution images of YFP-labeled RGC dendrites were acquired using a confocal microscope. Reconstruction of dendritic trees was carried out using the computer-aided filament tracing function of Imaris (Bitplane).

Synaptic markers and excitatory postsynaptic site density: Immunohistochemistry of VGLUT1 (pre-synaptic) and PSD95 (post-synaptic) was performed on retinal cross sections. Biolistic transfection of CMV:PSD95-YFP and CMV:tdTomato plasmids onto injured or control retinas was performed followed by analysis of PSD95 puncta on individual YFP-positive RGC dendrites.

RGC function: Two electroretinogram (ERG) components were analyzed: the positive scotopic threshold response (pSTR) and the photopic negative response (PhNR). Recordings were obtained by stimulating the retina at light intensities ranging between 10^{-6} to 10^{-4} cd s/m^2 (pSTR) or 10^{2} cd s/m^2 (PhNR) as described (Bu & Fortune, 2004).

RGC survival: Retinal wholemounts from control and experimental mice were labeled with the RGC-specific marker RBPMS and 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 twelve retinal areas.

RESULTS

1. Insulin promotes dendrite regeneration

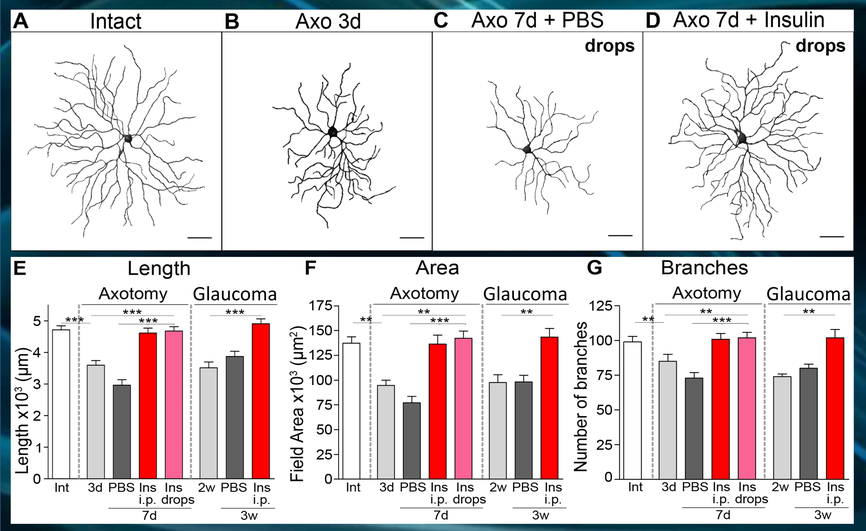


Figure 1. Insulin promotes dendritic regeneration in adult RGCs after axonal injury. (A to D) Representative examples of dendritic arbors obtained from intact retinas or after axonal injury (axotomy or ocular hypertension) and insulin treatment. Scale bars: 25 µm (all panels). (B) Three days after axotomy, RGCs had visibly smaller and simpler dendritic arbors relative to intact, non-injured neurons. (E to G) Quantitative analysis of dendritic parameters revealed that insulin-treated neurons had longer dendrites and markedly larger and more complex arbors than vehicle-treated controls (insulin i.p.: red, insulin eye drops: pink, PBS: dark grey). Similarly, systemic administration of insulin following ocular hypertension resulted in striking dendritic arbor regeneration **(E-G)**. RGC dendrites in retinas treated with vehicle did not regenerate **(E to G).** Data are presented as mean ± S.E.M. (ANOVA, *: p < 0,05 , **; p < 0,001, ***; p < 0,0001, N=4 to 6 mice/group, n=28 to 46 cells/group).

2. Synaptic density is restored by insulin

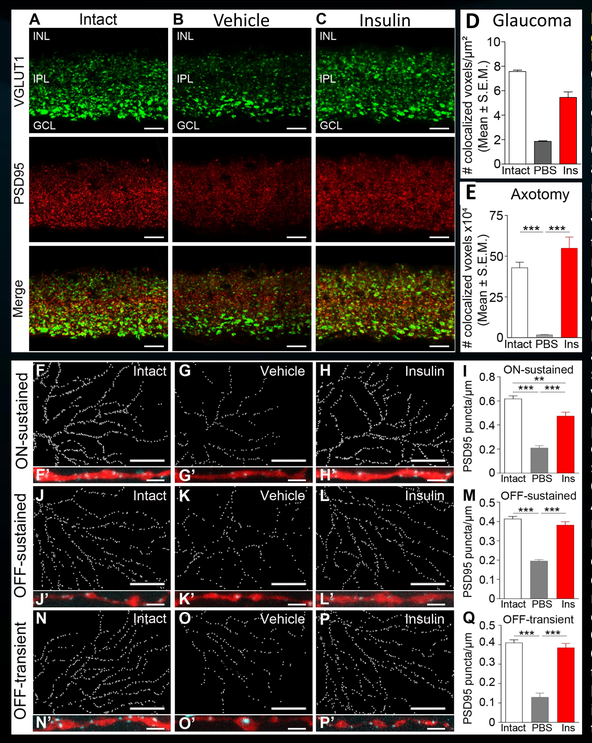


Figure 2. Insulin restores glutamatergic postsynaptic sites in injured neurons. (A) Glutamatergic synapses visualized in the inner plexiform layer (IPL) on retinal cross-sections using immunolabeling against VGLUT1 (green) and PSD95 (red), a pre- and a post-synaptic marker, respectively. (B, C, E) Axonal injury induced a pronounced loss of both VGLUT1 and PSD95 expression in the IPL, which was completely restored by insulin treatment (analysis at 7 days post-axotomy). (D) The same findings were obtained in mice with ocular hypertension in which insulin administration reestablished synaptic density compared to vehicle treated animals. Quantitative analysis of pre- and postsynaptic co-localized voxels confirmed that insulin promoted synaptic marker regeneration (Insulin: red bar, Vehicle: grey bar, ANOVA, ***=p<0.001, N=4-6 mice/group). (F-P) 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. (F'-P') 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 ± S.E.M. (ANOVA, ***=p<0.001, **=p<0.01, N=5-6 mice/group, n=3-6 RGCs/group). Scale bars: (A-C) =10 µm, (F-P) = 30 µm, (F'-P') = 2.5 µm. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

3. Insulin rescues retinal function

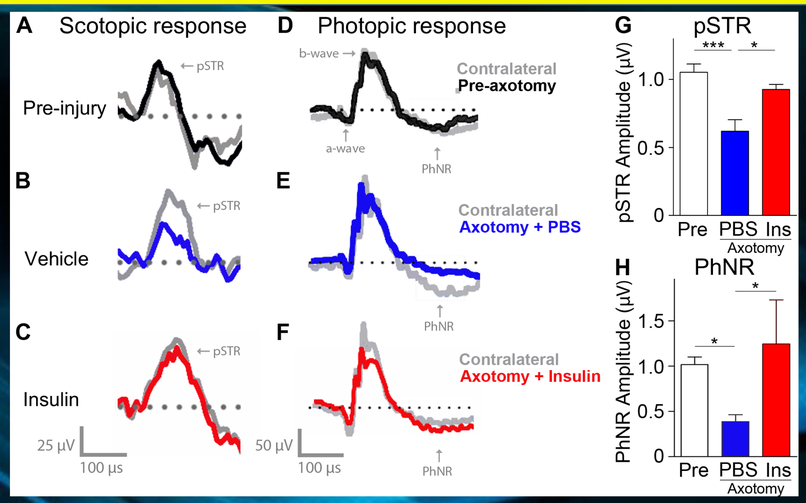


Figure 3. Insulin rescues retinal function after optic nerve axotomy. (A-F) Representative examples of ERG recordings elicited by dim scotopic (A-C) or photopic (D-F) light stimulation prior to axotomy (pre-injury, black trace), or after axotomy and treatment with PBS (blue trace) or insulin (red trace). All the recordings were normalized relative to the contralateral, non-injured eye (grey traces). (G, H) Quantitative analysis of the positive scotopic threshold response (pSTR) or photopic negative response (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).

4. Neuronal survival is robustly increased

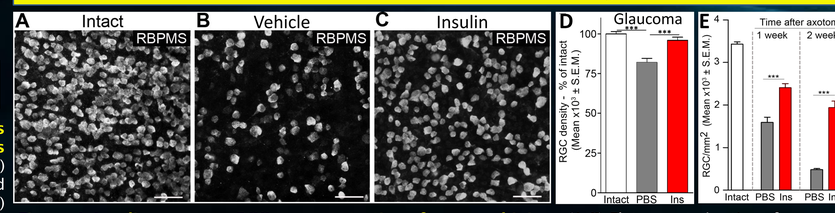


Figure 4. Insulin promotes RGC neuroprotection after axonal injury. (A-C) Flat-mounted retinas from eyes treated with insulin displayed higher densities of RBPMS-positive RGCs compared to control retinas treated with vehicle at one week after optic nerve axotomy. Scale bars: 50 µm. (D-E) Quantitative analysis of RBPMS-positive cells confirmed that insulin (red) promoted significant RGC soma survival at and 2 weeks after optic nerve axotomy and 3 weeks after ocular hypertension compared to control eyes treated with vehicle (grey). The densities of RGC soma in intact, non-injured animals is shown as reference (white, 100% survival). Values are expressed as the mean ± S.E.M. (ANOVA, ***=p<0.001, **=p<0.01, *=p<0.05, n=5-6 mice/group).

CONCLUSIONS

Our study reveals that adult RGCs are endowed with the ability to effectively regenerate dendrites and synapses once they have been lost. Importantly, we identify insulin as a powerful strategy to restore dendritic morphology and enhance the function and survival of RGCs after acute optic nerve injury and in experimental glaucoma.

IMPACT FOR GLAUCOMA PATIENTS

The relevance of our 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 in our experiments, were innocuous and produced no detectable clinical toxicity when applied topically (on the cornea) in humans. Collectively, our data support the rationale for using insulin and its analogues as pro-regenerative therapeutic targets to counter progressive RGC neurodegeneration and vision loss in glaucoma.

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