Axonal PPARγ Promotes Neuronal Regeneration After Injury

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ABSTRACT: **PPARγ is a ligand-activated nuclear receptor best known for its involvement in adipogenesis and glucose homeostasis. PPARγ activity has also been associated with neuroprotection in different neurological disorders, but the mechanisms involved in PPARγ effects in the nervous system are still unknown. Here we describe a new functional role for PPARγ in neuronal responses to injury. We found both PPAR transcripts and protein within sensory axons and observed an increase in PPARγ protein levels after sciatic nerve crush. This was correlated with increased retrograde transport of PPARγ after injury, increased association of PPARγ with the molecular motor dynein, and increased nuclear accumulation of PPARγ in cell bodies of sensory neurons. Furthermore, PPARγ antagonists attenuated the response of sensory neurons to sciatic nerve injury, and inhibited axonal growth of both sensory and cortical neurons in culture. Thus, axonal PPARγ is involved in neuronal injury responses required for axonal regeneration. Since PPARγ is a major molecular target of the thiazolidinedione (TZD) class of drugs used in the treatment of type II diabetes, several pharmaceutical agents with acceptable safety profiles in humans are available. Our findings provide motivation and rationale for the evaluation of such agents for efficacy in central and peripheral nerve injuries.** Develop Neurobiol 00: 000–000, 2015

Keywords: **PPARγ; regeneration; axon; retrograde transport; dynein**

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INTRODUCTION

The peroxisomal proliferator activated receptor γ (PPARγ) is a ligand-activated nuclear receptor best known for its roles in the regulation of lipid

metabolism, adipocyte differentiation, and maintenance of insulin sensitivity. Indeed PPARγ binds various fatty acids and oxidized lipids, and is the molecular target of the thiazolidinedione (TZD) class of drugs used in the treatment of type II diabetes. After binding a ligand, PPARγ heterodimerizes with the retinoid X receptor to increase gene transcription (Ahmadian et al., 2013).

A number of studies have also suggested that PPARγ may promote neuroprotection in a broad spectrum of pathologies, including Alzheimer's and Parkinson's diseases (Chen et al., 2012). The mechanism(s) underlying PPARγ-mediated neuroprotection are unclear, and might be associated with anti-inflammatory effects on glial cells (Garcia-Bueno et al., 2005) although other studies have shown that neuronal expression of PPARγ is protective in cerebral ischemia (Bernardo and Minghetti, 2008; Zhao et al., 2009). Moreover, TZDs can promote neuronal protection and axonal extension in neural cell lines and primary neuron cultures *in vitro* (Fuenzalida et al., 2005, 2007; Quintanilla et al., 2013; Chiang et al., 2014). Taken together, these studies suggest that PPARγ might be involved in axonal physiology in normal and pathological conditions.

Axonal expression of transcription factors has been implicated in retrograde signaling after nerve injury (Ji and Jaffrey, 2014; Rishal and Fainzilber, 2014). In this process, local axonal synthesis of importin β1 and other adaptor proteins enable linkage of diverse signaling molecules to the dynein retrograde motor in injured peripheral nerve (Perry et al., 2012). Analyses of the retrograde signaling proteome in injured sciatic nerve together with the transcriptome response in dorsal root ganglia has suggested a number of transcription factors, including the PPAR family, as candidate dynein-transported injury signals in axons (Michaelevski et al., 2010; Ben-Yaakov et al., 2012; Rishal and Fainzilber, 2014). We therefore examined here whether axonal injury affects levels and retrograde transport of PPARγ and, whether axonal PPARγ is required for axonal regeneration.

Here we show that PPARγ protein and mRNA are present in rat sensory neuron axons and that the protein is retrogradely transported from axons back to the soma. Upon injury, axonal PPARγ protein is upregulated and its association with the molecular motor dynein is increased. Pharmacological inhibition of PPARγ reduced axonal regeneration in mouse and stem cell-derived human neurons in culture. Consistently with these results, PPARγ antagonists also reduced axonal growth after axotomy of rat cortical neurons while the PPARγ agonist rosiglitazone increased the regeneration of axotomized cortical neurons. These findings indicate that axonal PPARγ is involved in the regenerative response triggered by axonal injury in peripheral and central neurons, and provide a rationale for future clinical testing of PPARγ-targeting drugs in nerve injuries.

METHODS

Reagents, Drugs, and Antibodies

Culture media, chemicals and serum were from Invitrogen. Rosiglitazone, GW9662 and T0070907 (T007) were provided by Cayman Chemical (MI). Stock solutions of drugs were prepared in Me_2SO and added to the culture medium (0.01% final Me_2SO concentration). For PPARγ immunofluorescence the chicken polyclonal antibody for PPARγ was used (GW21258, Sigma-Aldrich, MO). For immunofluorescence of sciatic nerve sections and neuronal cultures the following antibodies were used; mouse monoclonal anti-neurofilament 200 (from Sigma-Aldrich, MO), rabbit polyclonal anti-P0 was a kind gift from Dr. Alejandro Roth (Universidad de Chile), mouse monoclonal anti-β-III tubulin (Sigma-Aldrich, MO), Goat anti-calcitonin gene related peptide (CGRP) antibody (ab36001, Abcam, Cambridge, UK), rabbit anti-p75 antibody (Upstate, NY). Other antibodies used for immunoprecipitation and Western blot were the following; mouse monoclonal anti-PPARγ recognizing both isoforms (ab41928, Abcam, Cambridge, UK), rabbit polyclonal anti-S100 (Sigma-Aldrich, MO), mouse monoclonal anti-RCC1 (C6, Santa Cruz, CA), mouse monoclonal anti-Erk 1/2 (Abcam, Cambridge, UK), mouse monoclonal anti-dynein (ab23905, Abcam, Cambridge, UK). Goat serum (Cell Signaling, CA), cytosine arabinoside (AraC; Sigma-Aldrich, MO), deoxynucleotide probes were from Integrated DNA Technologies, Inc (IA). Terminal transferase, DIG-labeled deoxyuridine-triphosphate (DIG-dUTP) and anti-DIG antibodies conjugated to fluorescein (FITC) were from Roche (Germany). Prolong Gold Antifade Reagent was from Invitrogen (CA). Protein A-agarose (Santa Cruz Biotechnology, CA). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Life Technologies, CA). Polydimethylsiloxane (PDMS) was from Dow Corning Corporation (MI).

Sciatic Nerve Immunofluorescence

Rat (Sprague-Dawley) sciatic nerves were excised, fixed 30 min at room temperature with 4% paraformaldehyde, passed through a sucrose gradient (5%, 10%, and 20%) overnight, and frozen in Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan). Cryostat sections (10 μm or 8 μm for longitudinal and cross-sciatic nerve sections, respectively) were washed with cold PBS and then blocked and permeabilized with 0.25% Triton X-100 and 5% fish gelatin in PBS solution for 3 h at room temperature. Sections were incubated with primary antibodies overnight in the cold, washed and incubated with secondary antibodies for 1 h at room temperature. Finally,

sections were mounted in mounting medium and analyzed by fluorescent microscopy.

Immunofluorescence of DRGs Ganglia

Dorsal Root Ganglia (L4-L6) were excised from adult rat (200–250 g), fixed for 30 min, treated in sucrose gradient and frozen with Tissue-Tek (Sakura, Tokyo, Japan). DRG cross sections were obtained by cryostat sectioning (10 μm sections) and washed with PBS and then blocked and permeabilized with 0.25% Triton X-100 and 5% fish gelatin in PBS solution for 3 h at room temperature. Sections were incubated with the primary antibodies (chicken polyclonal antibody for PPARγ or mouse monoclonal anti-neurofilament 200 both from Sigma-Aldrich, St Louis, MO) and mounted as described for sciatic nerve immunofluorescent.

Rat Sciatic Nerve In Situ Hybridization

One hundred picomoles of each deoxynucleotide probe for rat PPARγ mRNA (NM_013124, Genbank) were labeled by incubation with 55 units of terminal transferase in 25 μL of tailing buffer, 9 mmol of ATP, and 1 mmol of DIG-labeled deoxyuridine-triphosphate. Sciatic nerve cross-sections were rinsed with PBS and then incubated at 50°C in a prehybridization solution containing Denhardt's 1× and 4× saline sodium-citrate buffer (SSC) consisting of 0.6 *M* NaCl and 60 m*M* sodium citrate at pH 7.0. Sections were then hybridized overnight at 56°C using 10 picomoles/mL of DIG-labeled probe in a buffer containing 50% formamide, 1 mg/mL dextran sulfate, and 10 m*M* dithiotreitol, 0.06 *M* Tris, pH 7.5. Following hybridization, tissue sections were rinsed with 2× SSC followed by 1× SSC, 10 min each at 42°C. Control experiments were performed in the presence of 100× excess nonlabeled probes or using a DIG-labeled random probe. The presence of DIG-labeling in rat sciatic nerve sections was detected after overnight incubation with anti-DIG antibodies conjugated to fluorescein (FITC). Finally tissue sections were mounted with Prolong Antifade Reagent (Life Technologies, USA) in slides and analyzed by fluorescence microscopy.

Transcription factor binding site analysis for genes containing PPAR response elements regulated by sciatic nerve injury was performed according to previous studies (Michaelevski et al., 2010; Ben-Yaakov et al., 2012).

Axonal Transport in Normal and Injured Rat or Mouse Sciatic Nerve

All surgical procedures were performed under deep anesthesia. Rats weighting approximately 250 g were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (50 mg/kg and 10 mg/kg, respectively). Two ligations 10 mm apart, were applied at mid thigh level of the sciatic nerve to allow accumulation of transported molecules at the ligation site (Delcroix et al., 1999). In this model, accumulation of a substance on the proximal side (closer to cell bodies) of the ligation provides evidence for anterograde transport and accumulation on the distal side of the double ligation indicates retrograde transport. The nonligated contralateral sciatic nerve served as control. Goat anti-calcitonin gene related peptide (CGRP) antibody and rabbit anti-p75 antibody were used as anterograde and retrograde transport markers, respectively.

Axonal injuries (crush) were generated in rat sciatic nerve by applying firm force with N°5 fine forceps for 10 s, twice, 2 mm distally from the fascia of the paraspinal muscles. Ligatures were applied at the mid-thigh level of the sciatic nerve, and the tissue was then processed for immunofluorescence at 2, 4, or 6 h after injury. In sham-operated rats the nerve was exposed, but not injured. For immunoprecipitations, 500 μg axoplasm obtained from control and injured nerve was precleared for 2 h with Protein A-agarose (Santa Cruz). Following overnight incubation with primary antibody (5 μg) the axoplasm was incubated with Protein A-agarose beads for 2 h at room temperature and then precipitated at 4°C and washed four times with ice-cold buffer (20 m*M* HEPES/KOH, pH 7.3; 110 m*M* KAc; 5 m*M* MgAc; 0.5 m*M* EGTA). Proteins were eluted by boiling in sample buffer and subjected to Western blotting.

Axoplasm Preparation, Protein Extraction, and Immunoblotting

Axoplasm-enriched samples were obtained immediately after dissection of the sciatic nerve, by gentle squeezing of sciatic nerve segments in nuclear transport buffer (NTB, 20 m*M* HEPES/KOH, pH 7.3; 110 m*M* KAc; 5 m*M* MgAc; 0.5 m*M* EGTA) as previously described (Rishal et al., 2010). For Western blot, axoplasm samples were resolved in 10% SDS-PAGE and transferred to nitrocellulose membrane. After reaction with the antibodies the membrane was developed with SuperSignal Femto West Chemiluminescent Substrate (Life Technologies).

Culture and Immunofluorescence of Mouse Primary Sensory Neurons

Dorsal Root Ganglia (DRG) were dissected from adult (8–12 weeks old) male C57BL/6 mice (Harlan Laboratories, Israel) and cultured on laminin-coated Costar 24-well plastic plates as previously described (Ben-Yaakov et al., 2012). Mouse DRG neuronal cultures were imaged using an ImageXpress microscope (Molecular Devices), followed by determination of morphological parameters by MetaXpress4 (Molecular Devices). The parameters examined include total neurite length, defined as the sum of lengths of all processes per neuron, percentage of growing neurons, defined as the percentage of neurons with maximal process length exceeding 50 μm, and number of branching points per neuron. At least 500 neurons were quantified per repeat for each treatment. Comparison of PPARγ accumulation in growing and

nongrowing DRG neurons was done using Cell Profiler 2.0 (Carpenter et al., 2006).

Culture and Regeneration Analysis of Human Axons

NP1 human neural precursors were purchased from Neuromics (MN) and passaged up to eight times before generation of neurons. For terminal neuronal differentiation, medium was changed to neurobasal medium, 2% B27 supplement, GIBCO, penicillin streptomycin, supplemented with 10 ng/mL NGF, 5 ng/mL NT3, and 10 ng/mL BDNF (all from Alomone Labs, Israel) one day after plating. After 12 days, neurons were transfected with a GFP expression plasmid (Addgene#11154) using Xfect reagent. Two days after transfection, cells were removed with a scraper (without trypsin) and passed through a P1000 tip 10 times to strip off axons. Cells were then re-plated in 48-well plates previously coated with ECM gel (matrigel equivalent, Sigma-Aldrich E1270, 1–3 h at 4°C) with 250 μL differentiation medium per well (Shin et al., 2006; Dhara et al., 2008). Cultures contained 95% neurofilament-immunopositive neurons, the majority of which were CNS-type.

Cultures and Immunofluorescence of Rat Primary Cortical Neurons

Primary cortical neurons were prepared from E18 rat embryos as described previously (Taylor et al., 2003). Briefly, the cortex was dissected and dissociated to single cells by gentle trituration, resuspended in MEM/HS (Minimum Essential Medium supplemented with 10% horse serum, 20% D-glucose, and 0.5 m*M* glutamine) and seeded on poly-L-lysine (1 mg/mL) for culture in microfluidic chambers devices. For immunostaining, coverslips were washed with PBS, fixed with 4% paraformaldehyde and processed for immunofluorescence labeling as previously described (Fuenzalida et al., 2005). To study axonal regeneration after axotomy in cortical neurons, axons of nine DIV cultures were lesioned by vacuum aspiration in the distal axons compartment as described previously (Taylor et al., 2009) and treatment was performed for 96 h with rosiglitazone (1 μM) or two different agonist for PPARγ, GW9662 (10 μM) and T0070907 (10 μM) added to the distal axons compartment.

Preparation of Compartmentalized Cultures from Cortical Neurons and Quantification of Axonal Growth after Axotomy

Microfluidic chambers for compartmentalized cultures were produced as described (Park et al., 2006) and fixed to coverslips coated with Poly-L-lysine (1 mg/mL). Approximately, 30,000 neurons were seeded in the cell body chamber. Total volume differential between the two compartments was maintained at 20 µL to ensure fluidic isolation during experiments. Four hours after seeding the cells, the culture medium was replaced with Neurobasal medium supplemented with 3% B27 and 0.5 m*M* glutamine. Proliferation of non-neuronal cells was limited by the use of cytosine arabinoside at 3 DIV. The neurons were grown for 9 to 10 days *in vitro* (DIV) to allow axons to project into the distal axons. Neurite growth in compartmentalized cultures of cortical neurons was determined by counting the number of axons positive for β-III-tubulin crossing through a line located every 100 μm distal to the microgrooves. The distance from microgrooves was calculated using ImageJ software (NIH). Quantifications were performed on three independent experiments, each one comprising three microfluidic chambers per treatment.

Immunofluorescence of Rat Primary Cortical Neurons

For immunostaining of cells, coverslips with the primary cultures were washed with PBS, cultures were fixed using 4% paraformaldehyde, permeabilized using 0.1% Triton X-100, blocked with 7% normal goat serum and immunostained with chicken polyclonal antibody for PPARγ (GW21158, Sigma-Aldrich, St. Louis, MO).

Statistical Analyses

Results are expressed as mean ± SE. Statistical analyses were carried out using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. All experiments were replicated independently (new animals for each repeat) at least three times.

RESULTS

PPARγ Protein is Present and Retrogradely Transported in Axons of Rat Sciatic Nerve

In order to evaluate the presence of PPARγ in axons, we examined the localization of PPARγ in rat sciatic nerve using a specific antibody. As shown in Figure 1(A,B), PPARγ expression is observed in neurofilament-positive axons in both cross-sections and dissociated fibers of sciatic nerve (see Supporting Information Fig. S1 for verification of antibody specificity). Co-localization with P0-positive sciatic nerve cells indicates that PPARγ is also present in Schwann cells. Western blots of axoplasm further confirmed the presence of PPARγ in axons [Fig. 1(C)].

Nerve ligation enables analysis of the net directionality of axonal transport of molecules of interest. The ligation separates proximal axonal segments that remain connected to the cell bodies from downstream distal regions. Proteins accumulating on the proximal side of the ligation are anterogradely transported, while those

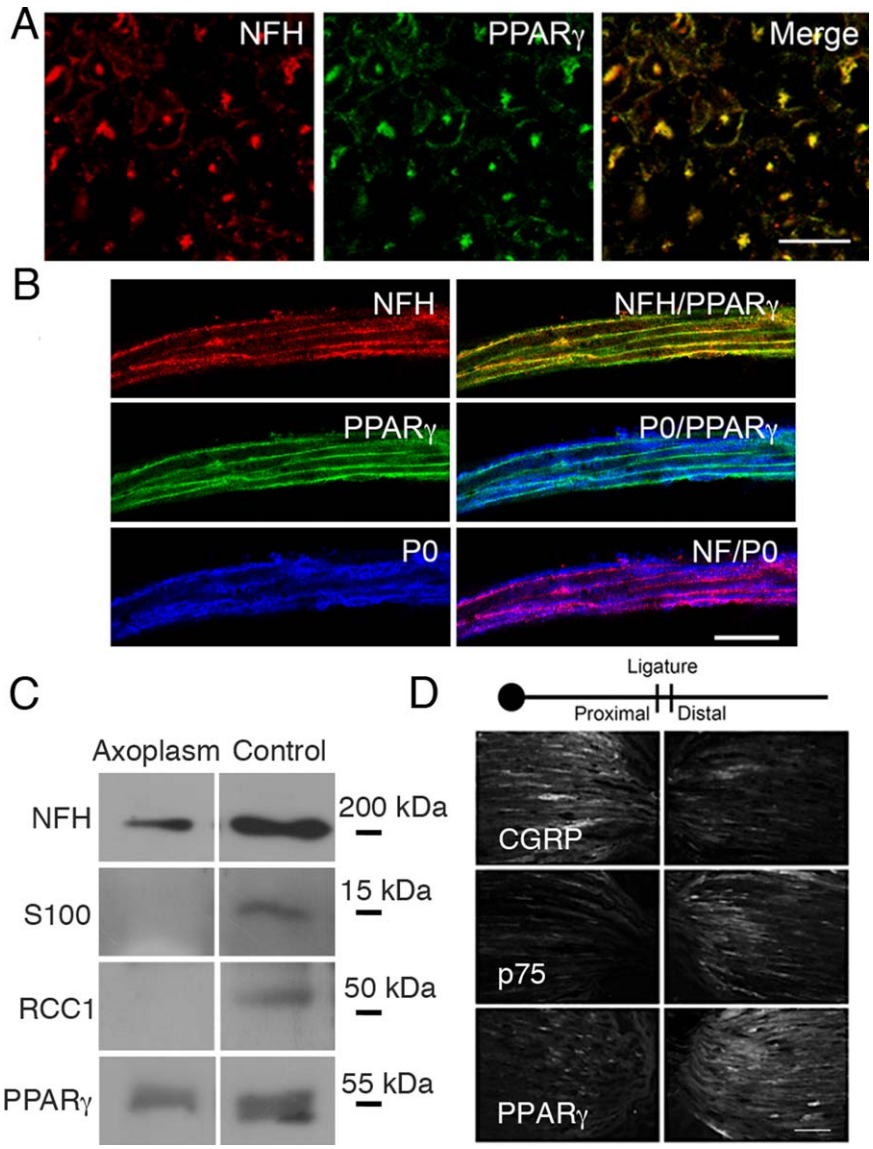

Figure 1 PPARγ is present in the axons of rat sciatic nerve. (A) Rat sciatic nerve was fixed in 4% paraformaldehyde and transverse sections were immunostained for PPARγ and neurofilament heavy chain (NFH). Colocalization of both proteins is evident in the merged image (Scale bar: 10 μm). (B) Nerve fibers from rat sciatic nerve were gently isolated, fixed as above and immunostained for PPARγ, NFH, and P0 (a Schwann cells marker). Co-localization of NFH with PPARγ is visible in the merged image. Co-localization between PPARγ/P0, and NFH/P0 is also shown (scale bar: 50 μm). (C) Isolated axoplasm from rat sciatic nerve was obtained by gentle squeezing the nerve segments in physiological buffer and immunoblotted with an antibody against PPARγ. Axoplasm purity was verified by the absence of S100 (Schwann cell marker) and RCC1 (nuclear marker) proteins. Positive controls were whole sciatic nerve extract for NFH, S100 and RCC1 and adipose tissue extract for PPARγ. (D) To assess axonal transport of PPARγ in the intact sciatic nerve, we used a ligation procedure that distinguishes between anterograde and retrograde flow. After 24 h of ligation, sciatic nerve segments were exposed, gently cut and fixed in 4% paraformaldehyde. Longitudinal sections were obtained by cryostat sectioning (scale bar: 200 μm). Axonal transport of endogenous PPARγ was compared with the neuropeptide calcitonin gene-related peptide (CGRP, anterograde transport marker), and with the p75 receptor (retrograde transport marker). CGRP immunoreactivity predominantly accumulates proximal; whereas, PPARγ and p75 accumulate distal to the double ligature. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

accumulating on the distal are retrogradely transported. To study whether PPARγ is retrogradely transported we performed ligation experiments that revealed accumulation of PPARγ protein on the distal side after 24 h of ligation, indicating that PPARγ is normally transported retrogradely along the axon towards the cell body [Fig.

1(D)]. In these experiments, the calcitonin gene-related peptide (CGRP) was used as a marker for anterograde transport (Kashihara et al., 1989) and p75 as marker for retrograde transport (Yano and Chao, 2004).

Axonal Injury Increases PPARγ Levels in Axons and Nucleus of Sensory Neurons

In order to evaluate the effect of axonal injury on PPARγ levels, we performed immunofluorescence for PPARγ on the region proximal to the lesion site in crushed rat sciatic nerve, 6 h after the lesion [Fig. 2(A)]. The immunofluorescence showed increased immunoreactivity for PPARγ compared with control uncrushed contralateral nerve [Fig. 2(A)]. Similar results were obtained on Western blots for PPARγ in axoplasm samples where the increased levels were quantified [Fig. 2(B)]. This increase in PPARγ levels is consistent with local translation of PPARγ in injured axons. We therefore used FISH to identify PPARγ mRNA in non-crushed sciatic nerve. We observed robust signal co-localized with neurofilament immunostaining [Fig. 2(C)], indicating that mRNAs encoding PPARγ are indeed present in sciatic nerve axons.

Axonal transport has an important role during the injury response and subsequent axon regeneration (Eva et al., 2012; Rishal and Fainzilber, 2014). Dynein is the molecular motor responsible for retrograde axonal transport in neurons, and association of transcription factors with the dynein complex has been reported in axonal retrograde injury signaling (Rishal and Fainzilber, 2014). In order to determine whether PPARγ associates with dynein, dynein was immunoprecipitated from control and injured (6 h postlesion) rat sciatic nerve axoplasm, followed by Western blotting for PPARγ. Consistent with the results described in Figure 2, we observed increased co-precipitation of PPARγ with dynein in axoplasm from injured-sciatic nerve [Fig. 3(A)] compared with noninjured conditions. This result was also confirmed by reciprocal co-immunoprecipitation of dynein with PPARγ [Fig. 3(B)]. Taken together, our results suggest that newly synthesized PPARγ associates with dynein for retrograde transport after injury. A time course analysis further revealed increased accumulation of PPARγ distal to the ligation over 2 to 6 h after lesion [Fig. 3(C,D)], supporting the notion of an increase in retrogradely transported PPARγ in nerve injury.

Injury-induced increases in PPARγ expression and retrograde transport should correlate with increased nuclear accumulation of PPARγ in the affected neurons together with a PPAR transcriptional response. Indeed, L4-L6 DRG sections exhibited an increase in the nuclear localization of PPARγ in injured sensory neurons [Fig. 4(A,B)]. Interestingly, recent studies identified the transcriptional signature of PPARs in microarray analysis of DRG tissue following sciatic nerve damage (Michaelevski et al., 2010; Ben-Yaakov et al., 2012). A heat map representation of the PPAR-responsive genes identified in those studies [Fig. 4(C)] shows a delayed kinetics of gene regulation after injury, consistent with the nuclear uptake kinetics observed for PPARγ [Fig. 4(A,B)]. Taken together these data indicate that axonal PPARγ is retrogradely transported to the soma and nucleus to influence the injury response.

In additional analyses, we observed that the vast majority of actively growing adult mouse DRG neurons in culture (defined as those with process lengths exceeding 50 μm) display PPARγ nuclear accumulation, while only about 20% of non-growing neurons have nuclear PPARγ (Supporting Information Fig. S2). Furthermore, adding the PPARγ antagonists GW9662 and T0070907 (Lee et al., 2002; Leesnitzer et al., 2002) to adult mouse DRG cultures reduced the percent of growing neurons by approximately half after 40 h *in vitro* [Supporting Information Fig. S3 and Fig. 5(A,C)]. In addition, we observed reductions in the total neurite length per cell in GW9662 and T0070907-treated cultures [Supporting Information Fig. S3 and Fig. 5(A,B)].

Inhibition of Axonal PPARγ Decreases Regenerative Responses in Different Cellular Models

All the data presented above suggest that increased activation of axonal PPARγ increases the regenerative response of sensory neurons after injury. We tested this hypothesis in a conditioning lesion paradigm, which is a well-established model for monitoring retrograde injury signaling in neurons (Smith and Skene, 1997). To perform this experiment, 28 ng of GW9662, 69 ng of T0070907 or DMSO as control (1:500) were injected at the lesion site in parallel with nerve crush and 48 h later neuronal cultures were performed from L4-L6 DRGs. Neurons were then allowed to grow for 18 h *in vitro* in normal F12 medium. As shown in Figure 6, both PPARγ antagonists reduced both total neurite outgrowth and the percent of growing neurons.

To extend the above-mentioned observation to other species and culture systems we asked whether PPARγ inhibition would also affect human neurite growth in culture, using an *in vitro* axotomy model of neurons differentiated from NP1 Neuronal Progenitors (hNP1 cells) (Dhara and Stice, 2008). After 12 days of terminal differentiation, human neurons were axotomized and re-plated to allow neuronal process regeneration.

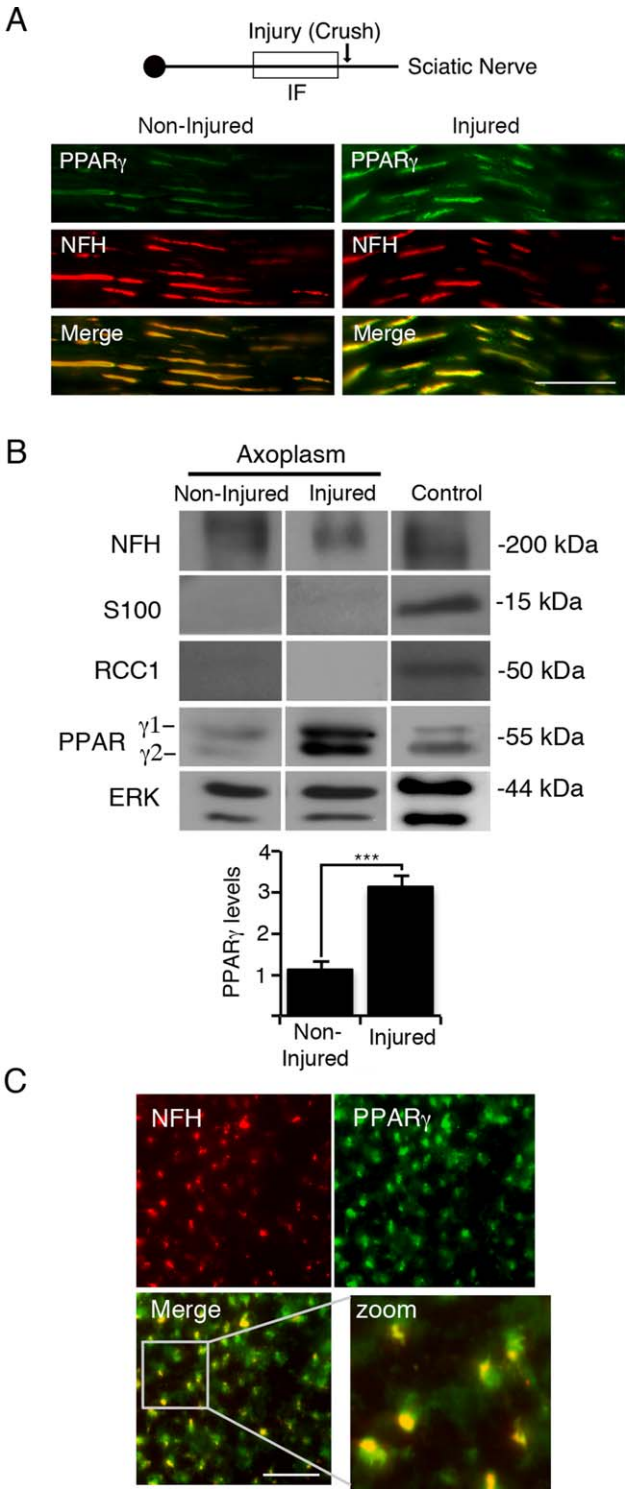

Figure 2 PPARγ expression increases after axonal injury. (A) Schematic representation of PPARγ immunofluorescence analysis. Injury was done by mechanical crush with fine forceps in the sciatic nerve. After 6 h, sciatic nerve was fixed and longitudinal sections from intact nerve and sections next to the proximal side of the injury (5–8 mm, rectangle) were immunostained for PPARγ and NFH (scale bar: 20 μm). (B) Pure axoplasm from intact and injured sciatic nerve was evaluated by Western blot for PPARγ and a specific axonal protein marker (NFH), a Schwann cell marker (S100), and a nuclear marker (RCC1) and quantified (lower panel). General Erk (Erk) was used as a loading control. Values represent the mean $\pm$ SEM of three independent experiments (***$p < 0.001$, Student's t-test). PPARγ protein levels are expressed relative to general Erk expression in axoplasm samples. (C) Fluorescence *in situ* hybridization against PPARγ mRNA in transverse sections (10 μm) of rat sciatic nerve is compared with neurofilament (NFH) immunostaining. Co-localization between PPARγ fluorescent-probe and NFH is shown in the merge picture (scale bar: 10 μm). Image at the bottom-right corner shows a magnified merge picture. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

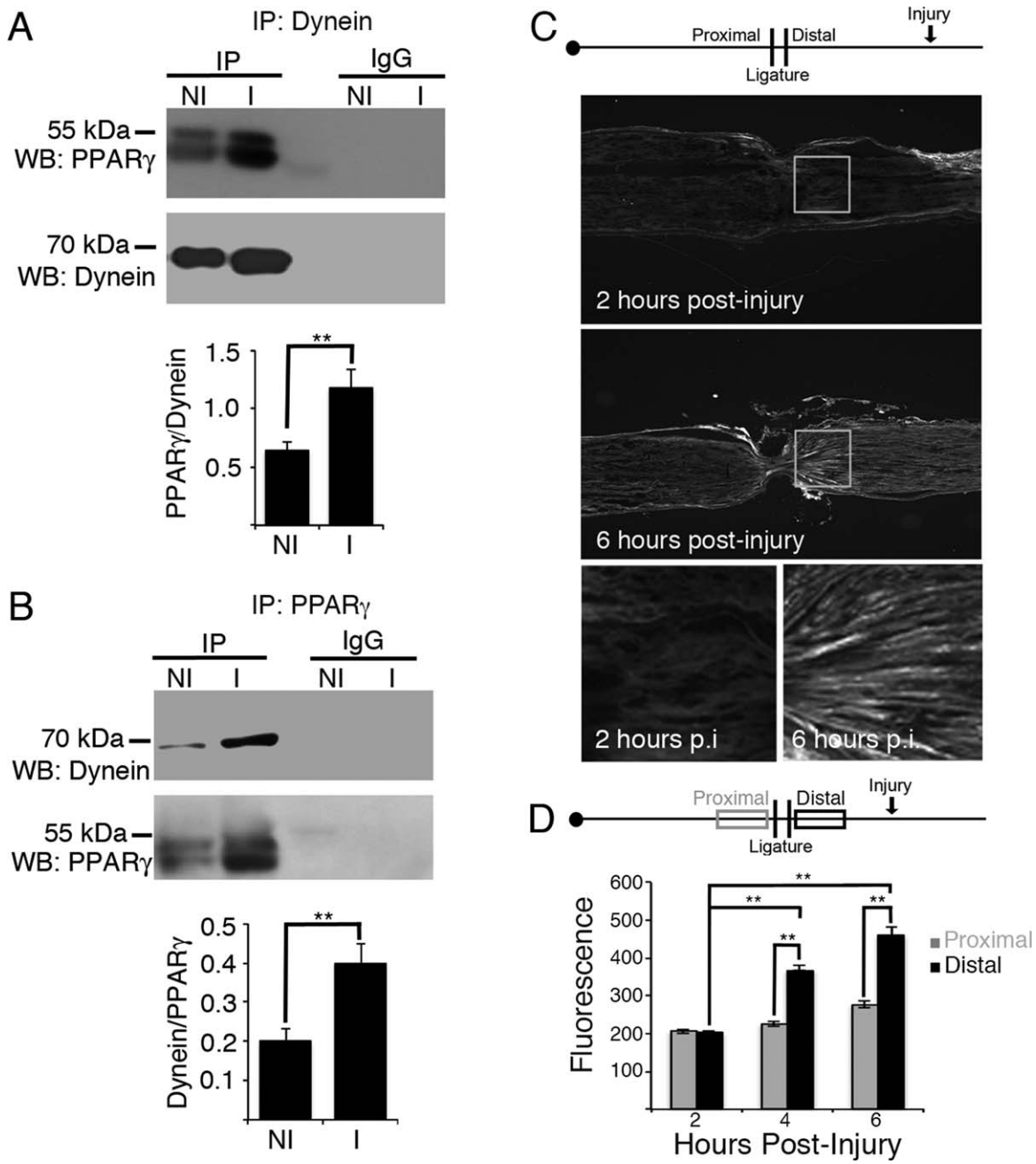

Figure 3 PPARγ interacts with dynein and its retrograde transport increases after nerve injury. (A and B) To study whether axonal PPARγ interacted with dynein, 500 μg axoplasm from naive and injured sciatic nerve (6 h after injury) was subjected to dynein (A) or PPARγ (B) immunoprecipitation and Western blotting against PPARγ (A) and dynein (B) was performed. Precipitation with an unrelated total IgG serves as a control. Levels of PPARγ or dynein co-immunoprecipitacion were quantified. There is an increased amount of PPARγ interacting with dynein or dynein interacting with PPARγ after injury consistent with the fact that PPARγ protein levels are increased after injury (see Fig. 2). Numbers were standardized against the levels of co-immunoprecipitated PPARγ (A) or dynein (B) measured by Western blotting. Values represent the mean +-SEM of three independent experiments (**$p < 0.01$, Student's t-test). NI, non-injury. I, injury. IP, immunoprecipitation. IgG, non-related IgG. WB, Western blot. (C) PPARγ retrograde transport is increased after injury. Sciatic nerve was ligated, and distal to ligation, an injury (mechanical crush) was performed. After 2, 4 and 6 h, longitudinal sections of sciatic nerve were assessed by immunofluorescence for PPARγ in both side of the ligature. (D) Quantification of PPARγ fluorescence in the proximal (site of ligature closer to cell bodies, grey bars) and distal side (black bars) of the ligature at 2, 4, and 6 h postinjury. Values represent the mean $\pm$ SEM of three independent experiments (**$p < 0.01$, Student's t-test).

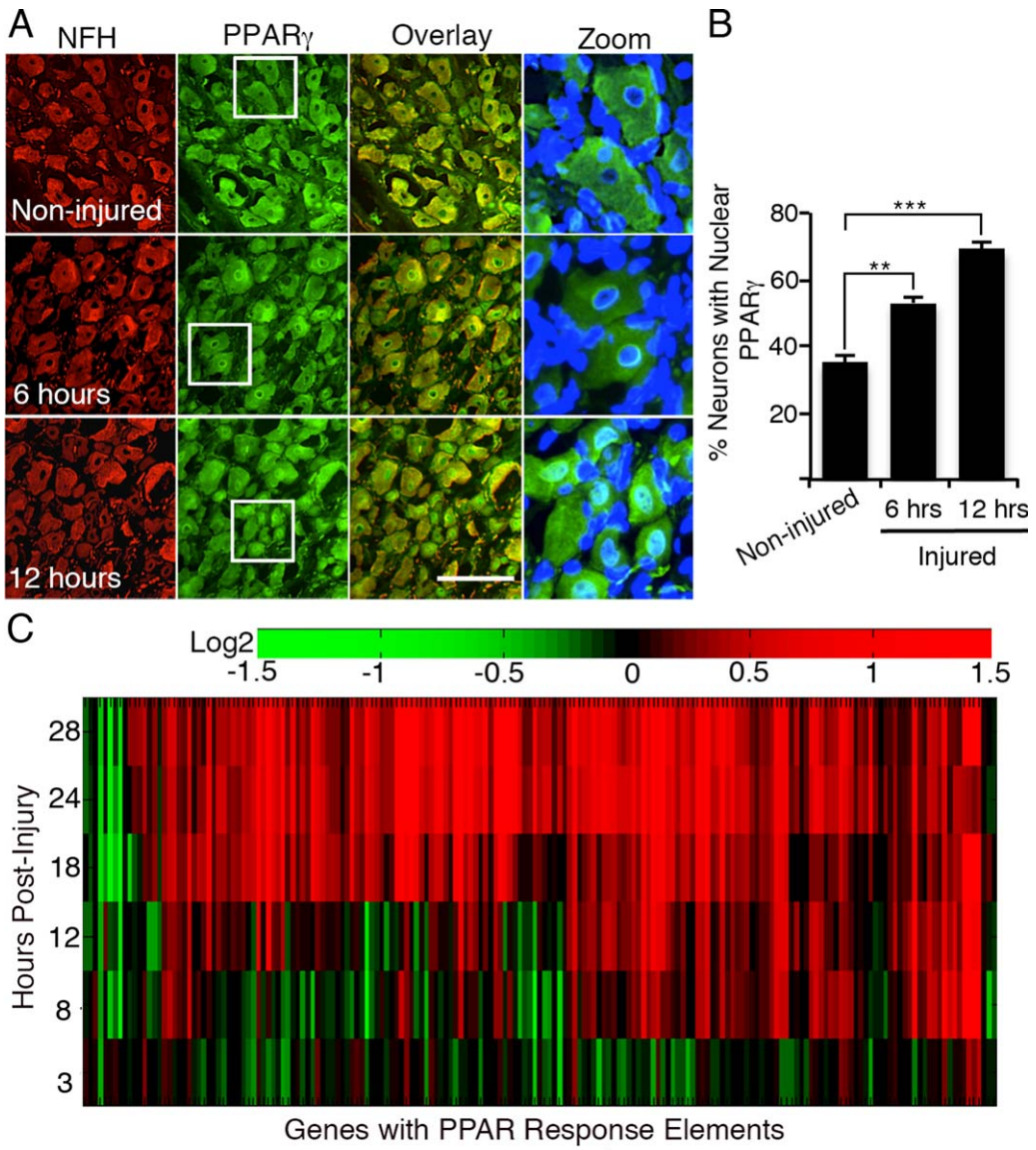

Figure 4 Nuclear localization of PPARγ in sensory neurons after sciatic nerve injury. (A) Rat L4-L5 DRGs were fixed 6 and 12 h after sciatic nerve crush and transverse sections were immunostained for NFH (red), PPARγ (green) and DAPI (blue). (Scale bar: 20 μm). The right column shows the nuclear localization of PPARγ in the highlighted section of the respective PPARγ picture. (B) Quantification of positive nuclei for PPARγ in DRGs tissue sections. Over 100 nuclei were quantified for each replicate. The experiment was repeated three times with similar results. Values represent the mean +-SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$, ANOVA followed by Tukey's multiple comparisons test). (C) Expression of DRG injury-regulated genes with PPAR response elements plotted from the microarray data of Michaelevski et al. (2010). Expression data is shown as a heat map of fold changes (color code is shown above). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Similar to results from rodent neurons, GW9962 treatment of axotomized human neurons reduced the number of neurite-extending cells and the lengths of neuronal processes (Supporting Information Fig. S4).

Finally, we examined whether PPARγ might play a role in regeneration of rodent CNS neurons as well. We used microfluidic chambers to study the presence of PPARγ in the axons of rat E18 cortical neurons. Immunofluorescence staining of compartmentalized cultures at 10 DIV revealed the presence of PPARγ in distal cortical axons. The axonal localization of PPARγ was confirmed by co-staining with β-III-tubulin (Supporting Information Fig. S5). Next, we evaluated whether axonal activation by the PPARγ agonist rosiglitazone (Wright et al., 2014) affects the regeneration of axotomized axons. Neurons were

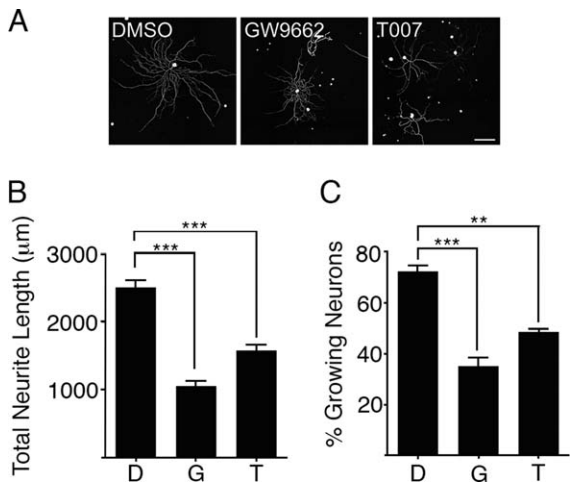

Figure 5 Assessing PPARγ antagonist effects on DRG neurons in culture. (A) Adult L4-L6 DRG neurons were cultured for 40 h in medium containing GW9662 (10 μM) or T0070907 (5 μM) or DMSO as control (scale bar: 200 μm). (B) Average of total neurite length and percent growing neurons (neurons with maximal process length exceeding 50 μm). Values represent the mean + SEM of three independent experiments (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ANOVA followed by Tukey's multiple comparisons test). D, DMSO. G, GW9662. T, T0070907.

axotomized by aspiration of the liquid in the axonal chamber (Supporting Information Fig. S6) and then allowed to regrow after supplementation of the axonal compartment with PPARγ agonists and/or antagonists as indicated (Fig. 7). Cultures treated with rosiglitazone for 96 h displayed extensive axonal regeneration compared with the control situation. This effect was abolished by treatment of distal axons with the PPARγ inhibitors GW9662 and/or T0070907, suggesting that rosiglitazone enhances axonal regeneration in a PPARγ-dependent manner (Fig. 7).

DISCUSSION

Activation of the transcriptional response to nervous system injury requires retrograde signaling from axonal lesion sites to neuronal cell bodies. These transcriptional changes enable an increase in the intrinsic growth capacity of injured neurons, hence are critical for functional regeneration. A variety of transcription factors have been implicated in neuronal regeneration, typically acting within the soma as part of the cell body response. However recent studies have suggested that transcription factors are also found within axons, and may traffic with retrograde signaling complexes to elicit and modulate events in the soma (Ji and Jaffrey, 2014; Rishal and Fainzilber, 2014).

Analyses of the responses of DRG sensory neurons to sciatic nerve lesion suggested that the PPAR family of transcription factors might be candidate retrograde injury signals (Michaelevski et al., 2010; Ben-Yaakov et al., 2012). Here we have confirmed this prediction by showing that PPARγ is located within axons in the sciatic nerve and in sensory and cortical neurons in cultures. Sciatic nerve axons contain both PPARγ protein and mRNA. Upon injury, increases in protein levels, dynein motor association and retrograde transport of PPARγ were observed, suggesting that it is recruited to motor-driven complexes for transport back to the cell body. This was further supported by observations of increased nuclear localization of PPARγ in the nucleus of DRG neurons after sciatic nerve crush and in growing sensory neurons in culture together with increased expression of genes bearing the PPAR response element. Furthermore, the PPARγ inhibitors GW9662 and T0070907 (Lee et al., 2002; Leesnitzer et al., 2002) both reduced the conditioning lesion response of sensory neurons subjected to *in vivo* axonal lesions in the sciatic nerve. We also observed inhibition of neurite regeneration in axotomized human neurons treated with GW9662, consistent with

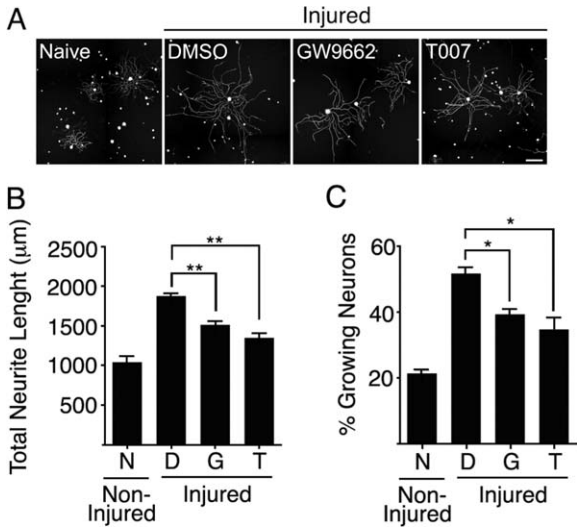

Figure 6 Inhibition of PPARγ reduces neuronal response to sciatic nerve injury. (A) Sciatic nerve of adult mice was injected with 28 ng GW9662, 69 ng T0070907 or DMSO as control in parallel with nerve crush. The contralateral side was left uninjured as a control. Two days after the crush L4-L6 DRG neurons were extracted and cultured for 18 h (scale bar: 200 μm). (B and C) Average of total neurite length (B) and percent growing neurons (neurons with maximal process length exceeding 50 μm) (C). Values represent the mean ± SEM of at least three experiments (*$p < 0.05$, **$p < 0.01$, ANOVA followed by Tukey's multiple comparisons test). D, DMSO. G, GW9662. T, T0070907.

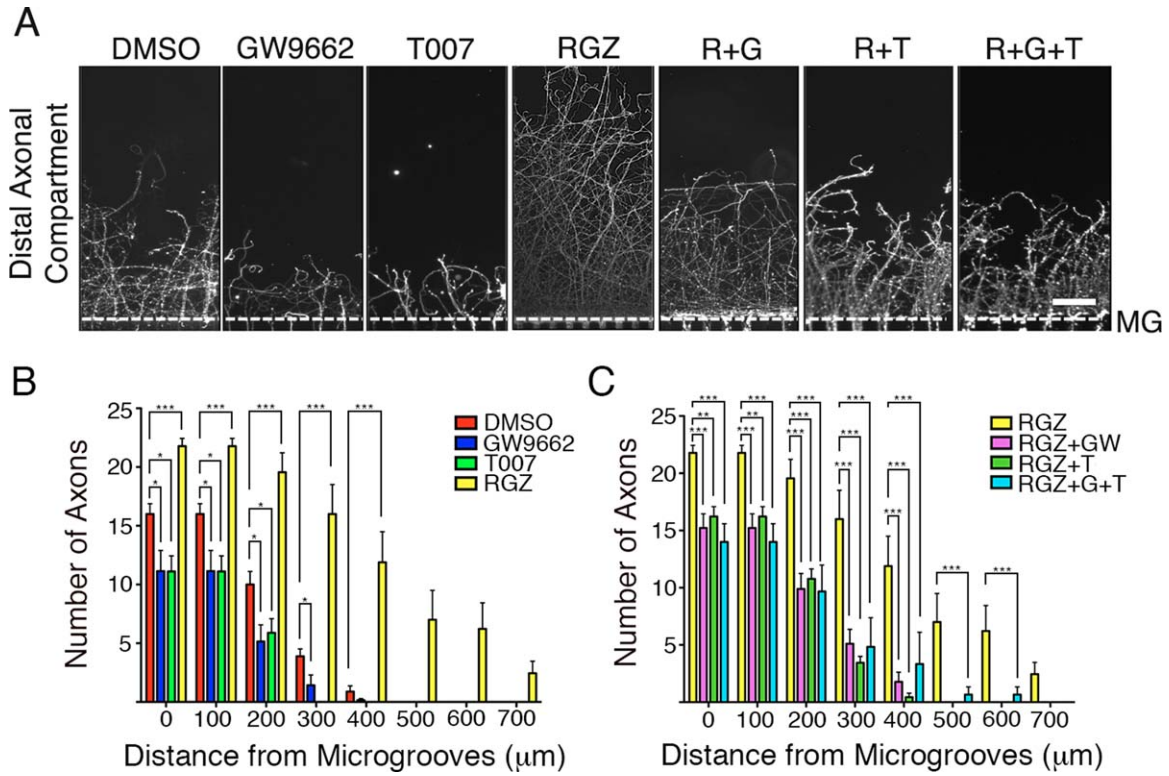

Figure 7 PPARγ promotes axonal regeneration after axotomy in primary cultures of cortical neurons. Axons from cortical neurons were axotomized by aspiration at nine DIV and then allowed to re-grow into axonal compartments supplemented with DMSO vehicle, or GW9662 (GW, 10 μM), T0070907 (T007, 10 μM), Rosiglitazone (RGZ, 1 μM) or RGZ + GW; RGZ + T007; and RGZ + GW + T007, as indicated. Ninety-six hours after treatment, growth of distal axons was assessed by immunofluorescence for β-III-tubulin. (A) Representative images from three independent experiments with three microfluidic chambers by treatment. Scale bar: 100 μm. (B and C) Quantification of three microfluidic chambers per treatment from three independent experiments. The number of axons crossing a line every 100 μm distal to the microgrooves (MG) was quantified using ImageJ software. Values represent the mean ± SEM of three independent experiments (*$p < 0.5$; **$p < 0.01$; ***$p < 0.001$; ANOVA followed by Tukey's multiple comparisons test). Dashed lines represents final part of the microgrooves (MG) to distal axonal compartment. D, DMSO. G, GW9662. T, T0070907. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

a role of PPARγ on axonal regeneration. Consistently with the above-mentioned results, treatment of axotomized cortical neurons with both PPARγ antagonists reduced axonal growth, and growth enhanced by rosiglitazone was also attenuated by the two antagonists. The effects of GW9662 and T0070907 on rosiglitazone-induced axonal growth were not additive or synergic, indicating that they both affect the same target. Taken together, these results suggest that axonal activation and retrograde transport of PPARγ are part of a pro-regenerative program in neurons.

A number of transcription factors and transcriptional modulators have been implicated in signaling from axon to soma in both the developing and the adult nervous system (Ji and Jaffrey, 2014). Examples for adult neurons include STAT3 as a retrograde survival factor in injured sensory neurons (Ben-Yaakov et al., 2012), CREB as a nociceptive transducer in pain models (Melemedjian et al., 2014), and ATF4 as a neurodegenerative signal in neurons challenged with β-amyloid (Baleriola et al., 2014). Axonal expression of transcription factors may also fulfill localized functions within the axon itself, as was reported for STAT3 in microtubule stabilization in motor neurons (Selvaraj et al., 2012). In most cases reported to date, retrograde transport of axonal transcription factors primarily affects neuronal survival or death pathways. In contrast, retrograde transport of PPARγ in axons has clear and robust effects on axonal outgrowth in diverse neuronal subtypes.

The PPAR family of nuclear receptors transcription factors consists of three members in mammals, PPARα, PPARβ/δ, and PPARγ. Upon ligand binding, PPARs enter the nucleus and bind PPAR responsive elements as obligate heterodimers with the retinoid X receptor (RXR), activating networks of genes related to glucose

and lipid metabolism as well as inflammation in different tissues (Ahmadian et al., 2013). The PPARs are known as receptors for different fatty acids and lipids metabolites, hence are mainly considered to be lipid sensors regulating cellular metabolism. Natural or endogenous ligands for PPARγ include unsaturated fatty acids, oxidized phospholipids, eicosanoids and nitroalkens. The relatively large size of the ligand-binding region in PPARγ allows the binding of different types of lipid molecules. For example, the PPARγ ligand-binding domain can accommodate at least two oxidized fatty acids, and different ligands are able to generate graded transcriptional responses by recruiting different subsets of the requisite co-activators (Hughes et al., 2012). These characteristics might enhance the versatility of PPARγ-dependent injury response mechanisms. For example, injury-induced elevations in axonal calcium could initially enhance the activity of calcium dependent lipases such as PLA2. Subsequent fatty acid release from membrane-associated phospholipids would then generate a variety of potential PPARγ ligands by oxidation or modification of fatty acids due to calcium-induced oxidative stress (Leslie, 1997; Villegas et al., 2014). Thus, axonal injury can lead to significant production of diverse PPARγ agonists, suggesting that axonal localization of PPARγ might facilitate efficient coordination of regeneration.

In additional to transcriptional effects in the nucleus, PPARγ might influence axonal growth via other downstream targets in axons or soma. For example, PPARγ and its co-activator PGC-1α are implicated in the promotion of mitochondrial biogenesis, and axonal or dendritic targeting of mitochondria is required for growth. Increased PGC-1α expression in neurons increases mitochondrial density and regulates mitochondrial transport and oxidation state after injury (O'Donnell et al., 2013; van Spronsen et al., 2013; Corona and Duchen, 2015). Another possibility is that downstream targets of PPARγ modulate the c-jun-NH2 terminal kinase (JNK) pathway to modulate axonal growth, as previously shown for long-term treatment of hippocampal neurons with TZDs (Quintanilla et al., 2013). The JNK pathway is known to affect a series of transcription factors and cytoskeleton substrates in axonal regeneration (Waetzig et al., 2006; Coffey, 2014).

The activation of PPARγ in axons upon nerve lesion and its effects on axonal outgrowth raise interest in the translational potential of targeting the PPARγ pathway for enhancing nerve growth and regeneration. PPARγ is a master regulator of metabolism and an important drug target in diabetes, as the specific target of thiazolidinediones (TZDs), a drug class of full agonists for PPARγ which includes rosiglitazone (Wright et al., 2014). Although TZDs are widely used in the clinic, a number of undesirable side effects have motivated significant efforts to develop new pharmaceutical agents targeting PPARγ (Ahmadian et al., 2013), including selective PPARγ modulators (SPPARMs) that act as PPARγ partial agonists (Grygiel-Gorniak, 2014). Thus, there is a rich pharmacological toolkit for PPARγ, including a number of agents with acceptable safety profiles in humans that could potentially be evaluated for efficacy in central and peripheral nerve injuries. Moreover, a series of recent studies have reported neuroprotective effects of TZDs in stroke and in a number of neurodegenerative disease models (Chen et al., 2012). These effects were attributed to anti-inflammatory activities reported for PPARγ in glial cells (Bernardo and Minghetti, 2008; Zhao et al., 2009). Our results however, strongly support direct effects of PPARγ on neuronal regeneration, and raise the possibility that TZD-induced neuroprotection might also be due to specific effects on neurons. We expect that the findings reported in this study will open new avenues for both basic understanding and translational exploitation of the roles of PPARγ in the nervous system.

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