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Hyperactivated Stat3 boosts axon regeneration in the CNS

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Abstract

Axonal regeneration after spinal cord injury (SCI) is intrinsically and extrinsically inhibited by multiple factors. One major factor contributing to intrinsic regeneration failure is the inability of mature neurons in the central nervous system (CNS) to activate regeneration-associated transcription factors (TFs) post-injury. A prior study identified TFs overexpressed in neurons of the peripheral nervous system (PNS) compared to the CNS; some of these could be involved in the ability of PNS neurons to regenerate. Of these, signal transducer and activator of transcription 3 (STAT3), as well its downstream regeneration-associated targets, showed a significant upregulation in PNS neurons relative to CNS neurons, and a constitutively active variant of Stat3 (Stat3CA) promoted neurite growth when expressed in cerebellar neurons (Lerch et al., 2012; Smith et al., 2011). To further enhance STAT3's neurite outgrowth enhancing activity, Stat3CA was fused with a viral activation domain (VP16). VP16 hyperactivates TFs by recruiting transcriptional co-factors to the DNA binding domain (Hirai et al., 2010). Overexpression of this VP16-Stat3CA chimera in primary cortical neurons led to a significant increase of neurite outgrowth as well as Stat3 transcriptional activity in vitro. Furthermore, in vivo transduction of retinal ganglion cells (RGCs) with AAV constructs expressing VP16-Stat3CA resulted in regeneration of optic nerve axons after injury, to a greater degree than for those expressing Stat3CA alone. These findings confirm and extend the concept that overexpression of hyperactivated transcription factors identified as functioning in PNS regeneration can promote axon regeneration in the CNS.

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Keywords

VP16; Activation domain; Transcription factor; Optic nerve; Neurite outgrowth; Axon regeneration

1. Introduction

Axons often fail to regenerate following insults to the central nervous system (CNS). Neurons in the CNS that suffer damage fail to re-connect to their targets due to a multitude of factors. Severed axons typically fail to grow past injury sites due to local environmental factors and an inhibitory inflammatory response (Huebner and Strittmatter, 2009; Wang et al., 2012). Additionally, mature CNS neurons generally fail to express many of the genes known to promote axon regeneration after injury. One way to promote regeneration in the CNS is by manipulating the expression of regeneration-associated genes (RAGs). Recent findings show that suppression or deletion of inhibitory factors such as PTEN, Klf4 or SOCS3, or overexpression of genes upregulated during PNS regeneration such as CREB, cJun, and Klf7 can induce axon growth in the adult CNS (Blackmore et al., 2012; Gao et al., 2004; Lerch et al., 2014; Moore et al., 2009; Park et al., 2008; Smith et al., 2009). Rather than individually targeting the genes dysregulated during PNS regeneration, it should be possible to target individual transcription factors (TFs) that control expression of many RAGs (Gao et al., 2004; Lerch et al., 2012; Smith et al., 2011). In this context, it is notable that several of the genes cited above are TFs. Although these treatments have shown some success, significant functional recovery remains elusive. Typically, less than 1% of retinal ganglion cells (RGCs) or corticospinal tract neurons regenerate axons after injury, even with therapeutic intervention. Moreover, the axons that do regenerate may be unable to connect with their original targets (Luo et al., 2013).

Our laboratory has used a variety of approaches to identify TFs potentially regulating successful regenerative responses (Lerch et al., 2012; Smith et al., 2011). In overexpression studies designed to assess the impact of these TFs on neurite outgrowth from primary neurons (Blackmore et al., 2010, 2012; Lerch et al., 2014), Stat3, which regulates the transcription of many RAGs, emerged as one possible candidate (Smith et al., 2011). Interestingly, overexpression of wild-type Stat3 was not sufficient to promote neurite outgrowth in CNS neurons. However, a constitutively active variant of Stat3 (Bromberg et al., 1999), which can homodimerize in the absence of phosphorylation by Janus kinases (Bareyre et al., 2011; Lang et al., 2013; Pernet et al., 2013), was able to promote neurite growth when overexpressed in cerebellar neurons (Smith et al., 2011).

Addition of exogenous transcriptional activation domains to specific TFs can be used to increase TF activities (activation or repression) or even to switch the dominant activities from activation to repression or vice versa (Hirai et al., 2010; Kobayashi et al., 1998; Milbradt et al., 2011) VP16 is an activation domain from Herpes simplex virus, which functions by recruiting basal transcription factors and histone acetyltransferases to the TF complex on which it resides. Fusion of VP16 to regeneration-associated TFs has been used

to manipulate their activities after spinal cord injury and to promote regeneration of spinal axons in vivo (Blackmore et al., 2012; Gao et al., 2004).

To study whether "hyperactivated" Stat3 could promote axon growth, we used two activation domains — we fused either the VP16 or the VP64 transactivation domain to the constitutively active form of Stat3 (VP16-Stat3CA or VP64-Stat3CA). VP64 is a tetramer of the 11 aa minimal activation domain of VP16, which has been shown in some cases to result in increased activation compared to VP16 (Beerli et al., 1998). To test hyperactivated Stat3 in vitro, we overexpressed variants of Stat3 – wild type Stat3 (Stat3WT), Stat3CA, VP16-Stat3WT, VP16-Stat3CA, and VP64-Stat3CA – in postnatal day 3 (P3) rat cortical neurons. We found that VP16-Stat3CA, but not the other constructs, promoted neurite outgrowth at 2 days in vitro (DIV). Furthermore, qPCR analyses show that overexpression of VP16-Stat3CA led to an increase in mRNA levels of Stat3 as well as downstream regeneration associated genes Sprr1a and ATF3. Finally, in an in vivo model of axon regeneration, overexpression of VP16-Stat3CA promoted significantly more axonal regeneration in the optic nerve at two weeks post-injury (WPI) than did Stat3CA alone. Thus hyperactivation of Stat3 can improve its ability to promote axon growth in CNS neurons.

2. Materials and methods

2.1. Cloning and virus construction

Stat3WT and Stat3CA mutants were fused to VP16 at the N-terminus to make VP16-Stat3WT and VP16-Stat3CA. VP64 was fused to the N-terminus of Stat3CA. All Stat3 mutants were cloned with a 3× flag tag on the N-terminus of either Stat3 or VP16 with a CMV promoter followed by a beta-globin intron in the pAAV-MCS plasmid (Stratagene). Viral particles for RGC transduction were produced by the Miami Project Viral Vector Core in AAV2 capsid plasmids using FPLC purification to achieve titers of $2.7–3 \times 10^{12}$ genome copies/mL.

2.2. Neuronal cell culture

Cortices were dissected from postnatal day 3 (PN3) rat pups (Sprague–Dawley; Charles River) in order to isolate primary cortical neurons for transfection as previously described (Blackmore et al., 2010). Briefly, neurons were dissociated and sequentially digested in 20 U/mL papain (Worthington) followed by 2.5% trypsin (GIBCO). 150,000 cells were electroporated with 1 μg of DNA using the AMAXA Nucleofector Technology (Program: Neurons, High Viability). Each of the plasmids was individually electroporated into freshly isolated primary cortical neurons, yielding transfection efficiencies of approximately 60%. Cells were cultured in 12-well Falcon plates (Catalog #353043) coated with 0.5 mg/mL poly-D-lysine (Sigma Aldrich; Catalog #P6407), in Proneural Growth Medium supplemented with PNGM SingleQuots (LONZA; Catalog #CC4461) conditioned overnight in cultured P5 rat glia. Cortical cultures were maintained at 37 °C with 5% CO_2 for 2 days.

2.3. Neurite outgrowth assays

Cells were fixed 2DIV in 4% paraformaldehyde in PBS (Sigma-Aldrich) and immunostained first with antibodies to neuronal specific beta-III tubulin (rabbit; 1:2000;

Abcam; ab18207) and flag tag (mouse; 1:750; Sigma-Aldrich; F1804) followed by AlexaFluor 488 (anti-rabbit) and 546 (anti-mouse) secondary antibodies. Images were captured using a Cellomics ArrayScanVTI automated microscope and neuronal morphology was assessed with the Neuronal Profiling algorithm (ThermoFisher). The total neurite length (NTL) and length of longest neurite (LOLN) were computed for each transfected neuron. For each experiment we measured a minimum of 100 neurons with three technical replicates per condition; 5 independent biological replicates were performed. Neurite outgrowth was normalized to controls transfected with a plasmid expressing the fluorescent protein mCherry. Cell survival was determined by quantifying cells in each condition with at least one neurite and a minimum NTL of 10 μM, normalized to control. One control replicate was set to 100% and the other controls were normalized to the replicate.

2.4. RNA isolation and qPCR

RNA was isolated from approximately 1×10^6 P3 rat cortical neurons transfected with mCherry, Stat3CA, or VP16-Stat3CA (Qiagen). Isolated RNA was reverse transcribed (iScript; Bio-Rad) to obtain 1 μg of DNA, and quantitative-PCR reactions (Power SYBR Green PCR Master Mix; Applied Biosciences) were run on a 7300 Real-Time PCR System (Applied Biosciences) with primers for Stat3, ATF3, Sprr1a, and GAPDH (Table S1). Each condition was run in three biological replicates as well as a no template control. Relative expression for each gene in each sample was calculated using the $2^{-\Delta Cq}$ method to reference gene GAPDH (Schmittgen and Livak, 2008).

2.5. Optic nerve experiments

Intravitreal injections were performed on 4–6 week old female C57BL/6 mice using a glass micropipette inserted in the peripheral retina, avoiding the lens. This method was used to deliver 2 μL of either AAV2 treatment (14 days and 7 days before injury) or cholera toxin B (CTB; 12 days post-injury). Optic nerve injury was performed by intraorbitally exposing the left optic nerve, which was crushed with Dumont #5 forceps (Roboz) for 10 s approximately 1 mm from the optic disc. CTB-Alexa 555 was injected 2 days before euthanasia as an anterograde tracer to visualize regenerating axons (2 μg/μL; Invitrogen). At 2 WPI, mice were euthanized and transcardially perfused and the optic nerve was dissected, post-fixed in 4% PFA in PBS overnight and cryoprotected with 30% sucrose. The tissue was embedded in OCT and sectioned at 12 μm. Regenerating axons were identified by CTB tracing and were counted at various distances from the site of injury (200 μm, 500 μm, 100 μm, and 1500 μm). At least four sections were counted at each distance for each animal (6 animals per condition). For RGC survival quantification, Neuron-specific tubulin+ RGCs in the injured and contralateral uninjured retinas were manually counted. For each flat-mounted retina, 10 fields of the central, intermediate, and peripheral regions were imaged. The percentage quantification is based on the average RGC count in the injured normalized to the uninjured contralateral retina. Quantification of regeneration and cell survival was performed by an investigator blinded to the treatment groups.

3. Results

3.1. VP16-Stat3CA promotes neurite outgrowth in postnatal cortical neurons

We overexpressed five STAT3 constructs via electroporation in P3 cortical neuron cultures: Stat3WT, Stat3CA, VP16-Stat3WT, VP16-Stat3CA, and VP64-Stat3CA (Fig. 1). Each construct contained a tag to allow identification of transfected cells; Fig. 1A shows representative images of neurons successfully transfected with each construct. A construct encoding the reporter gene mCherry was used as a normalization control as it has been shown to have no significant impact on neurite outgrowth in comparison to cells electroporated with an empty plasmid (Lerch et al., 2014). After 2 days in vitro (DIV) overexpression of VP16-Stat3CA promoted neurite outgrowth compared to controls by allowing the growth of longer neurites (Fig. 1A). Overexpression of other variants of Stat3, including the CA variant containing the VP64 tetramer, did not appear to promote substantial neurite outgrowth (Fig. 1A). We quantified neurite outgrowth from transfected cells by measuring, for each cell, the total length of all neurites, the length of the longest neurite, and the number of neurite branches. These data reveal that VP16-Stat3CA increased both total neurite length (159.5% ± 14.2 SEM, N = 5 experiments) and the mean length of the longest neurite (148.5% ± 10.4 SEM) compared to mCherry controls, but did not significantly affect neurite branching (104.3%±2.5 SEM) in cortical neurons. None of the other constructs significantly altered any measured parameter of neurite growth (Fig. 1B–D; N=5 experiments). Because changes in neuronal cell health and/or the selective survival of different populations of cortical neurons after expression of different TF variants could influence our results, we also measured neuronal survival in each condition. None of the Stat3 variants affected neuronal cell number compared to mCherry controls (Fig. 1E). The effects of VP16-Stat3CA on neurite outgrowth require fusion of VP16 to the TF, as overexpression of VP16 alone does not impact any of the measured parameters of neurite outgrowth (Fig. S1).

3.2. VP16-Stat3CA shows increased transcriptional activity

Stat3 is known to regulate the expression of dozens of genes, including genes associated with axon growth and regeneration (Smith et al., 2011; Tedeschi, 2011). Our hypothesis is that overexpression of hyperactivated Stat3 promotes axon growth through the strong upregulation of regeneration-associated neuronal genes (RAGs). If so, we should be able to identify increased expression of known Stat3-responsive genes after transfection with active Stat3 constructs, and in particular it should be possible to identify highly upregulated RAGs in neurons transfected with hyperactivated Stat3. To explore this issue, we used qPCR to look at gene expression changes in two of Stat3's regeneration-associated transcriptional targets, ATF3 and Sprr1a, after overexpression of Stat3CA and VP16-Stat3CA. We also examined expression of the Stat3 gene itself, because it is known that Stat3 strongly upregulates its own expression (Bonilla et al., 2002; Dauer et al., 2005). Overexpression of Stat3CA in cortical neurons led to 2–3 fold increases in expression of the Stat3 (2.55 ± 0.29 SEM, $p < 0.01$; N = 3) and ATF3 (3.66 ± 0.73, $p < 0.05$; N = 3) genes, and appeared to increase Sprr1a gene expression, though this did not reach statistical significance (Fig. 2). Interestingly, overexpression of VP16-Stat3CA increased gene expression for Stat3 (4.89 ± 0.08 SEM, $p < 0.001$; N = 3) as well as the known RAGs ATF3 (5.51 ± 0.40, $p < 0.01$; N =

3) and Sprr1a (5.58 ± 1.04 SEM, $p < 0.05$; N = 3) to a greater extent than Stat3CA alone (Fig. 2). These data are consistent with the idea that VP16-Stat3CA promotes neurite outgrowth by upregulating the expression of relevant RAGs.

3.3. Overexpression of activated Stat3 promotes axon regeneration in the optic nerve

We used the optic nerve crush model (Benowitz and Yin, 2010) to ask whether hyperactivated Stat3 can promote more efficient RGC axon regeneration in vivo. In a previous study, it was found that overexpression of Stat3WT or, somewhat more effectively, Stat3CA, is sufficient to induce some axonal regeneration after optic nerve crush (Pernet et al., 2013). Based on the finding that the hyperactivated version, VP16-Stat3CA, upregulates expression of Stat3 gene targets more effectively than Stat3CA, we hypothesized that overexpression of VP16-Stat3CA will also promote more substantial RGC axon regeneration in vivo. Fig. 3A shows the timeline used in these experiments. Adeno-associated viral serotype 2 (AAV2) particles encoding either negative control GFP, Stat3CA, or VP16-Stat3CA were injected into the intravitreal space of the left eye both 14 days and 7 days prior to the injury, and animals were sacrificed 2 weeks post-injury (WPI) to assess axon regeneration. Animals without injury were sacrificed after two weeks to assess AAV2-mediated overexpression of Stat3 constructs in RGCs (Fig. 3B). RGC's were successfully transduced with Stat3CA and VP16-Stat3CA at a transduction efficiency of 65%. Axon regeneration was assessed by imaging axons labeled with fluorescent cholera toxin subunit B (CTB) at 2WPI in tissue sections. As has been described, control RGC axons regenerated poorly. Overexpression of Stat3CA led to some regeneration for short distances, and considerably more regeneration was seen with overexpression of VP16-Stat3CA (Fig. 3C). We quantified regeneration by measuring the numbers of labeled axons able to reach specified distances from the injury site. These data show that overexpression of VP16-Stat3CA promoted significant axon regeneration up to 1 mm past the site of injury, while overexpression of Stat3CA did not promote significant regeneration at distances greater than 200 μm (Fig. 3D). These results cannot be explained by differential cell survival, since the numbers of surviving RGCs in injured animals overexpressing either Stat3 variant at 2 WPI did not differ from those seen in controls (Fig. 3E). Thus, hyperactivation of Stat3 by fusion of the constitutively active mutant to VP16 resulted in a significant increase in the regeneration of RGC axons.

4. Discussion

Stat3 has previously been implicated in the regeneration of both PNS and CNS axons. Stat3 is upregulated in motor neurons following peripheral axotomy (Schwaiger et al., 2000), and in dorsal root ganglion (DRG) neurons, inhibition of Stat3 signaling blocked the positive effects of peripheral conditioning lesions on regeneration of the central axonal branches (Qiu et al., 2005). Transcriptomic studies have identified Stat3 binding sites on a number of RAGs upregulated in PNS neurons following injury (Lerch et al., 2012; Smith et al., 2011). In the CNS, deletion of SOCS3, an inhibitor of Stat3 signaling, significantly enhances optic nerve regeneration, and overexpression of Stat3CA was also shown to promote modest regeneration of optic axons following injury (Dominguez et al., 2010; Pernet et al., 2013). In the corticospinal tract (CST), overexpression of Stat3 promotes axonal sprouting and

functional recovery following a unilateral pyramidotomy (Lang et al., 2013). These findings point to Stat3 as a key factor in promoting axon sprouting and regeneration, and our results confirm and extend this idea.

To date, the regeneration-promoting activity of overexpressed Stat3 has been modest; we therefore modified Stat3 in efforts to increase its transcriptional activity and ability to promote regeneration. First, we used a constitutively active Stat3 variant with cysteine substitutions in the SH2 binding domain (Stat3CA). This mutant can homodimerize by forming disulfide bridges in the absence of phosphorylation by Janus kinases (Bromberg et al., 1999; Shi et al., 1996). Additionally, we fused a transcriptional activator, VP16, to Stat3CA (VP16-Stat3CA) to test the idea that increased activation of Stat3-regulated genes would have a positive effect on neurite growth and regeneration. Our results, both in vitro and in vivo, support this idea.

Our results with Stat3 provide further data supporting the use of “transcriptional enhancement” to boost CNS axonal regeneration. Fusion with VP16 has been shown to increase the abilities of both CREB (in PNS neurons) and Klf7 (in CNS neurons) to promote regeneration (Blackmore et al., 2012; Gao et al., 2004). VP16 increases TF activity by recruiting acetyltransferases and chromatin remodeling proteins to TF binding sites (Beerli et al., 1998; Hirai et al., 2010). Although previous studies suggest that VP64, a tetramer of VP16's minimal activation domain, can increase transcriptional activation compared to VP16 (Beerli et al., 1998; Konermann et al., 2013; Sanchez, 2002), we did not find that VP64-Stat3CA promoted neurite growth in cortical neurons.

Overexpression of Stat3CA upregulated levels of Stat3-responsive mRNAs in cortical neurons, and these levels were further increased using VP16-Stat3CA, consistent with our initial hypothesis. The lack of effect of VP64-Stat3CA on neurite growth might reflect a lack of response to this specific activator in primary cortical neurons, or it may indicate that neurite growth and regeneration require optimal levels of RAG expression, which can be unmet (as for Stat3CA) or exceeded (in the case of VP64-Stat3CA).

We used the optic nerve crush model to assess regeneration in vivo. Although we found that overexpression of Stat3CA in RGCs promoted modest regeneration, overexpression of VP16-Stat3CA promoted substantial regeneration up to 1mmpast the injury. It is admittedly difficult to quantitatively compare effects observed in different studies. However, the regeneration we observed with overexpression of VP16-Stat3CA was comparable to that seen with either knockout or knockdown of PTEN in earlier studies (Park et al., 2008; Yungher et al., 2015), and appeared substantially greater than the regeneration resulting from knockdown of the Stat3 inhibitor SOCS3 (Smith et al., 2009). The modest regeneration seen with Stat3CA is generally consistent with a previous study using AAV2 to transduce RGCs with Stat3CA (Pernet et al., 2013). Whether the imperfect guidance of regenerating axons along the nerve seen by Pernet and colleagues is replicated or even exacerbated using VP16-Stat3CA is not known; this could be investigated using light sheet fluorescence microscopy (Luo et al., 2013). In this context, it will be important to investigate the effects of increased transcriptional activity by VP16 on functional outcomes of Stat3 overexpression (Lang et al., 2013). Although the use of hyperactivated TFs to aid axonal growth may improve functional

recovery, it seems likely that a combination of interventions will be necessary to fully realize the potential of these treatments (Lerch et al., 2014; Luo et al., 2013; Yungher et al., 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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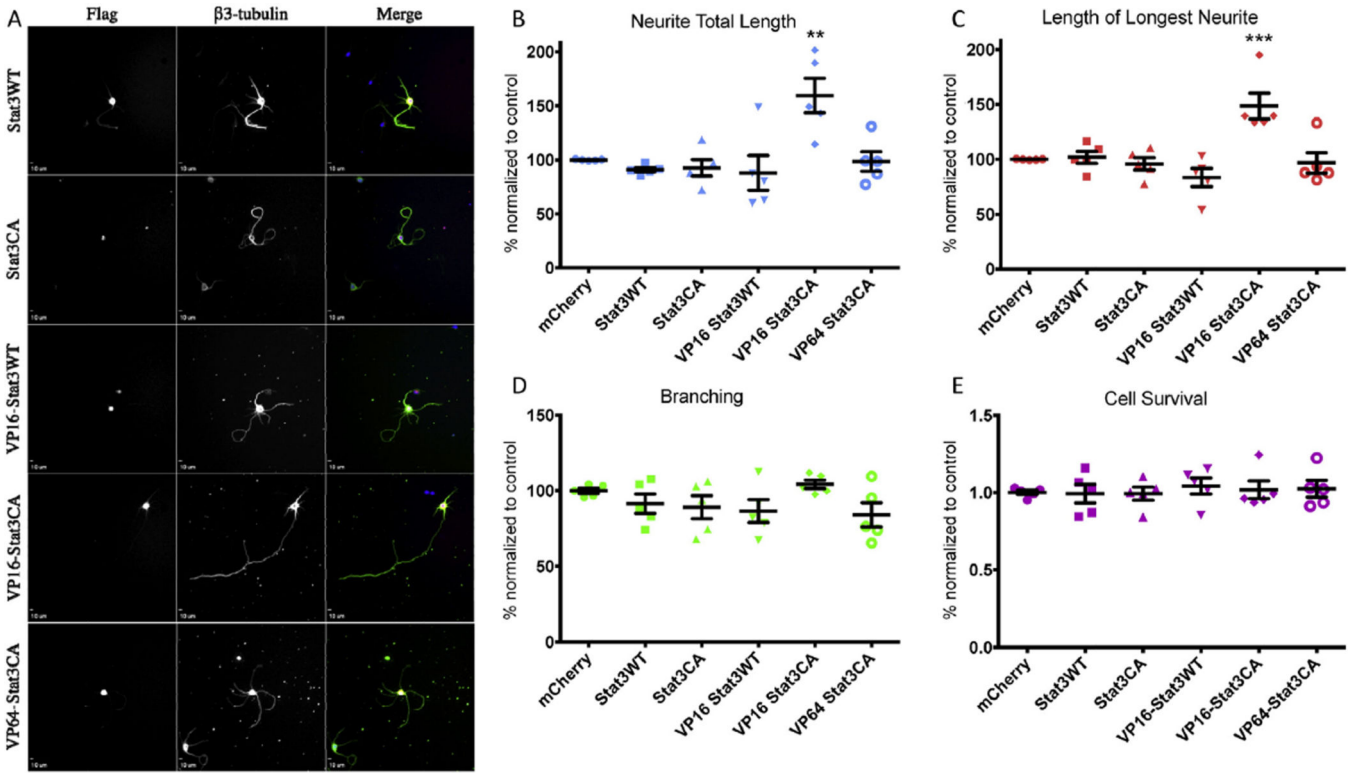

Fig. 1.

Overexpression of VP16-Stat3CA promotes neurite outgrowth in vitro (A) Representative images of P3 cortical neurons overexpressing Stat3 variants at 2DIV (blue, nuclear stain; green, beta-III tubulin; red, flag-tag). (B–D) Neurite outgrowth analyses from automated imaging and tracing (Cellomics ArrayScan VTI) show that overexpression of VP16-Stat3CA promotes neurite outgrowth by increasing total neurite length (B) and the length of the longest neurite (C) while having no impact on branching (D); data plotted shows the results from individual biological replicates and bars indicate mean ± SEM; N = 5. (E) The number of surviving cells in each condition is not different at 2DIV. **p < 0.05, ***p < 0.001 (one-way ANOVA with Dunnett's post-test).

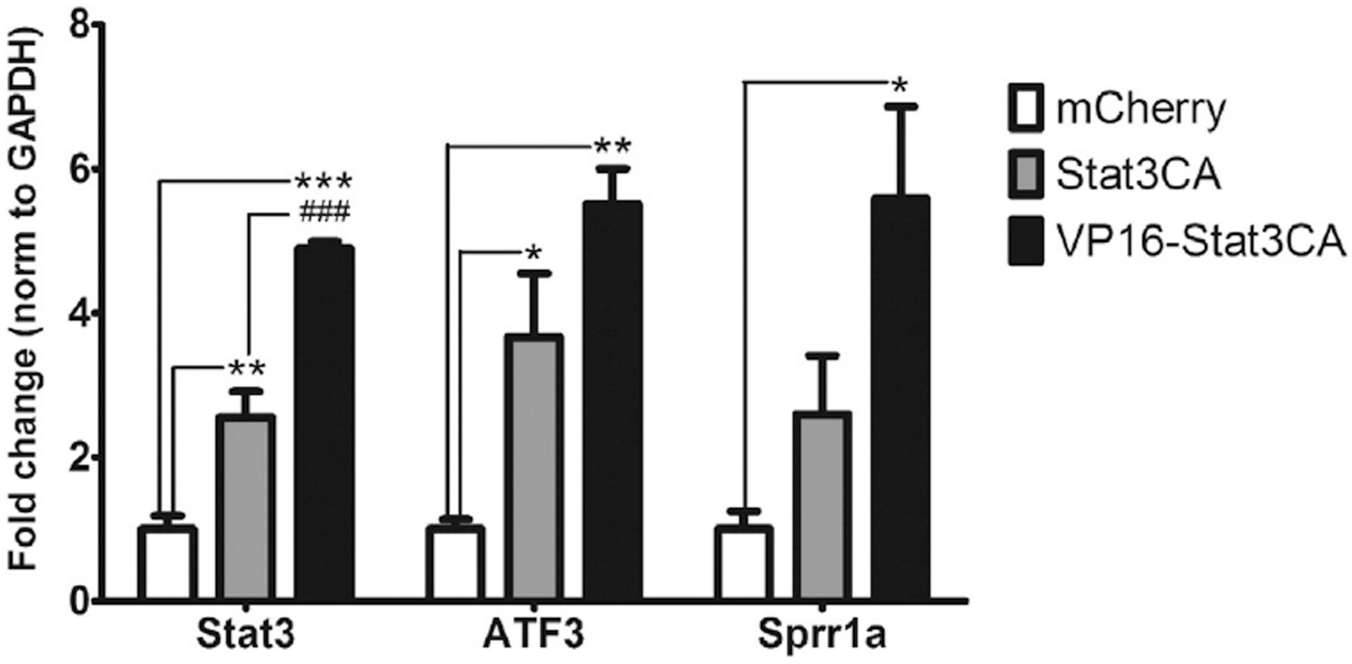

Fig. 2.

VP16-Stat3CA overexpression increases mRNA levels of Stat3 responsive genes. qPCR analysis showing fold change relative to mCherry of mRNA levels for Stat3, ATF3, and Sprr1a. Significance was quantified using one-way ANOVA with Bonferroni posttest. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control, ###$p < 0.001$ compared to Stat3CA; $N = 3$.

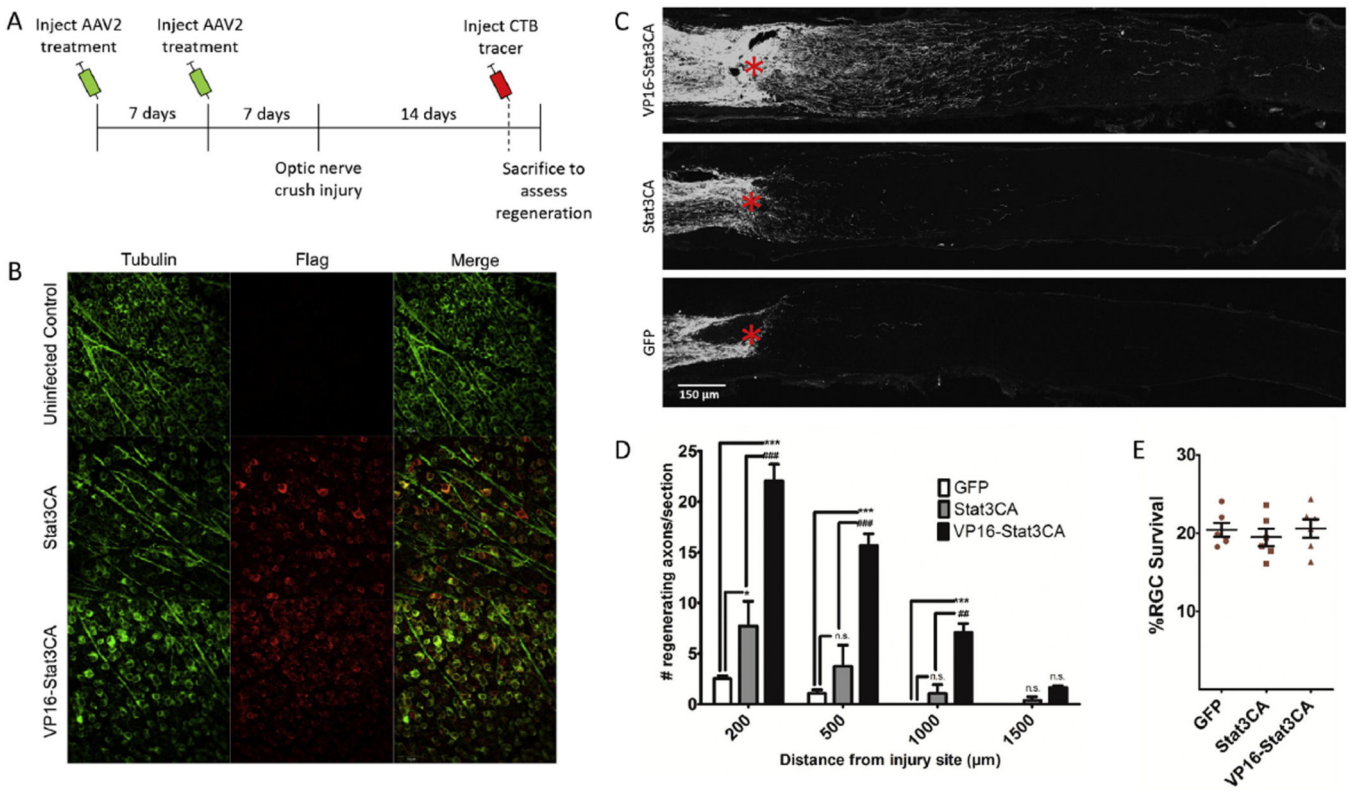

Fig. 3.

Hyperactivated Stat3 promotes axon regeneration in the optic nerve (A) Timeline followed for optic nerve experiments. (B) Overexpression of AAV2-Stat3CA and AAV2-VP16-Stat3CA in RGCs 2 weeks following first viral injection; Stat3 variants (red, flag tag); RGCs and their axons are stained for neuronal-specific tubulin (green). (C) Representative images of CTB labeled axons in the optic nerve 2WPI. * indicates site of injury. (D) Quantification of regenerating axons indicates that overexpression of VP16-Stat3CA significantly promotes axon regeneration up to 1 mm past the site of injury at 2WPI. ***$p < 0.01$ compared to GFP control; ###$p < 0.01$ compared to Stat3CA using two-way ANOVA with Bonferroni posttest; N = 6. (E) Quantification of RGC survival at 2WPI; number of RGCs does not differ between GFP controls and either Stat3 variant (one-way ANOVA with Dunnett's post-test).