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The histone acetyltransferase p300 promotes intrinsic axonal regeneration

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Axonal regeneration and related functional recovery following axonal injury in the adult central nervous system are extremely limited, due to a lack of neuronal intrinsic competence and the presence of extrinsic inhibitory signals. As opposed to what occurs during nervous system development, a weak proregenerative gene expression programme contributes to the limited intrinsic capacity of adult injured central nervous system axons to regenerate. Here we show, in an optic nerve crush model of axonal injury, that adenoviral (cytomegalovirus promoter) overexpression of the acetyltransferase p300, which is regulated during retinal ganglion cell maturation and repressed in the adult, can promote axonal regeneration of the optic nerve beyond 0.5 mm. p300 acetylates histone H3 and the proregenerative transcription factors p53 and CCAAT-enhancer binding proteins in retinal ganglia cells. In addition, it directly occupies and acetylates the promoters of the growth-associated protein-43, coronin 1b and Sprr1a and drives the gene expression programme of several regeneration-associated genes. On the contrary, overall increase in cellular acetylation using the histone deacetylase inhibitor trichostatin A, enhances retinal ganglion cell survival but not axonal regeneration after optic nerve crush. Therefore, p300 targets both the epigenome and transcription to unlock a post-injury silent gene expression programme that would support axonal regeneration.

Keywords: p300; histone acetyltransferase; optic nerve; axonal regeneration; transcription

Abbreviations: AVGFP = adenovirus green fluorescent protein; CBP = cyclic adenosine monophosphate responsive element binding protein; C/EBP = CCAAT-enhancer binding protein; GFP = green fluorescent protein; P/CAF = p300/CBP-associated factor

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Introduction

Mature neurons of the adult CNS lack axonal regeneration capacity following axonal injury. The reason for such a regenerative failure is 2-fold: (i) the presence of a non-permissive glial environment (Yiu and He, 2003, 2006); and (ii) an intrinsic lack of proregenerative ability (Lee *et al.*, 2010). This is in contrast to the potential for axonal regeneration and outgrowth present in the injured PNS (Huebner and Strittmatter, 2009) and in immature neurons during development (Cai *et al.*, 2001; Filbin, 2006).

The intrinsic properties of neurons are regulated by gene transcription, which regulates gene expression, and therefore tightly controls the neuronal intrinsic capacity to synthesize new proteins needed for pro-axonal regeneration signalling. Indeed, transcriptional regulation controls axonal outgrowth during development (Butler and Tear, 2007) as well as axon regrowth after injury in the adult (Goldberg *et al.*, 2002; Raivich *et al.*, 2004; Moore *et al.*, 2009). Intrinsic signals receive numerous inputs from extrinsic ones and are used here to describe those signals whose modulation is sufficient to promote axonal outgrowth without additional inhibition of the inhibitory environment.

Mature retinal ganglion cells fail to regenerate axons and undergo apoptosis following optic nerve damage; however, experimental evidence has shown that enhancement of the intrinsic properties of retinal ganglion cells can promote axonal regeneration of the injured optic nerve. Examples include the lens injury-dependent activation of a proregenerative state characterized by gene expression comparable with that seen after peripheral nerve injury (Leon *et al.*, 2000; Fischer *et al.*, 2001, 2004). In fact, a lens injury previous to the optic nerve crush induces, likely via inflammatory molecules (Yin *et al.*, 2006, 2009), the expression of progrowth genes such as Sprr1a and Narp as well as transcription factors such as cyclic adenosine monophosphate responsive element binding protein (CBP) and CCAAT-enhancer binding proteins (C/EBP). In addition to lens injury, the combined administration of several growth factors (Logan *et al.*, 2006), as well as the lens injury induced ciliary neurotrophic factor are other well-established means to enhance intrinsic axonal regeneration of the injured optic nerve (Lingor *et al.*, 2008; Leibinger *et al.*, 2009; Muller *et al.*, 2009).

Recent studies have demonstrated that the modulation of individual intrinsic molecules such as PTEN (phosphatase and tensin homologue) or the transcription factors KLF4 (Krupper-like factor 4) can promote axonal regeneration of retinal ganglion cells after optic nerve crush (Park *et al.*, 2008; Moore *et al.*, 2009). Both PTEN and KLF4 show repressive effects on neurite outgrowth, while their suppression in retinal ganglion cells strongly enhances axonal regeneration ultimately activating a proregenerative gene expression response. These lines of evidence suggest that as opposed to what occurs during development and in immature neurons, the gene expression programme in mature retinal ganglion cells does not allow mounting an axonal regenerative response unless modified by experimental manipulations.

We hypothesize that the proregenerative transcriptional machinery is silenced or repressed in adult CNS neurons after neuronal maturation and following axonal damage; however, it could be reactivated by modulating genes that regulate the proregenerative gene expression programme. Gene expression is controlled by the state of chromatin as well as by the presence of specific transcriptional complexes near gene promoters. The balance between the histone acetyltransferases and histone deacetylases regulates the level of histone and transcription factor acetylation, which modifies the state of chromatin and the activity of transcription factors, and overall contributes to the fine-tuning of gene expression (Yang and Seto, 2007). We have recently reported that chromatin relaxation and transcription factor activation via histone deacetylases inhibition by trichostatin A enhances neurite outgrowth on permissive and non-permissive substrates. Specifically, this was due to an increased expression of the histone acetyltransferases CBP/p300 and p300/CBP-associated factor (P/CAF) that enhanced acetylation of H3 and p53, which stimulated the expression of several proregenerative genes (Gaub *et al.*, 2010). However, this work was performed *in vitro* and the role of histone acetyltransferases in axonal regeneration *in vivo* is yet to be investigated.

In the present study, we investigated the regulation of expression of the specific histone acetyltransferases p300, CBP and P/CAF during retinal ganglion cell maturation and whether they could thus become potential candidates to control the ability of retinal ganglion cells to regenerate axons following optic nerve crush. Indeed, we found that histone acetylation and the expression of CBP and p300 are repressed in mature retinal ganglion cells and after optic nerve crush. Importantly, overexpression of p300 but not histone deacetylases inhibition, promotes axonal regeneration after optic nerve crush. P300 leads to hyperacetylation of histone H3 and the transcription factors p53 and C/EBP, as well as to increased p300 occupancy and H3 acetylation of selected pro-axonal outgrowth gene promoters.

This is a first report showing that a specific modification of the transcriptional and epigenetic environments can promote axonal regeneration *in vivo*, likely by redirecting the transcriptional programme on proregeneration promoters.

Materials and methods

Viral construction, production and infection

AVp300 vector was created by using the AdEasy^{TM} system (Luo *et al.*, 2007). p300 complementary DNA was purchased from Addgene (plasmid 10718) and subcloned into the pAdTrack-cytomegalovirus (CMV) shuttle vector (Addgene plasmid 16405). Preparation of adenovirus green fluorescent protein (AVGFP) has been described previously (Naumann *et al.*, 2001). The plasmid containing p300 and pAdTrack-CMV was then linearized and recombined with the viral backbone pAdEasy-1. All viruses were expanded in 293 cells (ATCC) and tested to be replication-deficient by polymerase chain reaction (primer: E1Afrwd GTTGGCGGTGCAGGAAGGGATTG and E1Arev CTCGGGCTCAGGCTCAGGTTCAGA) and by immunoblot of the E1A gene product (mouse-anti E1A, 1 : 10 000, BD Biosciences 554155). Viral titres were assessed using a hexon titre kit (Clontech). The efficacy of adenoviral gene delivery and expression was ascertained by green fluorescent protein (GFP) fluorescence or by polymerase chain

reaction for p300 messenger RNA expression. Infection with recombinant viruses *in vitro* was accomplished by exposing cells *in vitro* to 100 multiplicity of infection (MOI) of adenovirus immediately after plating. *In vivo*, AVGFP as a control and AVp300 were injected intravitreally immediately after optic nerve crush.

Retinal ganglion cell survival assay

Assessment of retinal ganglion cell survival was performed on flat-mounted retinae. Eyes were dissected and the retinae removed from the eye cup. The retinae were then washed in phosphate-buffered saline and blocked in a solution of 10% bovine serum albumin and 1% Triton X-100. After the blocking solution, the whole retina was incubated in the same solution with mouse anti-β-III tubulin (1 : 400) (Promega) overnight at 4°C. Retinae were then washed with phosphate-buffered saline, incubated with the secondary antibody anti-mouse Alexa 568 (1 : 1000, Pierce) and flat mounted on slides with Fluorsave™ (Calbiochem).

Quantification was performed by taking pictures in the central, intermediate and peripheral region for each quarter of flat mounted retina under fluorescent illumination ($n = 3$) as previously reported (Park *et al.*, 2008; Kurimoto *et al.*, 2010). β-III tubulin-positive cells were then counted on each picture using the Neurolucida software and normalized as a percentage to sham retinae ($n = 3$). Similarly, additional counting was performed by evaluating the number of GFP-positive infected cells (with a control AVGFP or AVp300) co-expressing β-III tubulin only. GFP/β-III tubulin-positive cells were then counted on each picture using the Neurolucida software and normalized as a percentage to sham retinae ($n = 3$).

Retinal cell culture

Primary culture of retinal cells was performed following a previously described protocol (Hauk *et al.*, 2010). Briefly, P6–P7 eyes were dissected, and retinae were incubated in Dulbecco's modified Eagle's medium with Papain (Cellsystem) and L-cystein (Sigma). After incubation, retinae were dissociated in Dulbecco's modified Eagle's medium with B27 (Life Technologies) and penicillin/streptomycin (Sigma) and $\sim 1 \times 10^6$ cells per 2 cm^2 were plated. Immediate infection by AV-GFP and AV-p300 was carried out using 100 MOI. Cells were then fixed with 4% paraformaldehyde for 30 min. Cells were washed with phosphate-buffered saline, then blocked with 8% bovine serum albumin, 0.2% TritonX-100 in phosphate-buffered saline and finally incubated with the primary antibodies overnight at 4°C: mouse anti-β-III tubulin (1 : 1000) (Promega). Cells were then washed with phosphate-buffered saline and incubated with an anti-mouse Alexa 564-coupled secondary antibody (1 : 1000) (Pierce) for 1 h at room temperature. As a control, we stained with Hoechst 33258 (Molecular Probes) and then washed in phosphate-buffered saline before mounting the coverslips on a slide with Fluorsave™ (Calbiochem).

Optic nerve crush surgery and intraocular injection

All animal experiments were conducted according to the European Union and German regulations under the allowance of the animal protocol number N03/07 and AK7/07 (University of Tübingen). Surgical procedures were based on those described previously (Berry *et al.*, 1996; Fischer *et al.*, 2000; Leon *et al.*, 2000). Adult (2–3 months old) Crl-CD1 rats (400–500 g) were anaesthetized intraperitoneally with 80 mg/kg of ketamine and 50 mg/kg of xylazine. After shaving the head, rats were immobilized in an apparatus and a 1.5- to 2-cm incision was made in the skin in the middle of the head. Under microscopic illumination, a longitudinal section above the right orbit was made to access the orbital space below the bones. The lachrymal glands and extraocular muscles were resected and retracted to expose 3–4 mm of the optic nerve. The epineurium was slit open along the longitudinal axis and the nerve was crushed 2 mm behind the eye with angled jeweller's forceps (Dumont #5, FST) for 10 s, avoiding injury to the ophthalmic artery. Nerve injury was verified by the appearance of a clearing at the crush site, while the vascular integrity of the retina was evaluated by funduscopic examination. Cases in which the vascular integrity of the retina was in question were excluded from the study. For intraocular injections, the eye was rotated to expose its posterior aspect. Injections were made through the sclera and retina with a 30 gauge needle 1–2 mm superior to the optic nerve head, inserting the tip of the needle perpendicular to the axis of the nerve to a depth of 2 mm without infringing on the lens (minimally invasive injection). Injection volumes were dependent upon the solution. In a subset of rats, we performed lens injury as described previously (Schnichels *et al.*, 2011). Survival times ranged from 1–3 days and 14 days after the surgery. Groups included sham controls ($n = 3$), animals with optic nerve crush ($n = 3$), animals with optic nerve crush and phosphate-buffered saline ($n = 4$) or trichostatin A (T-8552, Sigma) (10 ng/ml; $n = 4$); animals with optic nerve crush and AVGFP (7.5×10^7 pfu; $n = 5$) or AVp300 (7.5×10^7 pfu; $n = 5$). Animals showing signs of lens injury or intravitreal haemorrhage after puncture were excluded from the study. The surgical site was sutured and closed. Animals were observed for postoperative recovery and were housed with *ad libitum* access to food and water.

Evaluation of axonal regeneration

For evaluation of optic nerve axon regeneration following optic nerve crush, GAP-43 immunofluorescence was performed. Photomicrographs were taken with a fluorescence microscope using the Zeiss Axioplan microscope (Axiovert 200, Zeiss Inc.). Images of whole sections were assembled from single pictures taken with a ×20 objective. The number of regenerating axons at designated distances from the end of the crush sites was evaluated per section as previously reported (Planchamp *et al.*, 2008). The number of regenerating axons per nerve was then averaged over all sections of one nerve. The following experimental conditions after optic nerve crush were analysed ($n = 4$): AVGFP; AVp300; AV GFP + lens injury; and AVp300 + lens injury.

Tissue extraction

Postnatal CD rats at Days P0, P7 and P21, and adult rats were deeply anaesthetized using 100 mg/kg of ketamine and 80 mg/kg of xylazine and transcardially perfused with 100 ml of ice-cold phosphate-buffered saline followed by 50 ml of ice-cold 4% paraformaldehyde. The eyes were enucleated with the optic nerve and post-fixed overnight in 4% paraformaldehyde followed by cryoprotection using 30% sucrose in water. The eyes were later stored at −80°C. Three different retinae were sacrificed at each time point.

Immunohistochemistry

Eyes and optic nerves were embedded in freezing medium and longitudinal serial sections (10 μm) were cut and mounted on glass slides. The sections were washed once with phosphate-buffered saline and incubated in 4% sucrose for 30 min followed by ice-cold 100%

methanol treatment for 15 min. For antigen retrieval, we used citrate buffer [2.1 g citric acid (monohydrous); 0.74 g EDTA; 0.5 ml Tween-20; in 1000 ml distilled water; pH 6.2] after the sucrose treatment and the slides were heated at 98°C. The sections were then washed with phosphate-buffered saline and blocked with 8% bovine serum albumin, 0.2% TritonX-100 in phosphate-buffered saline and then incubated in 2% bovine serum albumin, phosphate-buffered saline with the primary antibodies overnight at 4°C: rabbit anti-acetyl H3K18 (1 : 1000, Millipore); mouse anti-CBP (AC238, Abcam) (citrate buffer treatment, 1 : 50); mouse anti-p300 (3G230, Abcam), (citrate buffer treatment, 1 : 200); mouse anti-β-III tubulin (1 : 1000) (Promega); rabbit anti-acetyl-p53 lys373 (1 : 200, citrate buffer treatment) (06-916, Millipore); rabbit anti-p53 (1 : 200) (sc-6243, Santa-Cruz); rabbit anti-C/EBP acetylated 215-216 (1 : 200) (09-037, Millipore); rabbit anti-GAP-43 (1 : 500) (Chemicon, Schwalbach, Germany). Sections were then washed with phosphate-buffered saline and incubated with the respective secondary antibodies for 1 h at room temperature: Alexa 488, 546 or 564-coupled secondary antibodies (goat anti-rabbit IgG, goat anti-mouse IgG, Pierce). As a control, we stained with Hoechst 33258 (Molecular Probes) and then washed in phosphate-buffered saline before mounting on slides with Fluorsave™ (Calbiochem). For all experiments, a negative control was performed by immunostaining with the secondary antibody only.

Controls for anti-CBP and p300 antibody specificity were carried out previously by immunostaining after CBP and p300 gene silencing in both cell lines and primary neurons (Gaub *et al*., 2010), which showed reduced signal intensity in agreement with gene silencing. Specificity for anti-p53 antibodies has been tested previously by both immunoblotting and immunocytochemistry after overexpression of p53 in both cell lines and primary neurons (Di Giovanni *et al*., 2006; Tedeschi *et al*., 2009; Gaub *et al*., 2010). Specificity for antibodies anti-H3Ac has been supported by immunoblotting. In addition, the immunofluorescence signal has always been found specifically in the nucleus and to change as expected whenever we modified acetylation levels with either trichostatin A (T-8552, Sigma) or overexpression of CBP or p300 (Gaub *et al*., 2010).

Assessment of fluorescence intensity

A high-resolution image was obtained at ×40 magnification using the Zeiss Axioplan microscope (Axiovert 200, Zeiss Inc.). Images for the same antigen groups were processed with the same exposure time. Assessment of fluorescence intensity was performed using AlphaEaseFC 4.0.1 software by measuring the intensities specifically within the retinal ganglion cell layer. Care was taken that the area analysed for each cell was the same for each set, 20 cells per section and two sections per retina were quantified.

The intensity values of each cell were normalized to the 4′,6′-diamidino-2-phénylindole signal and mean values of intensities were calculated for each animal (three animals per condition). For statistical analysis, ANOVA with Bonferroni test was performed using Origene software. At least 100 cells were analysed in triplicates at each time point and *P*-values of $\leqslant 0.05$ (*) were considered significant.

Reverse transcriptase polymerase chain reaction and quantitative reverse transcriptase polymerase chain reaction

After the eyes were enucleated from the animal under deep anaesthesia, unfixed retinae were dissected and RNA was extracted. RNA was extracted using TRIzol® reagent (Invitrogen) and complementary DNA was synthesized from 1 μg of RNA using oligo dT and random hexamers from the SuperScript™ II Reverse Transcriptase kit (Invitrogen). Complementary DNA (1 μl) was used in a reverse transcriptase polymerase chain reaction using Master Mix (Invitrogen) and for quantitative reverse transcriptase polymerase chain reaction, SYBR-greenER (Invitrogen) was used.

The *RPL13A* gene was used for normalization. The sequences of the primers used were p300 forward 5′-GGGACTAACCAATGGTGGTG–3′ and reverse 5′–ATTGGGAGAAGTCAAGCCTG–3′ (386 bp), GAP-43 forward 5′-AAGCTACCACTGATAACTCGCC-3′ and reverse 5′-CTTCT TTACCCTCATCCTGTCG-3′ (246 bp); coronin 1 b forward 5′-GACCTG TGCCCACATAACGATCAGG5C-3′ and reverse 5′-CACGATGCCGACT CTCTTTGA-3′; α-tubulin 1 a forward 5′-GCTTCTTGGTTTTCCACA GC-3′ and reverse 5′-TGGAATTGTAGGGCTCAACC-3′ (162 bp); SCG10 forward 5′-CCACCATTGCCTAGTGACCT-3′ and reverse 5′-GAAGCACACACTCCACGAGA-3′ (202 bp); Chl1 forward 5′-CGC CTACACAGGAGCTAAGG-3′ and reverse 5′-TTCTTTTGGAAGGCAGT GCT-3′ (231 bp); L1cam forward 5′-CATCGCCTTTGTCAGTGCTA-3′ and reverse 5′-CTGTACTCGCCGAAGGTCTC-3′ (162 bp); Lgals1 forward 5′-GCTGGTGGAGCAGGTCTCAGGAATCT-3′ and reverse 5′-A AGGTGATGCACTCCTCTGTGATGCTC-3′ (314 bp); Sprr1A like forward 5′-CTGATCACCAGATGCTGAGG-3′ and reverse 5′-TCCTGAGC CATGGAAAGATT-3′ (202 bp); RPL13A forward 5′–CCCTCCACCCTAT GACAAGA-3′ and reverse 5′–CCTTTTCCTTCCGTTTCTCC-3′ (167 bp). All primers were initially tested for their specificity by running reverse transcriptase polymerase chain reaction samples on an agarose gel. Only primers that under specific polymerase chain reaction conditions gave a single band of the appropriate molecular weight were then used for real-time polymerase chain reaction experiments. For quantitative reverse transcriptase polymerase chain reaction, fold changes were calculated following manufacture instructions (Invitrogen) and normalized to the levels of a housekeeping gene (*RPL13A*).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation assays were performed according to the manufacturer's recommendations (Upstate). Briefly, three retinae per conditions (AVGFP versus AVp300 at 24 h) were dissected and subsequently fixed in a 1% formaldehyde solution for 10 min at 37°C. Following cell lysis (0.5% sodium dodecyl sulphate, 100 mM NaCl, 50 mM Tris–HCl, pH 8.0, 5 mM EDTA), extracts were sonicated to shear DNA to lengths of 200–600 bp.

Chromatin solutions were incubated overnight with rotation using 4 μg of rabbit polyclonal anti-acetyl histone H3 K9-14 antibody (Upstate) and mouse anti-p300 antibody (Abcam). The following day protein A agarose beads, which had been blocked with salmon sperm DNA, were added to each reaction to precipitate antibody complexes. The precipitated complexes were washed and then incubated for 4 h at 65°C in parallel with input samples to reverse the cross-link. DNA was isolated by phenol chloroform iso-amyl alcohol extraction, which was followed by ethanol precipitation in the presence of sodium acetate.

'Input', 'IP' and 'Mock' fractions were then analysed by quantitative polymerase chain reaction (ABI 7000) analysis with appropriate primer pairs. The primers used were as follows: coronin 1 b 5′ site <1 kb forward 5′-CTCCCAGCGTTATCATGTCA-3′ and reverse 5′-GGGAGA CTCGAATGTCCTCA-3′; GAP-43 5′ site <1 kb forward 5′-GCAGCTG TAACTTGTGTGCA-3′ and reverse 5′-GGTCCAGATTGGAGGTG TTTA-3′; Sprr1al 5′ site <200 bp forward 5′-ACCCTCTCACAAC ACAAGCA-3′ and reverse 5′- GAAACACACTTGCCCCAGAT-3′. For real-time quantitation of polymerase chain reaction products and fold-change measurements after chromatin immunoprecipitation,

each experimental sample was normalized to 'input' and 'Mock' fractions in triplicate from three independent samples, following the manufacturer instructions (Upstate).

Results

The expression of the acetyltransferase p300 is regulated during retinal ganglion cell maturation, and is repressed following optic nerve crush

Active gene expression is essential for axonal growth during development (Condron, 2002). On the contrary, an active proregenerative gene expression programme is deficient after nerve injury in the adult CNS, contributing to the lack of axonal regeneration (Cai *et al.*, 2001). First, we analysed the expression profile of selected epigenetic markers for active gene expression including H3 lysine K18 acetylation (H3AcK18), p300, CBP and P/CAF during retinal ganglion cell maturation, as these three histone acetyltransferases are responsible for H3K18 acetylation. Importantly, in these initial experiments, although retinal ganglion cells are organized in a clearly distinguishable layer of the retina, the identity of retinal ganglion cells was confirmed by β-III tubulin immunostaining (Supplementary Fig. 1). To tag retinal ganglion cell maturation, we used sequential maturation steps of retinal ganglion cells leading to full myelination of the optic nerve (Tennekoon *et al.*, 1977). Within the retina, the retinal ganglion cell layer was stained by immunohistochemistry for H3AcK18, p300, CBP and P/CAF before (P0), during (P7 and P21) and after (adult) full myelination of the optic nerve (Fig. 1A). Assessment of fluorescence intensity showed an increase of H3AcK18 at P7 and P21 followed by a decrease in the adult stage (Fig. 1B). All fluorescence signal measurements for the protein of interest were normalized to the nuclear 4′,6′-diamidino-2-phénylindole signal (data not shown). The expression pattern observed for H3AcK18 correlates with the expression of p300, which increases during retinal ganglion cell maturation to decrease in the adult (Fig. 1A and B). Conversely, CBP expression was stable throughout the maturation of retinal ganglion cells, while P/CAF appeared at very low and even expression levels along the time course (data not shown).

Hence, H3 K18 acetylation seems to be regulated similarly to the corresponding HAT p300 during retinal ganglion cell maturation and to decrease in adult cells.

We then investigated the expression of H3K18 acetylation and its acetyltransferases p300 and CBP by immunofluorescence at 24 and 72 h following optic nerve crush to investigate the post-injury regulation of this developmental epigenetic signature, potentially involved in axonal outgrowth. We chose a time window between 24 and 72 h for this experiment as optic nerve crush induces the expression of early genes as early as at 24 h after injury (Robinson, 1994; Bormann *et al.*, 1998), although the pro-regenerative programme is not spontaneously triggered. In addition, proregenerative gene expression is activated at ~72 h in case of axonal regeneration after optic nerve crush mediated by lens injury (Fischer *et al.*, 2004).

By immunofluorescence, we did not observe any change in H3K18 acetylation level in the retinal ganglion cell layer after optic nerve crush compared with sham neither at 24 nor at 72 h (Fig. 1C and D). However, p300 and CBP expression decreased significantly at 72 h after optic nerve crush (Fig. 1C and D). Importantly, we also observed decreased acetylation of the transcription factor p53 at lysine 373 (p53 K373) (Fig. 1C and D), which is acetylated specifically by CBP/p300 at K373, and together with CBP/p300 can regulate neurite outgrowth in cultured neurons (Tedeschi *et al.*, 2009; Gaub *et al.*, 2010). Significantly, p53 basal level was not modified after optic nerve crush at neither 24 nor 72 h compared with sham (Fig. 1C and D).

Double immunofluorescence experiments with antibodies anti-β-III tubulin/p300, anti-β-III tubulin/CBP or anti-β-III tubulin/H3AcK18 confirmed that the expression observed in the granular cell layer is indeed localized almost exclusively in retinal ganglion cells (Supplementary Fig. 2). In brief, optic nerve crush does not modify the chromatin environment through histone H3 acetylation, which remains at similar lower levels in the adult as compared with retinal ganglion cells during maturation even after injury. However, optic nerve crush further downregulates the enzymes responsible for lysine acetylation such as CBP and p300, likely leading to deacetylation of p53 at K373.

The histone deacetylases inhibitor trichostatin A enhances CBP expression, induces retinal ganglion cell survival, but not axonal regeneration

We have previously demonstrated that the histone deacetylases I/II inhibitor trichostatin A induces CBP and p300 expression as well as p53 acetylation leading to an increase of p53 binding on specific progrowth gene promoters, thereby inducing neurite outgrowth in cultured neurons on permissive and non-permissive substrates (Gaub *et al.*, 2010). In order to explore whether the administration of trichostatin A would enhance axonal regeneration after optic nerve crush via similar mechanisms, we injected either trichostatin A (1, 10 or 100 ng/ml) or vehicle into the vitreous at the time of injury. Optic nerves as well as retinae were subsequently analysed 14 days post-optic nerve crush. Trichostatin A injection resulted in a significant increase of retinal ganglion cell survival compared with vehicle 14 days post-injury based upon the number of β-III tubulin-positive cells (Fig. 2A and B). Then we performed immunohistochemistry for GAP-43 on optic nerve sections to quantify axonal regeneration between trichostatin A versus vehicle-treated animals. Trichostatin A-treated rats showed a very limited non-significant increase of labelled axons past the lesion site independently of the dose delivered, while control animals receiving vehicle showed as expected no axonal regeneration past the lesion site (Fig. 2C). As opposed to what we observed previously in cultured cerebellar granule cells (Gaub *et al.*, 2010), trichostatin A did not induce p300 expression and p53 K373-associated acetylation in the retinal ganglion cell layer following optic nerve crush (Fig. 3A and B). Importantly, however,

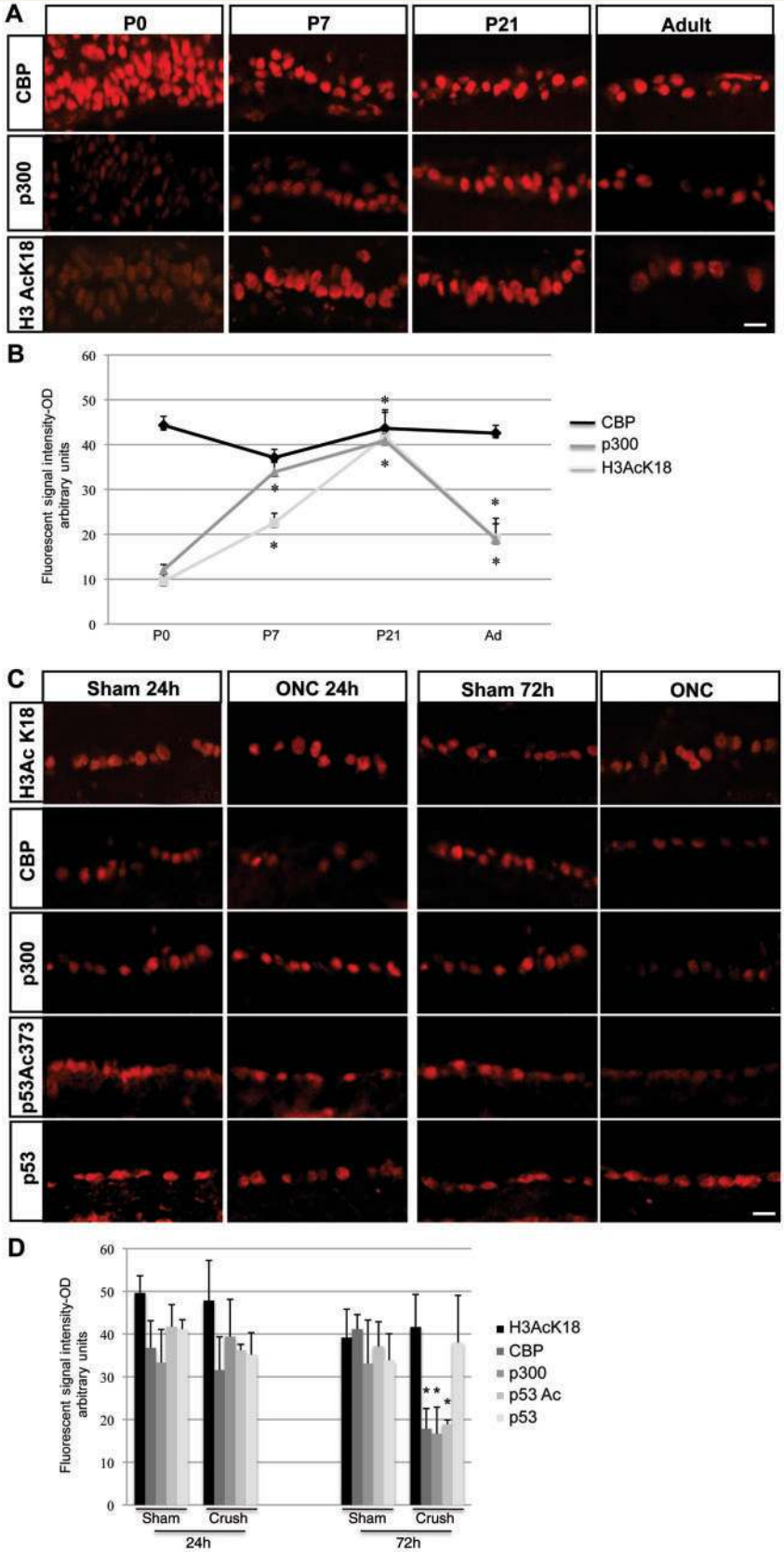

Figure 1 Maturation and optic nerve crush are associated with a decrease of histone acetyltransferase p300 in the retinal ganglion cell layer. (**A**) Representative pictures of the retinal ganglion cell layer at different time points during retinal ganglion cell maturation (P0, P7, P21 and adult) immunostained against CBP, p300 and H3AcK18. Scale bar = 20 μm. (**B**) The level of protein expression was quantified by analysis of fluorescence intensity and represented on the graph. The graphs show an increase of H3AcK18 and p300 between P0 and P21

(continued)

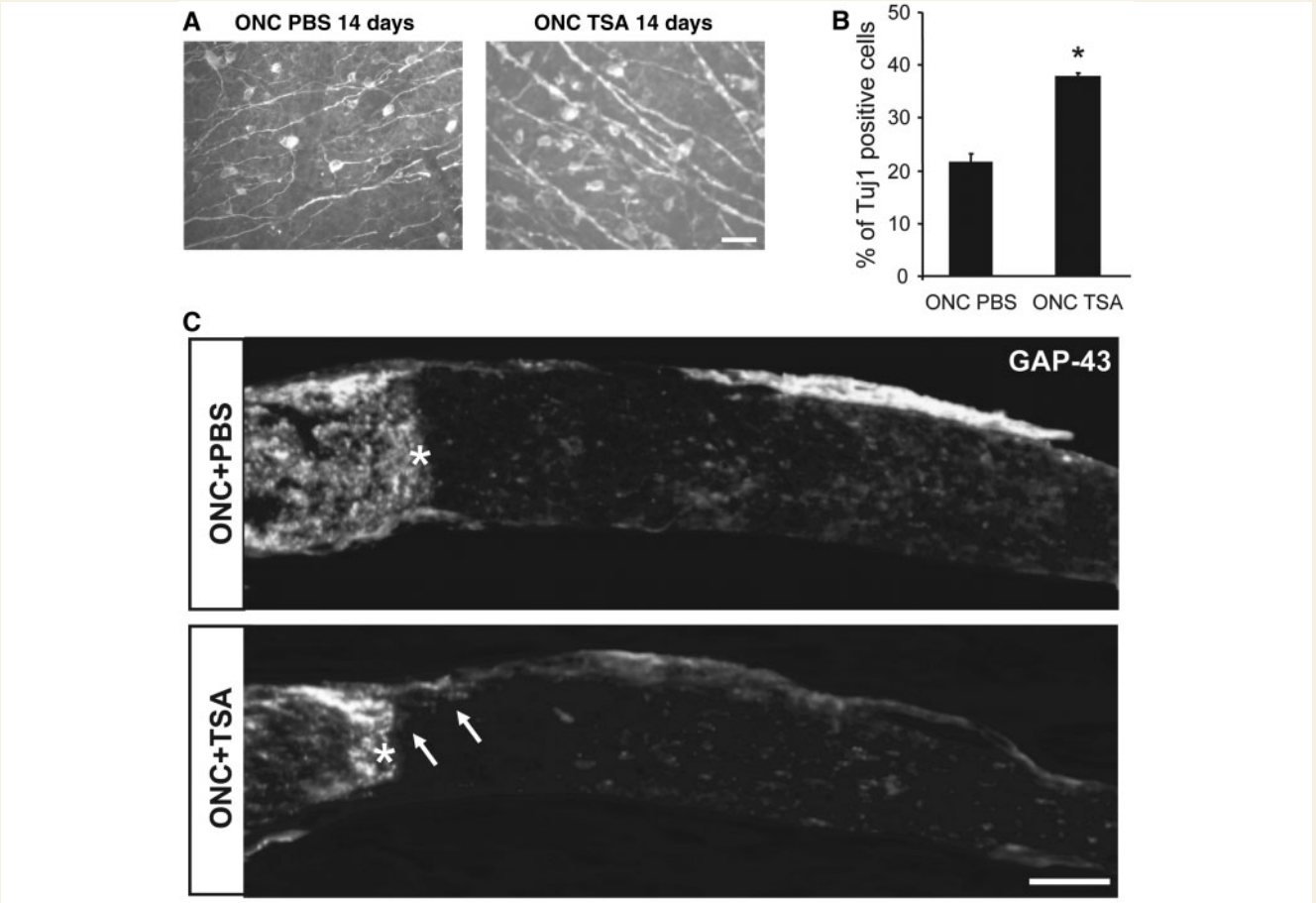

Figure 2 Histone deacetylases inhibition induces survival of retinal ganglion cells but not a significant enhancement of axonal regeneration. (**A**) Representative pictures of whole mount retina immunostained against β-III tubulin showing an increase of retinal ganglion cell survival 14 days after optic nerve crush (ONC) and injection of trichostatin A (TSA) 10 ng/ml, compared with optic nerve crush with phosphate-buffered saline (PBS). Scale bar = 50 μm. (**B**) The bar graph shows quantification of retinal ganglion cells β-III tubulin (Tuj1)-positive cells after optic nerve crush with phosphate-buffered saline or trichostatin A injection compared with sham. Asterisk = unpaired two-tailed *t*-test, **P*-value < 0.05; $n = 3$. Error bars represent SD. (**C**) Optic nerve longitudinal sections were immunostained against GAP-43 14 days after optic nerve crush with phosphate-buffered saline or trichostatin A 10 ng/ml. Representative pictures show sporadic short axons past the lesion site after trichostatin A stimulation. Scale bar = 100 μm.

trichostatin A did increase H3 acetylation, which is considered a read-out of the activity of histone deacetylases I/II inhibitors as well as of CBP (Fig. 3C and D). Hence, trichostatin A promotes the survival of retinal ganglion cells concomitantly with induction of histone acetylation and CBP expression. However, it is not able to stimulate axonal regeneration at any of the doses employed and does not promote the expression of p300 and of p53 acetylation, previously shown to enhance neurite outgrowth in cerebellar neurons cultured on inhibitory substrates (Gaub *et al.*, 2010).

p300 induces axonal regeneration and modifies the epigenome on select proregeneration promoters

Since intravitreal trichostatin A administration fails to promote axonal regeneration and is able to neither increase p300 expression nor p300-related p53K373 acetylation after optic nerve crush, we decided to overexpress p300 in order to enhance axonal

Figure 1 Continued
and a decrease in adult, whereas CBP expression was not altered. P300 and H3 AcK18 level show a similar expression pattern during retinal ganglion cell maturation ($n = 3$). Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.01; $n = 3$. Each average value per time point was measured against the average value of all time points together. Error bars represent SD. (**C**) Immunohistochemistry of retinae shows immunostaining of retinal ganglion cell layer against H3 AcK18, CBP, p300, p53 Ac373 and p53, 24 h and 72 h after optic nerve crush (ONC) compared with sham. No change is observed for H3K18 acetylation at either 24 h or at 72 h after optic nerve crush compared with sham, whereas a decrease of p300 and CBP expression is shown along with a decrease of p53 Ac373, while p53 basal level was stable. Scale bar = 20 μm. (**D**) The graph represents quantification of the protein level obtained by measurement of the fluorescence signal. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.01; $n = 3$. Error bars represent SD. OD = optical density.

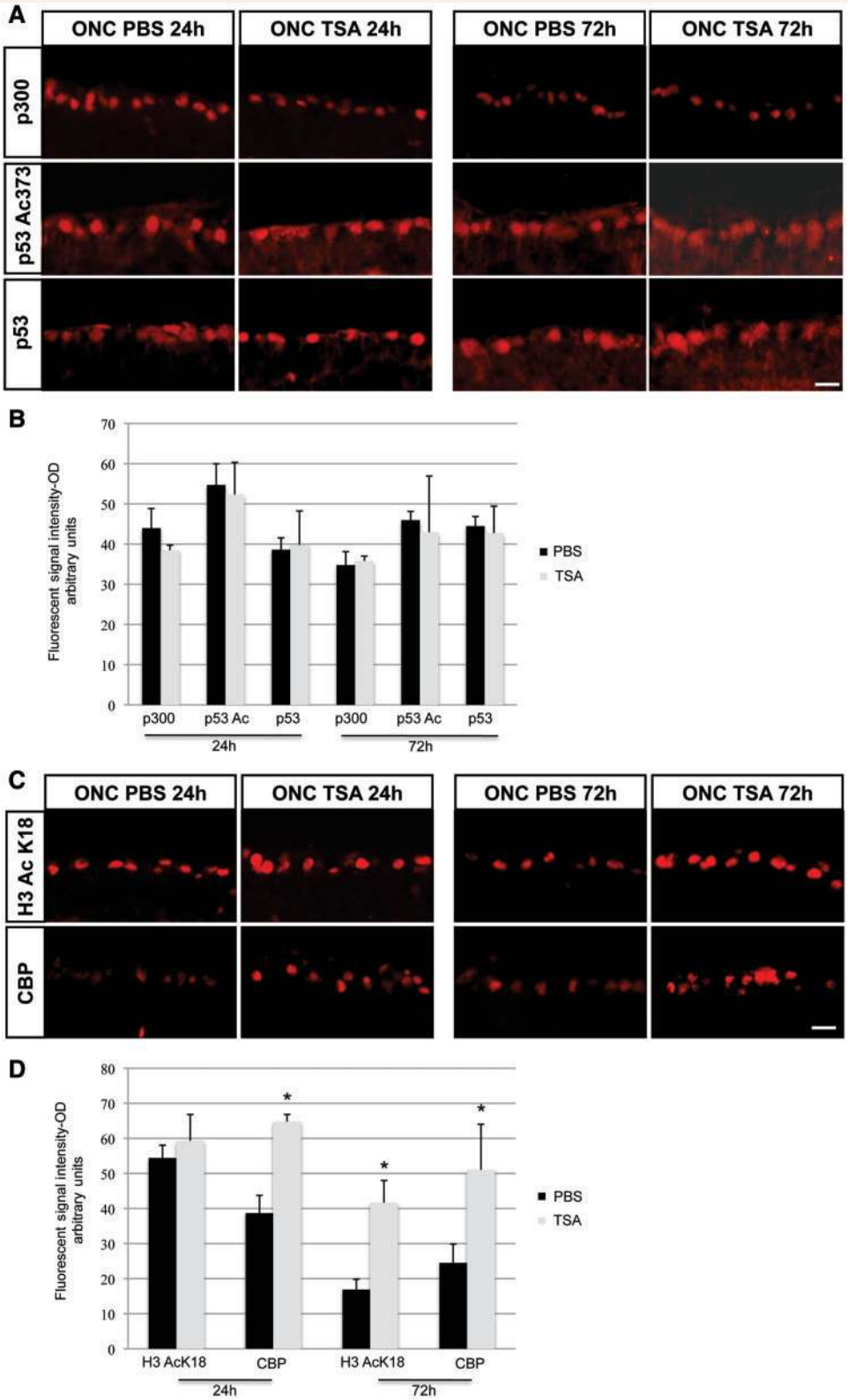

Figure 3 Histone deacetylases inhibition does not modify p300 expression or p53-dependent acetylation. (**A**) Retinae were immunostained against p300, p53Ac373 and p53 24 h and 72 h after optic nerve crush (ONC) with or without trichostatin A (TSA; 10 ng/ml). Shown are representative pictures of retinal ganglion cells showing no change for p300, p53 or p53Ac373 expression at 24 h or at 72 h after trichostatin A, compared with phosphate-buffered saline (PBS)-injected animals. Scale bar = 20 μm. (**B**) The bar graphs show quantification of p300, p53Ac373 and p53 protein level analysed by measurement of the fluorescence signal. (**C**) Immunostaining against H3AcK18 and CBP on retinal ganglion cells 24 h and 72 h after optic nerve crush with phosphate-buffered saline or trichostatin A represented in the pictures show a significant increase of H3AcK18 and CBP 72 h after trichostatin A injection compared with phosphate-buffered saline. Scale bar = 20 μm. (**D**) Quantification of expression levels of H3AcK18 and CBP are represented in the bar graphs. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.05. Error bars represent SD.

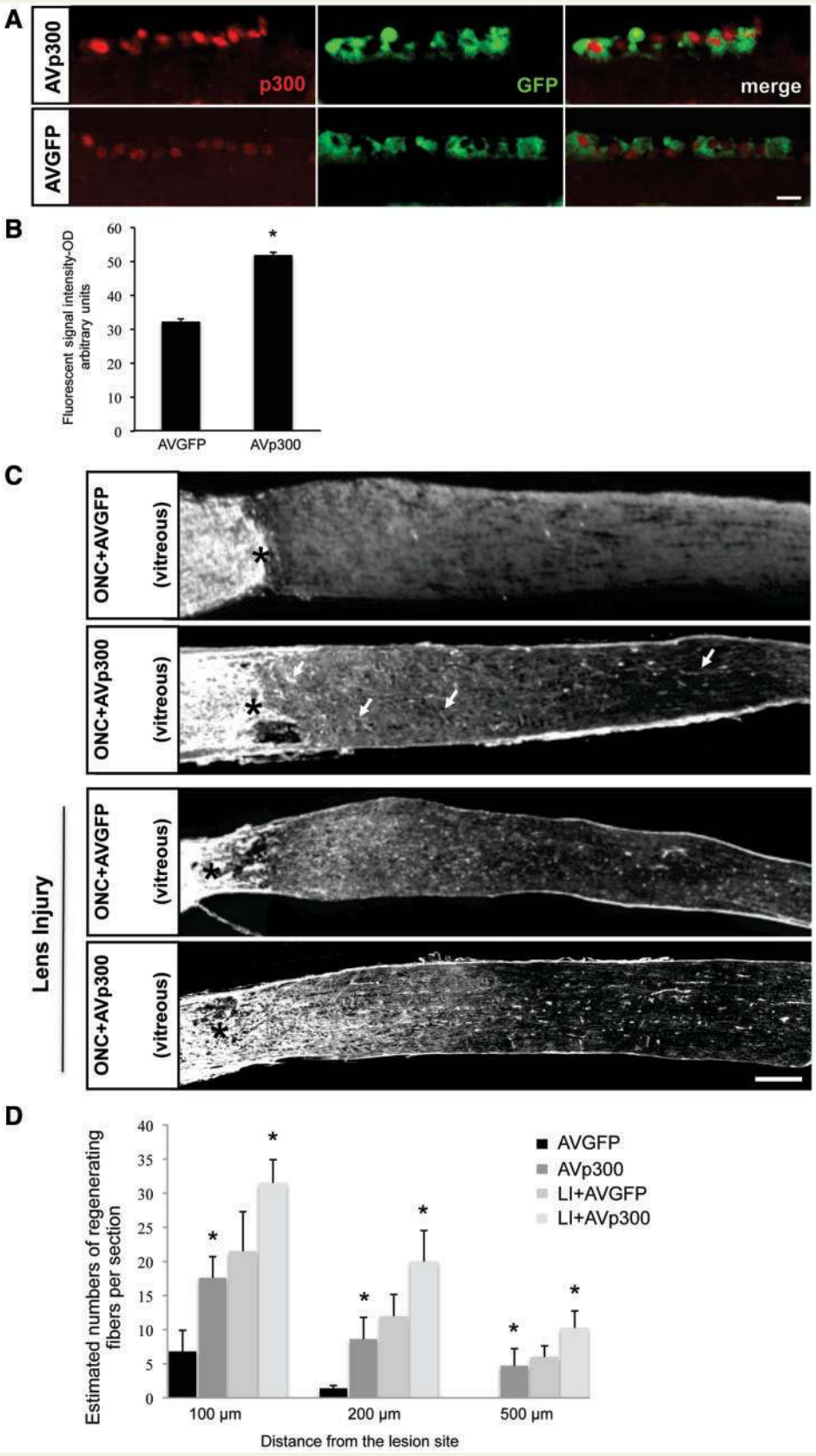

Figure 4 p300 over-expression by adenovirus infection induces axonal regeneration of the optic nerve. (**A**) Representative pictures of retinal ganglion cell layer after immunostaining in the retina against p300 shows expression of p300 in green fluorescence protein (GFP)-positive cells 24 h after optic nerve crush (ONC) and AVp300 or AVGFP infection. An increase of p300 expression in the retinal ganglion cell layer is shown following AVp300-GFP versus AVGFP infection. Scale bar = 20 µm. (**B**) Bar graph represents quantification of p300

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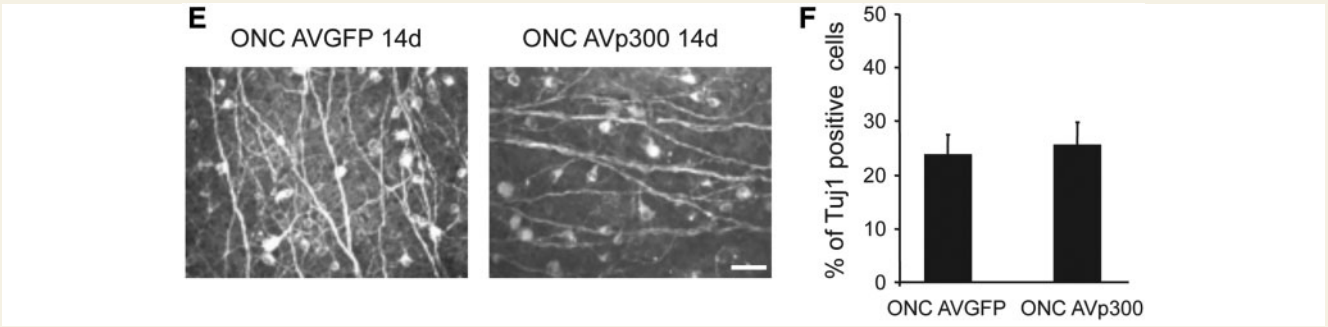

Figure 4 Continued.
protein levels analysed by measurement of the fluorescence signal. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.01; $n = 3$. Error bars represent SD. (**C**) Representative pictures of longitudinal optic nerve sections immunostained against GAP-43 14 days after optic nerve crush and infected with AVGFP or AVp300-GFP (alone or in combination with lens injury) show axonal regeneration in AVp300-infected rats, which is enhanced by lens injury. Scale bar = 100 μm. (**D**) Adenoviral overexpression of p300 alone or in combination with lens injury induces a significant increase in the number of axons past the lesion site compared with AVGFP-infected nerves alone or in combination with lens injury as shown in the bar graph ($n = 4$ per condition). Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.05. Error bars represent SD. (**E**) Representative pictures of whole flat retina immunostained against β-III tubulin (Tuj1) 14 days after optic nerve crush with AVGFP or AVp300 infection. Scale bar = 50 μm. (**F**) Bar graphs show quantification of retinal ganglion β-III tubulin-positive cells on whole flat retina ($n = 3$) that reveals no difference in retinal ganglion cell survival (as compared with sham) 14 days after optic nerve crush with AVGFP or AVp300. OD = optical density.

regeneration via both increased proregenerative transcription and histone acetylation on select target promoters. Due to the large size of p300 (8 kb), we decided to clone full-length p300 in a size-compatible adenoviral vector carrying two cytomegalovirus promoters driving either p300 or GFP for intravitreous *in vivo* infection experiments. AVGFP virus was employed as a control. AVp300/GFP (AVp300) or AVGFP were injected into the vitreous at the time of injury. Optic nerves were extracted 14 days post-injury and immunostained for GAP-43 to identify regenerating axons. Infection of p300 significantly increased p300 expression as early as at 24 h after infection (Fig. 4A and B) in the retinal ganglion cell layer. More importantly, it resulted in a significant increase in the number of regenerating axons compared with control GFP (Fig. 4C and D). Additionally, the combination of lens injury, a well-known strategy to enhance neuronal intrinsic-dependent axonal regeneration after optic nerve crush, and p300 overexpression led to further enhancement of axonal regeneration as compared with lens injury or p300 overexpression alone (Fig. 4C and D). However, we observed that AVp300 does not induce survival of retinal ganglion cells compared with AVGFP when counting the overall number of β-III tubulin-positive neurons (Fig. 4E and F), therefore the pool of regenerating axons stems from the limited pool of spontaneously surviving retinal ganglion cells. This was confirmed by evaluating the number of double β-III tubulin/GFP-positive cells in p300 and control virus-infected retinae, which showed no difference (Supplementary Fig. 3).

A percentage of retinal ganglion cells ($17.7 \pm 3.4\%$ SE of β-III tubulin-positive cells, $n = 3$) were successfully infected as shown by co-localization of GFP with β-III tubulin within the ganglion cell layer *in vivo* (Supplementary Fig. 4). A number of cells were also infected in the retina inner nuclear layer, corresponding presumably to bipolar/amacrine and Müller cells (Supplementary Fig. 4). In order to prove the cell autonomous effects of p300 overexpression specifically in neurons, we cultured primary retinal cells and infected them with either AVGFP or AVp300. Retinal ganglion cells were infected in culture as shown by expression of GFP in β-III tubulin-positive cells (Fig. 5A). More importantly, we found that overexpression of p300 induced a significant increase in neurite outgrowth as compared with control-infected neurons (Fig. 5B and C). All together, these data suggest that p300 overexpression can promote axonal regeneration but not survival of retinal ganglion cells following optic nerve crush and that these effects are at least in part mediated by neuronal intrinsic mechanisms.

Immunofluorescence experiments further showed that overexpression of p300 induced both pro-axonal regeneration transcription factor and histone H3 hyperacetylation in the retinal ganglion cell layer following optic nerve crush. At both 24 and 72 h post-optic nerve crush, we observed a significantly increased p53K373 acetylation in the retinal ganglion cell layer in AVp300 versus AVGFP infection, while total p53 levels remained unchanged (Fig. 6A and B). Similarly, we found that the acetylation of the pro-axonal regeneration transcription factor C/EBP, which can be acetylated on lysine 215 and 216 (Cesena *et al.*, 2007; Wang *et al.*, 2007), was enhanced at 24 and 72 h after optic nerve crush by p300 overexpression (Fig. 6A and B). Lastly, we confirmed as expected that p300 overexpression was able to induce H3K18 acetylation (Fig. 6A and B).

Therefore, induction of p300 resulted in an increased acetylation of p53 and C/EBP, which is associated with their increased

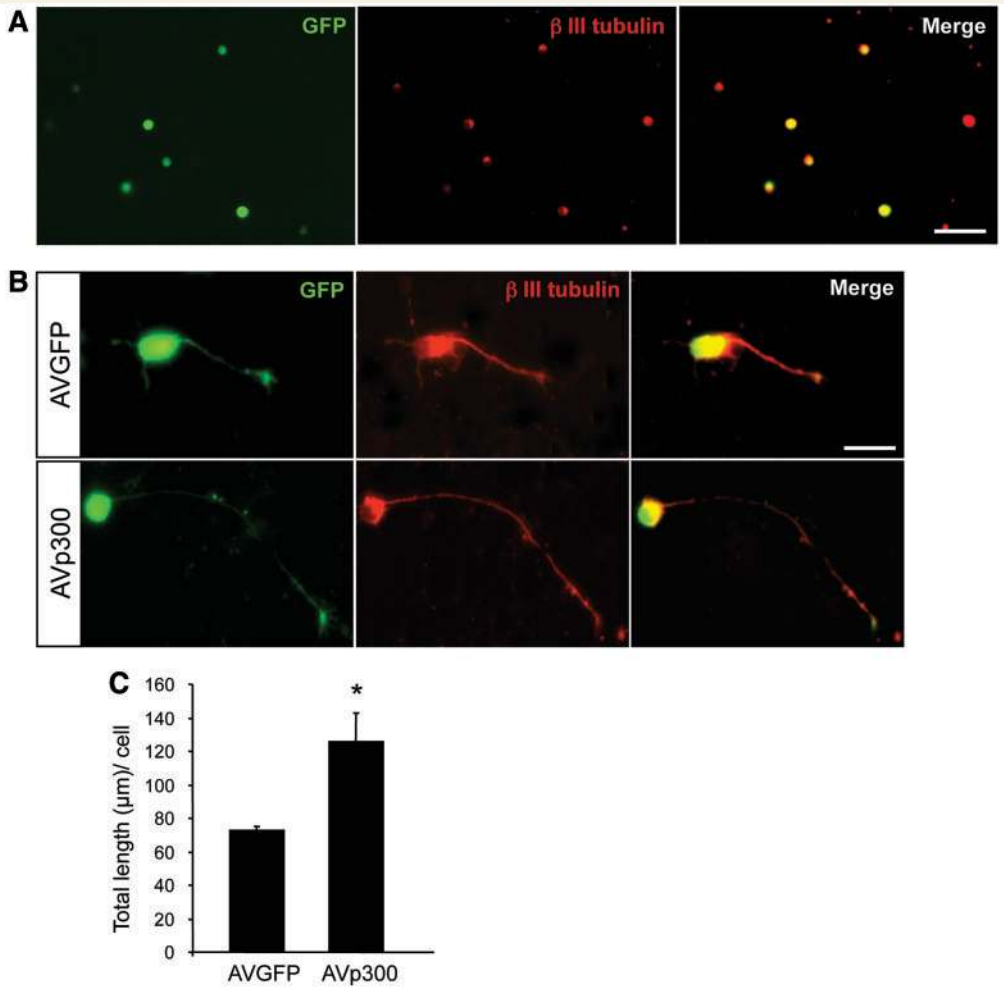

Figure 5 Overexpression of p300 induces neurite outgrowth in cultured cells. (**A**) Retinal cells were cultured on poly-D-lysine for 24 h and infected with AVGFP or AVp300 at MOI 100. Immunostaining against β-III tubulin for retinal ganglion cells shows a colocalization with infected green fluorescence protein (GFP)-positive cells. Scale bar = 20 μm. (**B**) Representative pictures of dissociated retinal primary culture immunostained against β III-tubulin show enhanced neurite outgrowth in p300-infected GFP-positive cells compared with control virus infection. Scale bar = 20 μm. (**C**) Quantification of neurite length shows an increase in neurite outgrowth 72 h after infection of AVp300 compared with AVGFP-infected cells. Asterisk = unpaired two-tailed *t*-test, **P*-value < 0.01; $n = 3$. Error bars represent SD. MOI = multiplicity of infection.

transcriptional activity, and with H3 hyperacetylation, signature of active chromatin. However, in order to assess whether AVp300, in addition to enhancing axonal regeneration, is also directly capable of occupying and acetylating the promoters of proregenerative gene targets, we performed chromatin immunoprecipitation assays from dissected retinae after optic nerve crush and infection with either AVp300 or AVGFP. Selected gene targets included Sprr1a and GAP-43 as markers of pro-regenerative state of retinal ganglion cells (Benowitz and Routtenberg, 1997; Fischer *et al.*, 2004), and coronin 1B as a pro-neurite outgrowth gene and target of p53-dependent acetylation (Di Giovanni *et al.*, 2006). Following p300 overexpression, we found a significant increase of p300 proximal promoter occupancy on GAP-43, coronin 1 b and Sprr1a (Fig. 7A), which was paralleled by a strongly enhanced promoter acetylation of H3 (Fig. 7B). Importantly, as p300 promoter occupancy and p300-dependent promoter acetylation are associated with gene transcription, we measured gene expression by real-time reverse transcriptase polymerase chain reaction post-optic nerve crush and AVp300 or AVGFP infection. Indeed, we observed an increase in messenger RNA expression of several pro-axonal outgrowth genes, including GAP-43, Sprr1a and coronin 1 b (Fig. 7C), as well as α-tubulin 1 a, Chl1 and Lgals1 (Fig. 7D). Interestingly, all of these genes contain p300-related p53 putative binding sites, and their induction is likely to contribute to the pro-axonal regenerative properties of p300. In summary, overexpression of p300 induces axonal regeneration upon optic nerve crush, acetylates the proregenerative transcription factors p53 and C/EBP, directly occupies and acetylates the promoters of the regeneration-associated genes GAP-43, coronin 1 b and Sprr1a and drives the gene expression programme of several regeneration-associated genes.

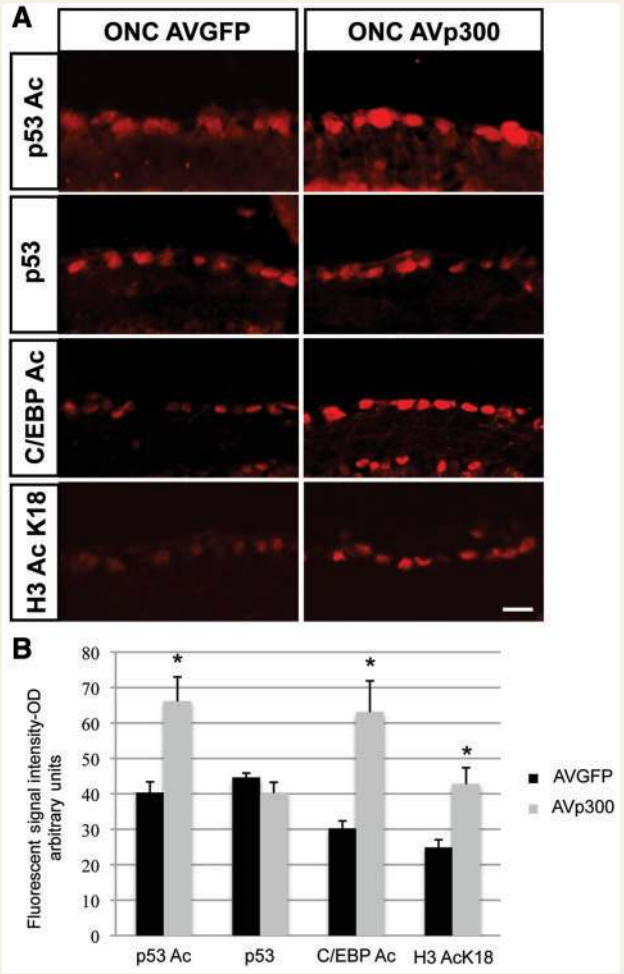

Figure 6 p300 overexpression leads to increased acetylation of p53, C/EBP and H3 K18. (**A**) Immunohistochemistry of retinae against p53 Ac373, p53, C/EBP Ac215/216 and H3AcK18 shows expression in the retinal ganglion cell layer 24 h after optic nerve crush (ONC) and AVGFP or AVp300 infection. Shown is an increase of H3AcK18, p53 and C/EBP acetylation. The basal level of p53 is unchanged. Scale bar = 20 μm. (**B**) The bar graphs represent assessment of fluorescence signal in retinal ganglion cells for the different antigens. Asterisk = unpaired two-tailed *t*-test, **P*-value < 0.01; *n* = 3. Error bars represent SD. OD = optical density.

Discussion

Variable degrees of axonal regeneration of the optic nerve have been achieved by both inhibiting the extrinsic environment or by enhancing the intrinsic capacity of retinal ganglion cells (Bertrand *et al.*, 2005, 2007; Park *et al.*, 2008; Moore *et al.*, 2009). As far as the intrinsic strategies are concerned, lens injury, the pro-inflammatory molecule oncomodulin, the Bcl-2 inhibitor BAG-1 or ciliary neurotrophic factor have all led to substantial axonal regeneration (Yin *et al.*, 2006, 2009; Planchamp *et al.*, 2008). More recently, direct modifications of transcription or of protein synthesis via KLF4 or PTEN deletion, respectively, promoted axonal regeneration after optic nerve crush (Park *et al.*, 2008; Moore *et al.*, 2009), and to a substantial distance in the case of combinatory treatment with PTEN deletion, cyclic adenosine monophosphate and oncomodulin (Kurimoto *et al.*, 2010).

Here, we show for the first time that intrinsic axonal regeneration of the optic nerve can be achieved by a different class of molecules, via overexpression of a transcriptional coactivator and epigenetic modifier, the acetyltransferase p300. Overexpression of p300 induces axonal regeneration of the optic nerve following crush, hyperacetylates histone H3, acetylates the promoters of several regeneration-associated genes and induces their gene expression. In addition, overexpression of p300 results in the acetylation of the pro-axonal outgrowth transcription factors p53 and C/EBP. p53 K373 acetylation has been previously shown to promote neurite outgrowth in primary neurons and to be a signature of active p53 that is required for axonal regeneration (Tedeschi *et al.*, 2009; Gaub *et al.*, 2010). Acetylated C/EBP, whose acetylation enhances its transcription potential, has been shown to be induced in retinal ganglion cells during lens injury-mediated axonal regeneration, and has been reported to be required for axonal regeneration in the PNS (Nadeau *et al.*, 2005).

It is therefore conceivable that p300 may unlock a silent pro-regenerative gene expression programme by driving the expression of several regeneration-associated genes via enhanced transcription.

We found initially that p300 was regulated during retinal ganglion cell maturation to decrease in the mature retinal ganglion cells as well as following optic nerve crush. Importantly, the signal for p300 and the related proteins does not follow the same pattern of expression in the inner nuclear layer (data not shown), suggesting that it is specific to the retinal ganglion cell layer. In addition, in the ganglion cell layer, the expression of histone acetyltransferases is largely restricted to retinal ganglion cells, and is only sporadically found in neighbouring glial cells.

Since mature adult neurons are known to be less plastic and to express a less vigorous pro-regenerative gene expression programme, we wondered whether p300 downregulation might be in part responsible for the lack of intrinsic neuronal proregenerative capacity. Indeed, after ruling out the pro-regenerative potential of a more general epigenetic strategy with the histone deacetylase inhibitor trichostatin A, which does not enhance p300 expression, we found that overexpression of p300 was able to promote axonal regeneration of surviving retinal ganglion cells. This supports the model where reactivating a silenced developmental programme in the adult may favour axonal regeneration.

P300 is a transcriptional coactivator and histone-modifying enzyme (Ogryzko *et al.*, 1996), thus contributing to epigenetic changes responsible for enhanced transcriptional activity. Recently, we have shown that a transcriptional complex formed by CBP/p300 and p53 occupies the promoter of GAP-43 driving its expression during axonal regeneration following facial nerve axotomy (Tedeschi *et al.*, 2009). Subsequently, we also observed that overexpression of CBP and p300 was able to promote neurite outgrowth on permissive and inhibitory myelin substrates in

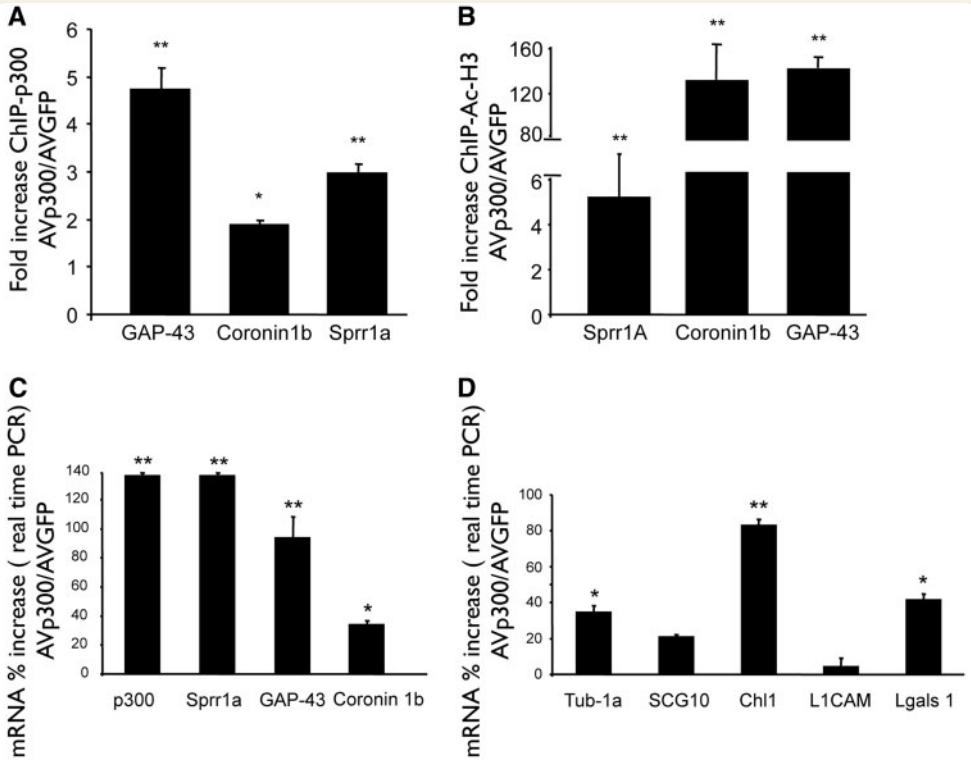

Figure 7 Infection of AVp300 enhances promoter occupancy of p300 and histone acetylation on specific proregenerative genes along with an increase of their gene expression level. (**A**) Chromatin immunoprecipitation (ChIP) assay from dissected retina shows increased occupancy of the GAP-43, coronin 1 b and Sprr1a promoters by p300 following 24 h of optic nerve crush plus AVp300 injection versus AVGFP. Fold change was calculated as a ratio of promoter occupancy between AVp300 treated versus AVGFP in three independent animals in triplicate samples. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.05, ***P*-value < 0.01. Error bars represent SD. (**B**) Bar graph shows an increase of histone H3 acetylation on Sprr1a, coronin 1 b and GAP-43 promoter 24 h after optic nerve crush with AVp300 compared with AVGFP infection. Fold change was calculated as a ratio of promoter occupancy between AVp300 treated versus AVGFP in three independent animals in triplicate samples. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.05, ***P*-value < 0.01. Error bars represent SD. (**C** and **D**) Bar graphs show real-time reverse transcriptase polymerase chain reaction (PCR) messenger RNA (mRNA) expression data for p300 and a number of regeneration-associated genes including Sprr1a, GAP-43 and coronin 1 b (**C**) or for α-tubulin1a, SCG10, Chl1, L1CAM and Lgals1 (**D**). Optic nerve crush with AVp300 induces an increase of several of these genes compared with optic nerve crush with AVGFP in three independent animals. Asterisks = unpaired two-tailed *t*-test, **P*-value < 0.05, ***P*-value < 0.01. Error bars represent SD.

primary cerebellar neurons (Gaub *et al.*, 2010). Here we show for the first time that p300 can promote neurite outgrowth in retinal ganglion cells, supporting the neuronal intrinsic effect of p300 in axonal regeneration. We used adenoviral infection to achieve p300 overexpression due to the large size of p300 (~8 kb), which is too large for other viral vectors such as adeno-associated virus (maximum insert size <5 kb) that have become the gold standard for retinal ganglion cell infection *in vivo* in recent years (Dinculescu *et al.*, 2005). However, adenoviruses have been extensively used to infect both non-neuronal and neuronal cells in the eye, both via intravitreal (Jomary *et al.*, 1994; Li *et al.*, 1994; Weise *et al.*, 2000; Zhang *et al.*, 2008) or axonal retrograde injection (Cayouette and Gravel, 1996; Isenmann *et al.*, 2001), and our findings suggest that our adenovirus is able to infect primary neurons at very high efficiency in culture and at a lower efficiency *in vivo*. It is possible that infection of bipolar/amacrine cells also plays an important role in determining the intrinsic growth ability of retinal ganglion cells (Goldberg *et al.*, 2002), and that the infection of glial cells may contribute to stimulating intrinsic axonal regeneration of retinal ganglion cells. Conceptually, the specificity of p300-dependent axonal regeneration is supported by the negative findings following trichostatin A treatment, where overall pro-transcriptional epigenetic changes do not enhance axonal regeneration. Interestingly, trichostatin A does induce survival of retinal ganglion cells 14 days after optic nerve crush, as well as increased CBP expression and H3K18 acetylation, but fails to promote p300 expression and p53 acetylation. Conversely, overexpression of p300 does not induce retinal ganglion cell survival but promotes axonal regeneration in surviving retinal ganglion cells, suggesting that histone deacetylases inhibition and p300 activate two independent pathways. Axonal regeneration is not always linked to neuronal survival, as in the case of deletion of the transcription factor KLF4 (Moore *et al.*, 2009), which results in a significant increase in axonal regeneration

from surviving retinal ganglion cells but not in increased retinal ganglion cell survival. Here, neuronal survival was assessed by β-III tubulin staining, which although it cannot discern among specific cell death mechanisms, is widely used to count retinal neurons. If lack of enhanced p300-dependent retinal ganglion cell survival is disappointing, it highlights the efficacy and specificity of p300 in promoting the axonal regeneration programme.

We have in fact shown, for the first time, that a selective modification of the transcriptional environment is capable of promoting axonal regeneration in the CNS by enhancing the intrinsic proregenerative programme. Moreover, the enhanced axonal regeneration achieved by the overexpression of p300, along with lens injury, suggests that p300 may further stimulate the intrinsic gene expression programme known to be activated by lens injury. Therefore, future combinatory experiments with molecules such as oncomodulin, deletion of PTEN or delivery of ciliary neurotrophic factor are also expected to enhance the level of p300-dependent axonal regeneration by boosting the intrinsic retinal ganglion cell regeneration potential.

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Supplementary material

Supplementary material is available at *Brain* online.

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