Biochemical and Biophysical Research Communications 454 (2014) 381–386

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Brain derived neurotrophic factor is involved in the regulation of glycogen synthase kinase 3β (GSK3β) signalling

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ARTICLE INFO

Article history:
Received 10 October 2014
Available online 24 October 2014

Keywords:
Brain-derived neurotrophic factor
TrkB receptor
7,8 Dihydroxyflavone
Protein phosphorylation
Glycogen synthase kinase 3β

ABSTRACT

Glycogen synthase kinase 3β (GSK3β) is involved in several biochemical processes in neurons regulating cellular survival, gene expression, cell fate determination, metabolism and proliferation. GSK3β activity is inhibited through the phosphorylation of its Ser-9 residue. In this study we sought to investigate the role of BDNF/TrkB signalling in the modulation of GSK3β activity. BDNF/TrkB signalling regulates the GSK3β activity both *in vivo* in the retinal tissue as well as in the neuronal cells under culture conditions. We report here for the first time that BDNF can also regulate GSK3β activity independent of its effects through the TrkB receptor signalling. Knockdown of BDNF lead to a decline in GSK3β phosphorylation without having a detectable effect on the TrkB activity or its downstream effectors Akt and Erk1/2. Treatment with TrkB receptor agonist had a stimulating effect on the GSK3β phosphorylation, but the effect was significantly less pronounced in the cells in which BDNF was knocked down. The use of TrkB receptor antagonist similarly, manifested itself in the form of downregulation of GSK3β phosphorylation, but a combined TrkB inhibition and BDNF knockdown exhibited a much stronger negative effect. *In vivo*, we observed reduced levels of GSK3β phosphorylation in the retinal tissues of the $BDNF^{+/-}$ animals implicating critical role of BDNF in the regulation of the GSK3β activity. Concluding, BDNF/TrkB axis strongly regulates the GSK3β activity and BDNF also exhibits GSK3β regulatory effect independent of its actions through the TrkB receptor signalling.

1. Introduction

Glycogen synthase kinase 3β (GSK3β) is a ubiquitously expressed and evolutionarily conserved intracellular protein serine/threonine kinase. It is constitutively active in most cells and plays a pivotal role in key cellular functions ranging from glycogen metabolism to important regulatory effects on the neural plasticity and survival. It is important regulatory protein that is subject to phosphorylation by growth factor-stimulated signalling pathways [1]. Phosphorylation at Ser^9 residue is the most well defined post-translational modification of GSK3β which regulates its activity [1]. GSK3β activity is negatively regulated by several signal transduction cascades that protect neurons against apoptosis, including the phosphatidylinositol-3 kinase (PI-3K) pathway. Over-expression of GSK3β in specific regions of the brains in animals results in region specific neuronal cell death [2].

Previous work has shown that Brain-derived neurotrophic factor (BDNF) mediated signalling modulates GSK3β activity in retinoic acid differentiated SH-SY5Y cells [1]. BDNF exerts neurotrophic effects primarily through its high affinity receptor tropomyosin receptor kinase B (TrkB), which upon stimulation undergoes dimerization and phosphorylation of its specific intracellular tyrosine residues and activates various cell signalling pathways linked to growth, differentiation, and survival [3,4]. BDNF binds to the TrkB receptor to initiate multiple signalling cascades, including the PI-3K which in turn leads to the activation of the Ser/Thr kinase Akt [3,5]. Akt is a major upstream regulator of GSK3β and regulates GSK3β signalling by its phosphorylation at Ser^9, thereby inactivating it [6]. GSK-3β activity changes have been reported to be associated with several psychiatric and neurodegenerative diseases, such as Alzheimer's disease, schizophrenia and autism spectrum disorders, in addition to several cellular proliferative disorders and it is becoming increasingly clear that GSK3β might serve as a potential therapeutic target in several of these disorders [7,8]. It is also possibly associated with ageing related cellular effects. GSK-3β phosphorylation inhibits CRMP-2 binding to

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http://dx.doi.org/10.1016/j.bbrc.2014.10.087
0006-291X/

tubulin and promotes microtubule dynamics along with axonal transport and outgrowth in neurons. It also plays a role in regulating the polarity of the neurons [7]. Enhanced GSK3β activity is involved in the CREB protein inactivation and promotes cell death and degeneration [9].

In retina, BDNF has been shown to play a vital role in maintaining the health of retinal ganglion cells (RGCs) and protecting them from apoptosis caused by glaucoma or injury [10]. BDNF is shown to stimulate the growth of neurites from regenerating RGCs [11], and protect optic nerve and RGCs from damage [10,12]. The higher susceptibility of $BDNF^{+/-}$ mice to development of neurodegenerative changes in the inner retina with age indicates that these might be arising due to the disruption of BDNF/TrkB axis leading to possible activation of GSK3β [13]. An enhanced GSK3β activity has been observed in the inner retinas of animal models of experimental glaucoma as well as post-mortem samples of human glaucoma subjects [6]. The present study investigates the regulatory effects of BDNF impairment on GSK3β activity *in vivo* using wild type and $BDNF^{+/-}$ animals. As GSK3β phosphorylation and activation can be regulated in a cell and tissue dependent manner; to rigorously investigate the role played by BDNF in regulating the GSK3β activity we used two neuronal RGC-5 and PC12 cell lines and evaluated the role of BDNF and TrkB in regulating the GSK3β activity and also determined the relevance of the findings *in vivo*. The results provide novel data that raise the prospects of using TrkB agonist treatment to regulate the potential deleterious effects of GSK3β activity. Further, we show here for the first time that *BDNF* regulates Ser^{9} residue phosphorylation of GSK3β independent of its effects through high affinity receptor TrkB and subsequent PI3K/Akt and Erk1/2 activation.

2. Materials and methods

2.1. Chemicals

Anti-BDNF (sc-546) and anti-TrkB (sc20542) antibodies were obtained from Santa Cruz Biotechnology, CA. pTrkB (Abcam, ab51187), pAkt (Ser473) (Cell Signalling, 193H12), Akt (Cell Signalling, 11E7), p44/42 MAPK (Erk1/2) (Cell Signalling, 137F5), p-p44/42 MAPK (Erk1/2 Thr202/Tyr204) (Cell Signalling D13.14.4E), GSK-3β (Cell Signalling, 27C10), pGSK-3β (Ser9) (Cell Signalling 5B3) and β-actin (Sigma, AC-40) antibodies were used for Western blotting. 7,8 Dihydroxyflavone (7,8DHF) was obtained from Tocris Bioscience, UK and cyclotraxin-B (CTX-B) was obtained from Life research Ltd, Australia. All other reagents were of analytical grade (Sigma, St. Louis, MO).

2.2. Cell culture and treatments

RGC-5 and PC12 neuronal cells were maintained in DMEM medium containing 10% foetal bovine serum (FBS) at 37 °C with 5% CO_2. Approximately 2.0×10^5 cells were seeded in each 60 mm culture dish 6–12 h before subjecting them to transient transfections. After transfections and subjecting the cells to serum starvation for a period of another 12 h, the cells were either treated with TrkB receptor agonist, 7,8DHF (100nM, 6 h) or a cyclic peptide CTX-B, which is a TrkB antagonist with 1–11 Cys residue disulphide linkage (CNPMGYTKEGC; 5 μM, 6 h). 7,8-DHF was dissolved in phosphate-buffered saline containing 17% dimethylsulfoxide. Subsequently, cells were harvested, lysed and the cell lysates subjected to Western blotting for biochemical analysis.

2.3. Transfections

RGC-5 and PC12 cells were subjected to transient BDNF knockdown using the BDNF siRNA (Santa Cruz Biotechnology; sc42122) which is a combination of 3 target-specific 20–25 nucleotide siRNA sequences designed to knockdown gene expression. It was resuspended in RNAse free water to obtain a 10 μM solution in Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA. Briefly the siRNA was mixed with the Lipofectamine RNAiMAX (Invitrogen) reagent and cells transfected [14]. Control cells were treated with the control siRNA sequences (Santa Cruz biotechnology; sc37007). It consisted of a scrambled sequence that is not known to lead to specific degradation of any known cellular mRNA. The cells were subjected to serum starvation by excluding FBS from the culture media, approximately 12 h after the transient transfections were performed and allowed to grow for another 18–24 h.

2.4. Animals and drug treatments

All procedures involving animals were conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes and the guidelines of the ARVO statement for the use of animals in ophthalmic and vision research. $BDNF^{+/-}$ and wild-type (WT) control mice (both male/female) were obtained from Mental Health Research Institute (Parkville, VIC, Australia) and genotyped using standard PCR methodology. All animals were maintained in our vivarium in cyclic light (12 h on; 12 h off; ~ 300 lux), with controlled temperature (21 ± 2 ℃) and with free access to water and rodent chow. The animals were treated chronically (2 mg/kg) with a TrkB agonist 7,8DHF. 7,8-DHF is a potent and selective TrkB receptor agonist that provokes receptor autophosphorylation and dimerisation and has been shown to activate the TrkB signalling in the RGCs [15–17]. 7,8DHF was administered fortnightly for a period of 2 months through intraperitoneal injections. Animals were sacrificed and tissues harvested 2 h after the last drug treatment. $BDNF^{+/-}$ mice were healthy with normal behaviour, and had no visible phenotype different from WT mice.

2.5. SDS–PAGE and Western Blot analysis

Following enucleation of the eyes and retinal dissection, ONH regions of the retina were excised under a surgical microscope, lysed in lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM EDTA) containing 10 μg/ml aprotinin, 10 μM leupeptin, 1 mM PMSF and 1 mM $NaVO_3$, 100 mM NaF, 1 mM Na_2MoO_4 and 10 mM $Na_4P_2O_7$ [18]. Proteins were resolved by 10% SDS–PAGE and transferred to PVDF membranes. The blots were washed 3 times (5 min each) with TTBS (20 mM Tris–HCl [pH 7.4], 100 mM NaCl, and 0.1% Tween 20) and blocked with 5% non-fat dry milk powder (Bio-Rad) in TTBS for 1 h at room temperature [19]. Blots were then incubated with anti-BDNF (1:1000), anti-TrkB (1:1000), anti-pTrkB (Tyr^{515}) (1:1000), anti-Akt (1:1000), anti-pAkt (Ser^{273}) (1:1000), anti-Erk (1:1000), anti-pErk (Thr^{202}/Tyr^{204}) (1:1000), anti-Gsk3β (1:1000), anti-pGsk3β (Ser^{9}) (1:1000) or anti-actin (1:5000) either for 1 h (actin) at room temperature or overnight at 4 °C. Actin was used to ensure a comparable loading was made in each case [20]. Following primary antibody incubations, immunoblots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies and after extensive washing, antibody detection was accomplished with supersignal west pico chemiluminescent substrate (Pierce). Signals were detected using an automated luminescent image analyser (ImageQuant LAS4000). Band intensities were quantified using ImageJ software (NIH, USA).

2.6. Statistical analysis

Data were analysed and graphed using commercially available Graphpad Prism software (version 6.0) (GraphPad Software, San Diego, CA). All values with error bars are presented as mean ± SD

and compared by Student's *t* test for unpaired data. Grouped data was analysed using ANOVA. The significance was set at $p < 0.05$.

3. Results

3.1. BDNF negatively regulates GSK3β activation in RGC-5 and PC12 cells

The role of BDNF knockdown on the TrkB and its downstream signalling comprising Akt, Erk1/2 and GSK3β was investigated in the RGC-5 and PC12 neuronal cells. The cells were also subjected to either treatment with the TrkB receptor agonist 7,8DHF or incubated with 7,8DHF subsequent to BDNF knockdown and downstream signalling changes analysed (Fig. 1A and E). Quantification of the band intensities revealed that siRNA mediated knockdown was successful in significantly reducing the BDNF expression in both cell types ($p < 0.03$) (Fig. 1B and F). A basal level of TrkB phosphorylation indicated that it was constitutively active in both cell lines. BDNF knockdown was not observed to have any significant effect on the phosphorylation levels of TrkB receptor. To decipher the relative contributions of BDNF and its high affinity receptor TrkB, the cells were treated with TrkB receptor agonist 7,8DHF. Treatment of cells with 7,8DHF produced significant activation of the TrkB receptor in both cell types ($p < 0.05$) (Fig. 1C and G). We also observed a corresponding activation of the Akt ($p < 0.005$) and Erk2 proteins ($p < 0.01$) which are downstream of the TrkB receptor. BDNF knockdown by itself did not have any detectable effect on the phosphorylation levels of any of these proteins. Interestingly, BDNF knockdown resulted in marked downregulation of GSK3β phosphorylation in the normal untreated cells ($p < 0.05$) as well as those treated with 7,8DHF ($p < 0.05$) indicating that BDNF also has a regulatory effect on GSK3β signalling independent of its effects through the TrkB activation (Fig. 1D and H). 7,8DHF treatment was not shown to have any effect on the expression of BDNF, TrkB, Akt, Erk1/2 or GSK3β proteins.

3.2. BDNF$^{+/-}$ mice depict reduced GSK3β phosphorylation with age

In order to determine whether BDNF mediated regulation of GSK3β is reflected under *in vivo* conditions, we investigated changes in the GSK3β phosphorylation in optic nerve head (ON) of the retinas of WT and BDNF$^{+/-}$ animals. No changes in the phosphorylation status of GSK3β were observed at 2 m time point (Fig. 2A). Our previous studies have shown that aged BDNF$^{+/-}$ animals depict reduced levels of BDNF in the ONH at 1 year [13]. We therefore also evaluated any changes in GSK3β phosphorylation levels at this time point and interestingly observed a significant level of GSK3β activation ($p < 0.01$) (Fig. 2B). There was no detectable change in the expression levels of total GSK3β protein. Actin was used as loading control in each case. These results indicate that BDNF loss modulates the GSK3β signalling *in vivo* by promoting its activation.

3.3. TrkB agonist enhances GSK3β phosphorylation in vivo

BDNF is a high affinity ligand for the TrkB receptor. We explored the role of TrkB receptor activation in mediating GSK3β phosphorylation *in vivo* by chronic administration of TrkB receptor agonist to the WT and BDNF$^{+/-}$ mice (2 mg/kg) fortnightly for 2 months. At the end point, ONH tissues of the mice retina were excised, lysates subjected to Western blotting and probed for changes in the GSK3β phosphorylation profile. 7,8DHF treatment was able to

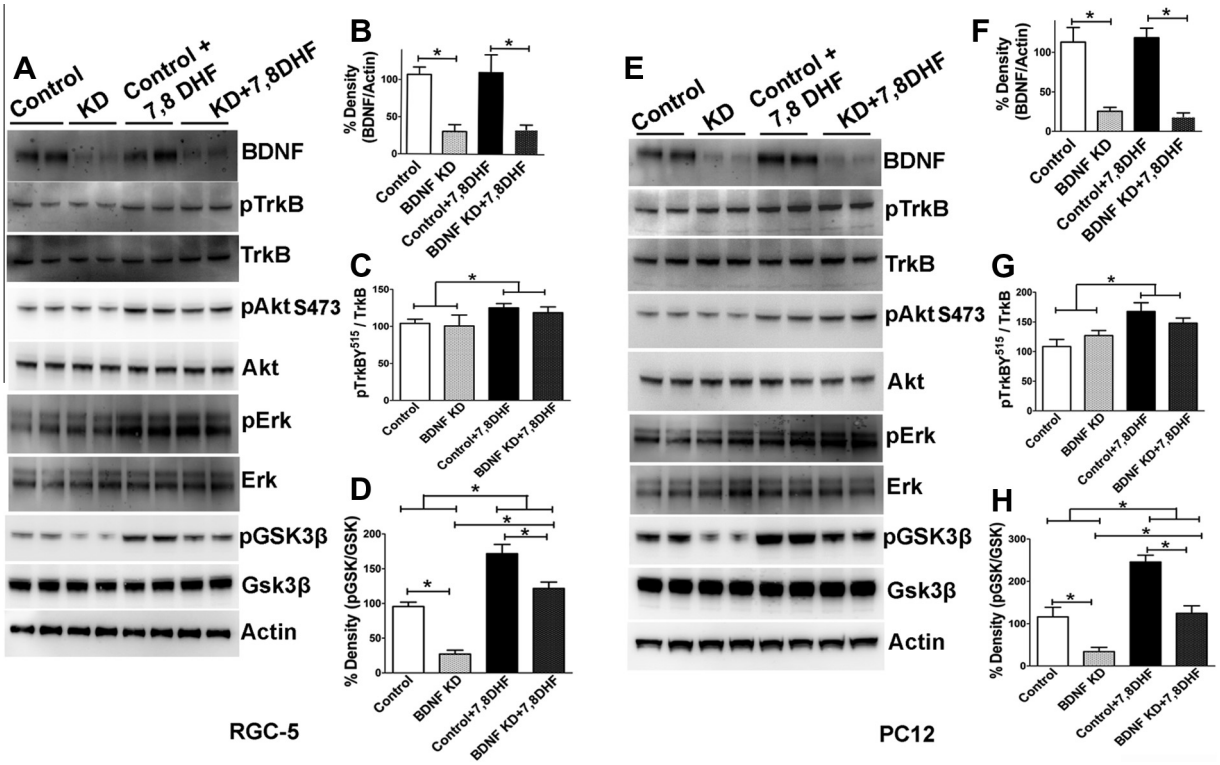

Fig. 1. BDNF knockdown leads to GSK3β activation by reducing its phosphorylation levels. (A) RGC-5 cells were subjected to BDNF knockdown, treatment with 7,8DHF (100 nM) or BDNF knockdown and then incubation with 7,8DHF and Western blotting performed. Band intensities were analysed and plotted for (B) BDNF (C) pTrkB (Tyr515) and (D) pGSK3β (Ser9) proteins using actin, TrkB and GSK3β as controls respectively. (E) PC12 cells were subjected to either knockdown of BDNF, treatment with 7,8DHF (100 nM) or BDNF knockdown and subsequent incubation with 7,8DHF followed by Western blotting. Band intensities were quantified and plotted for (F) BDNF (G) pTrkB (Tyr515) and (H) pGSK3β (Ser9) proteins using actin, TrkB and GSK3β as controls respectively. $^{*}p < 0.05$ in each case.

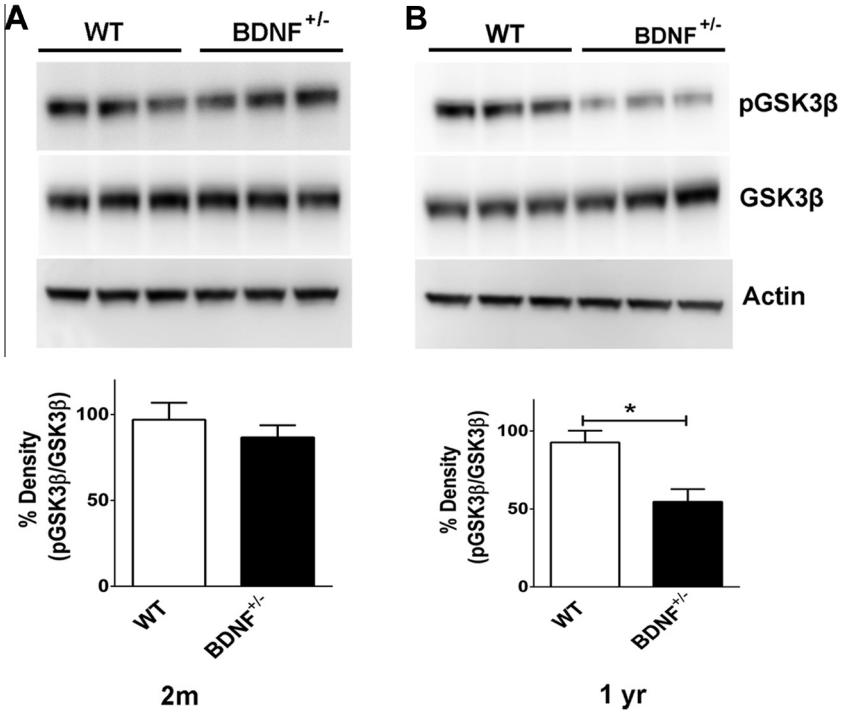

Fig. 2. BDNF$^{+/-}$ animals depict a decrease in GSK3β phosphorylation with age. (A) Optic nerve head region was excised from the retinas of 2 month old WT and BDNF$^{+/-}$ mice and subjected to Western blotting. Changes in the phosphorylation levels of GSK3β protein (Ser9) were plotted by quantification of the band intensities. (B) Optic nerve head region was excised from the retinas of 1 year old WT and BDNF$^{+/-}$ mice and subjected to Western blotting. GSK3β was assessed for any changes in the phosphorylation levels (Ser9) by quantification of the band intensities ($^{*}p < 0.01$). Actin was used as loading control in each case.

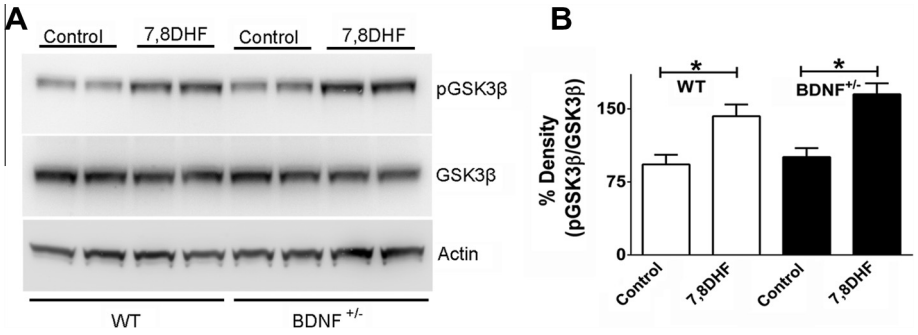

Fig. 3. Treatment with TrkB agonist leads to enhanced GSK3β phosphorylation *in vivo*. (A) ONH tissues from WT and BDNF$^{+/-}$ mice which were treated with 7,8DHF (2 mg/kg) or vehicle control, were excised, lysed and subjected to Western blotting. Blots were probed for changes in GSK3β phosphorylation using anti-pGSK3β and GSK3β antibodies. Actin was used as loading control. (B) Band intensities were quantified and values plotted as shown ($^{*}p < 0.05$).

significantly promote the GSK3β phosphorylation in both the WT ($p < 0.05$) and BDNF$^{+/-}$ ($p < 0.05$) mice (Fig. 3A and B). The results suggest that TrkB agonist action can lead to inhibition of GSK3β activity *in vivo* independent of the status of *BDNF* impairment. We did not observe any alterations in TrkB or BDNF expression in the ONH in response to 7,8DHF treatment. No changes in the total protein levels of GSK3β were observed. Actin served as the loading control.

3.4. BDNF knockdown along with TrkB inhibition augments GSK3β activation

Our experimental paradigm suggested that BDNF knockdown can produce GSK3β dephosphorylation (Ser^{9}) without producing corresponding changes in phosphorylation of TrkB receptor (Tyr^{515}) or its downstream effectors Akt (Ser^{473}) and Erk1/2 (Thr^{202}/Tyr^{204}) (Fig. 1). The involvement of TrkB in BDNF induced regulation of GSK3β activity was further elucidated by treating the cells with TrkB antagonist and evaluating changes in GSK3β phosphorylation as a consequence of BDNF knockdown. The levels of knockdown achieved by BDNF siRNA were consistent (Fig. 4A and C) with that observed in previous experiment ($p < 0.05$) (Fig. 1). Treatment with CTX-B resulted in significant reduction in the TrkB activation in both cell types ($p < 0.02$). CTX-B treatment also produced a significant downregulation of GSK3β phosphorylation in both the RGC-5 ($p < 0.03$) and PC12 ($p < 0.05$) cells. Importantly, when the cells were subjected to treatment with CTX-B subsequent to BDNF knockdown, there was a further reduction in the GSK3β phosphorylation in both the neuronal cell types ($p < 0.05$) (Fig. 4A–D). CTX-B treatment did not result in any changes in the BDNF, TrkB or GSK3β expression compared to the corresponding controls. BDNF knockdown also did not produce any alterations in the TrkB or GSK3β protein expression. This experiment further suggested that BDNF may have a regulatory

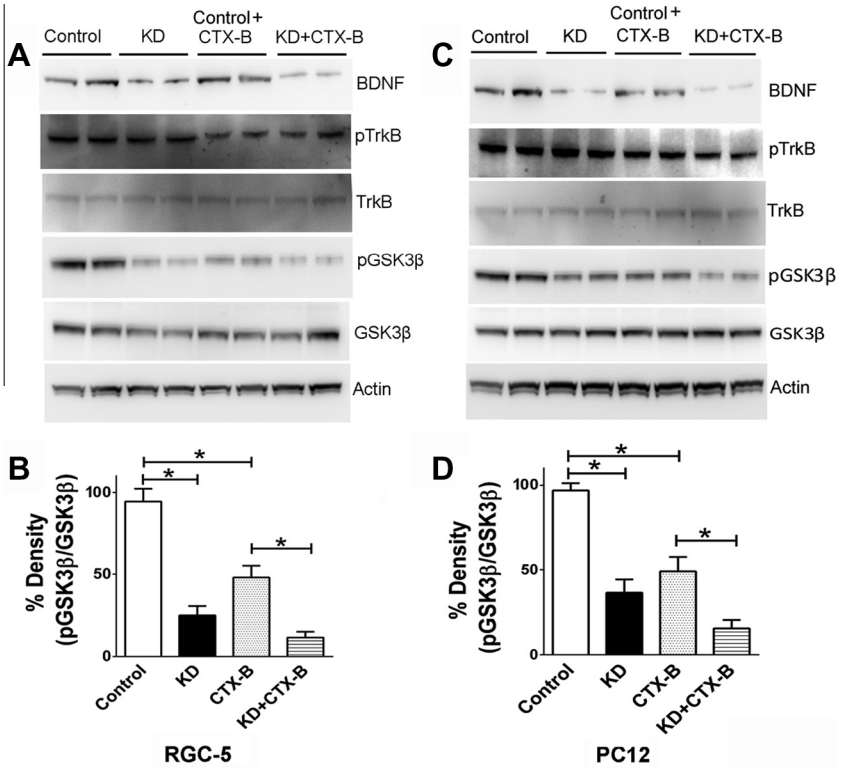

Fig. 4. Inhibition of TrkB receptor promotes dephosphorylation of GSK3β. (A) RGC-5 cells were subjected to either BDNF knockdown, treatment with CTX-B (5 μM) or BDNF knockdown followed by incubation with CTX-B and immunoblotting performed. The blots were probed to evaluate changes in BDNF, pTrkB and pGSK3β reactivity. (B) Quantification of the pGSK3β band intensities normalised to total GSK3β protein was plotted (*$p < 0.03$). (C) In a similar experiment, PC12 cells were subjected to BDNF knockdown, incubation with CTX-B (5 μM) for TrkB inhibition or BDNF knockdown followed by CTX-B treatment paradigms. Cell lysates were analysed by Western blotting using BDNF, pTrkB (Tyr515), TrkB, pGSK3β (Ser9), GSK3β and actin specific antibodies. (D) Quantification of the pGSK3β band intensities normalised to total GSK3β protein in each case (*$p < 0.05$).

effect on GSK3β activity independent of its effects on TrkB and that combined BDNF knockdown and TrkB inhibition has a reinforcing outcome on the GSK3β activation.

4. Discussion

An interruption in BDNF availability and transport has been proposed to be responsible for RGC apoptosis in various models of optic nerve injury including glaucoma [13,21,22]. This study confirmed that BDNF/TrkB signalling plays an important role in regulation of the GSK3β activity both in the neuronal cells in culture as well as in the retina *in vivo*. Gsk3β is a key protein involved in neuronal survival and has been regarded as a converging point for N-methyl-D-aspartate, BDNF and P2X7 purinergic receptor signalling [23]. We found that BDNF regulates the GSK3β activity independent of its effects *via* the high affinity receptor TrkB and downstream Akt and Erk1/2 signalling modules. A downregulation of the GSK3β (Ser^9) phosphorylation, which is generally downstream of PI3K/Akt pathway has previously been observed in animal models of glaucoma as well as in human post-mortem glaucoma tissues [13]. Activation of GSK3β has been shown to promote the phosphorylation and degradation of β-catenin [24]. Its activation inhibits mitochondrial pyruvate dehydrogenase and thereby promotes neuronal death [25]. GSK3β activity has also been implicated in PI3K inhibition dependent apoptosis induced in neuronal like PC12 cells [26].

Here we show that siRNA mediated knockdown of BDNF in the RGC-5 and PC12 cell lines lead to significant downregulation of the GSK3β phosphorylation. Cellular knockdown of BDNF did not produce any detectable effect on the TrkB, Akt or Erk1/2 activation or expression (Figs. 1 and 4). This indicated that BDNF plays a novel role in the regulation of GSK3β activity independent of these signalling pathways. In further experimental paradigms, we treated the cells independently with a known TrkB agonist 7,8DHF (Fig. 1) or antagonist CTX-B (Fig. 4), which resulted in the modulation of the TrkB activity [15] and also induced changes in the GSK3β phosphorylation. Results corroborate our previous observations that 7,8DHF and CTX-B treatments affect TrkB receptor phosphorylation in the RGCs [15]. Treatment of cells with a TrkB agonist following BDNF knockdown rescued the GSK3β phosphorylation to a significant extent while combined treatment with TrkB antagonist induced a further decline in the Ser^9 phosphorylation of GSK3β. Overall, these results support the hypothesis that GSK3β Ser^9 phosphorylation is regulated through multiple molecular pathways and is common target of both the TrkB receptor mediated signalling as well as BDNF effects independent of the TrkB and downstream Akt and Erk1/2 signalling.

We sought to further correlate our findings *in vivo* by investigating alterations in GSK3β activity in the $BDNF^{+/-}$ animals. $BDNF^{+/-}$ mice are more prone to inner retinal degeneration when exposed to high intraocular pressure (IOP) [13]. A higher vulnerability of $BDNF^{+/-}$ mice to degenerative changes in the inner retina caused by elevated IOP may be attributed to possible exacerbation of GSK3β activation subsequent to *BDNF* insufficiency. The regulatory effects of BDNF impairment were not evident in the younger animals but follow up revealed that GSK3β was significantly activated in the retinal tissues in aged $BDNF^{+/-}$ mice compared to their WT counterparts (Fig. 2). These findings indicated that *BDNF* impairment has a progressive age-related effect on the GSK3β activation. This corresponds with our previous observations that decreased BDNF levels were observed in the ONH of aged $BDNF^{+/-}$

animals [13]. Because BDNF/TrkB signalling has a noticeable effect on the GSK3β activity, its activation under various conditions may reflect an impaired status of BDNF/TrkB signalling. In previous studies, exogenously applied BDNF was shown to induce the dephosphorylation of collapsin response mediator protein-2 (CRMP2) and phosphorylation of GSK3β in hippocampal neurons. BDNF through its interactions with dedicator of cytokinesis-3 (Dock3) protein was also shown to be involved in recruitment of GSK3β to cell membrane and induce its phosphorylation and inactivation [7].

Long term chronic treatment of the animals with the TrkB agonist 7,8DHF resulted in significantly enhanced phosphorylation of the GSK3β in the ONH tissues in both the WT and $BDNF^{+/-}$ animals further confirming that this pathway regulates the GSK3β signalling (Fig. 3). The efficacy of 7,8DHF is supported by previous observations that a single dose of 7,8DHF could produce a significant reduction in β-secretase-1 (BACE1) expression in the brain [27]. 7,8DHF treatment induced phosphorylation of the GSK3β highlighted the pharmacological potential of TrkB agonists to enhance the neuroprotective biochemical signalling pathways in the retina. Increased RGC survival observed with the use of highly specific TrkB agonistic antibodies both *in vitro* and *in vivo* conditions, may be attributed to its effects on the GSK3β signalling observed in this study [28,29]. These findings provide a proof of concept that chronic pharmacological activation of retinal and optic nerve TrkB receptors with non-peptide agonists such as 7,8-DHF may represent an efficacious therapeutic approach to promote GSK3β inhibition, which may find therapeutic uses in several neurodegenerative diseases including glaucoma and other optic nerve disorders. In summary, this study sheds new light on the complex network of pathways through which GSK3β activity is regulated by the BDNF/TrkB signalling axis, and identifies it as a potential therapeutic target.

Acknowledgments

Supported by funds from Ophthalmic Research Institute of Australia (ORIA) and MQRDG (9201301278) grant. We thank Prof. Maarten Van Den Buuse for providing the $BDNF^{+/-}$ mice. V.G. supported by funding from Allergan Australia.

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