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Transcription factor Brn-3b overexpression enhances neurite outgrowth in PC12 cells under condition of hypoxia

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Abstract

Background—Transcription factor Brn-3b plays a key role in retinal ganglion cell differentiation, survival and axon outgrowth during development. However, the precise role of Brn-3b in the normal adult retina as well as during neurodegeneration is unclear. In the current study, the effect of overexpression of Brn-3b was assessed in vitro, in PC12 cells under conditions of normoxia and hypoxia.

Results—Immunoblot analysis showed that overexpression of Brn-3b in PC12 cells as well as 661W cells produced significant increase in the growth cone marker, growth associated protein-43 (GAP-43) and acetylated-tubulin (ac-TUBA). In addition, an increased immunostaining for GAP-43 and ac-TUBA was observed in PC12 cells overexpressing Brn-3b, which was accompanied by a marked increase in neurite outgrowth, compared to PC12 cells overexpressing empty vector. In separate experiments, one set of PC12 cells transfected either with a Brn-3b expression vector or an empty vector was subjected to conditions of hypoxia for 2h, while another set of similarly transfected PC12 cells was maintained in normoxic conditions. It was found that the upregulation of GAP-43 and ac-TUBA in PC12 cells overexpressing Brn-3b under conditions of normoxia was sustained under conditions of hypoxia. Immunocytochemical analysis revealed not only an upregulation of GAP-43 and ac-TUBA, but also increased neurite outgrowth in PC12 cells transfected with Brn-3b as compared to PC12 cells transfected with empty vector in both normoxia and hypoxia.

Conclusions—The findings have implications for a potential role of Brn-3b in neurodegenerative diseases in which hypoxia/ischemia contribute to pathophysiology of the disease.

Keywords

Transcription factor Brn-3b; Hypoxia; Neurodegeneration; Neurite outgrowth

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Conflict of Interest:

The authors declare that they have no conflict of interest

Background

Neuronal injury and degeneration contribute to pathological changes in a number of neurological disorders including Alzheimer's and Parkinson's disease, as well as various ocular neurodegenerative diseases, such as glaucoma and retinal degeneration. The limited regeneration capacity of neurons constrains the recovery from neuronal damage. Thus, better understanding of endogenous and exogenous factors facilitating neurite outgrowth, will aid in the development of therapeutic approaches for neurological diseases (Wu et al. 2012).

POU-domain proteins are generally associated with the development and differentiation of neuronal cell types (Mu et al. 2004). The POU (Pit-Oct-Unc) family of transcription factors are classified on the basis of a common region of 150–160 amino acids in mammalian transcription factors Pit-1, Oct-1, Oct-2 and nematode factor Unc-86. The POU domain common to these factors consists of a bipartite DNA binding domain, which comprises of POU-specific domain (POUS) and a POU homeodomain (POUHD) separated by a linker region (Latchman 1999). A search for POU domain transcription factors in mammalian retina lead to the identification of three closely related genes which were named as Brain-specific homeobox/POU domain protein 3 (Brn-3) including Brn-3a, Brn-3b, and Brn-3c (Xiang et al. 1995). Brn-3 genes are expressed in distinct and overlapping pattern in the developing and mature mammalian nervous system (Turner et al. 1994; Gerrero et al. 1993; Wang et al. 2002; Xiang et al. 1997; Gan et al. 1999; Fedtsova et al. 1995; Ninkina et al. 1993; Xiang et al. 1993; Xiang 1998). However, deletion of each of the Brn-3 proteins affects a specific population of neurons in which it is first expressed during development of the nervous system. It has been shown that Brn-3a knockout mice have defects in peripheral sensory ganglia, motor and sensory components of the hindbrain (Erkman et al. 2000; Wang et al. 2002; Huang et al. 1999). In Brn-3c knockout mice, deafness and balance problems have been observed due to failure in the differentiation of cochlear and vestibular hair cells (Xiang et al. 1997; Erkman et al. 2000; Erkman et al. 1996).

A prominent phenotype observed in Brn-3b-deficient mice is the loss of nearly 70% of retinal ganglion cells (RGCs) without discernible defects in other neurons of the central or peripheral nervous system (Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Camp et al. 2011). Mice lacking Brn-3b have RGCs with abnormal processes, having short and spiny projections reminiscent of dendrites rather than axons, consequently resulting in the formation of a thinner optic nerve (Gan et al. 1996; Wang et al. 2000). Gan et al. (1999) have shown that processes emanating from retinal explants from Brn-3b null embryos are shorter and less bundled than wild type retinas. Marked apoptosis observed in Brn-3b-mutant RGCs appears to be a secondary manifestation to initial defects in differentiation, which results in abnormalities in axonal growth and pathfinding (Mu et al. 2004; Erkman et al. 2000). Thus, Brn-3b is necessary for normal RGC differentiation, polarity, axonal outgrowth and survival (Gan et al. 1999; Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Wang et al. 2000; Camp et al. 2011). There is a considerable amount of information about the role of Brn-3b in RGC development. However, the role of transcription factor Brn-3b in adult retinas and genes influenced by Brn-3b in adult RGCs remains to be elucidated.

Mu et al., (2001) have shown a substantial decrease in GAP-43 expression in the E14.5 retina in Brn-3b knockout mice, compared to wild type mice, suggesting a regulatory linkage between Brn-3b and GAP-43. Corroborative evidence from many labs indicates that GAP-43 plays a key role in guiding the growth of axons and modulating the formation of new connections. Some of these effects of GAP-43 appear to involve regulation of cytoskeletal organization by transducing intra- and extracellular signals in the nerve terminals. GAP-43 is also an intrinsic determinant of neuronal development and plasticity (Benowitz and Routtenberg 1997) and is widely used in neurological studies as a marker of neurite growth (Benowitz and Routtenberg 1997; de Lima et al. 2012; Cho et al. 2005; Yin et al. 2009; Kurimoto et al. 2010; Sun et al. 2011).

Previous studies have linked acetylated α -tubulin (ac-TUBA) expression with neurite elongation in vivo as well as in vitro. Increased acetylation of α -tubulin has been observed in stable microtubules, compared to dynamic microtubules. While some studies have shown that acetylation promotes microtubules stability, other studies have claimed the acetylation itself does not stabilize microtubules and may enhance binding of molecular motors to microtubules (Dompierre et al. 2007; Fournier and McKerracher 1995; Gaub et al. 2010; Sengottuvel et al. 2011).

We carried out our preliminary work using the 661W cell line (SV40 middle T antigen transformed mouse photoreceptor cells) which is easily propagated, hence can be used to rapidly screen for Brn3b mediated changes in specific protein expression. However, it is very difficult to differentiate the 661W cell line for the purpose of experimentation. Hence, we decided to switch over to the rat pheochromocytoma PC12 cells, which can be differentiated by addition of NGF making it suitable for many applications including studying the effects of overexpression of exogenous genes of interest. PC12 cells have been widely used in neurobiological studies as a model of neuronal differentiation and neurite outgrowth in response to nerve growth factor (NGF) treatment (Lee et al. 1977; Cowley et al. 1994; Pang et al. 1995; Takebayashi et al. 2002; Das et al. 2004). NGF-treated PC12 cells cease proliferation, extend neurites and acquire properties characteristic of sympathetic neurons (Das et al. 2004). PC12 cells are exquisitely sensitive to hypoxia which makes it a useful model to study the effects of hypoxia on overexpression of Brn-3b (Alvarez-Tejado et al. 2001; Czyzyk-Krzeska et al 1994).

The goal of this study was to evaluate the role of Brn-3b in neurite outgrowth and assess the expression of growth cone marker GAP-43 and ac-TUBA following overexpression of Brn-3b in PC12 cells under conditions of normoxia and hypoxia.

Methods

Cell culture

661W cells (SV-40 transformed mouse photoreceptor cells) were seeded in T75 flasks with Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). The cells were grown to confluence in T75 flasks, trypsinized and seeded for various experiments on 100 mm dishes (200,000 cells per dish).

Rat pheochromocytoma (PC12 cells) cells were obtained from American Type Culture Collection (ATCC) and cultured as described by Adler et al., (2006). The cells were grown undifferentiated in T75 flasks with Dulbecco's modified Eagle's Medium (DMEM) containing 10% normal horse serum (Life technologies, NY), 5% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)). Cultures were maintained according to ATCC specified culture conditions at 37°C in a humidified incubator containing 95% air and 5% CO₂. For differentiation, cells were dissociated and plated on poly-D-lysine (Sigma-Aldrich) coated 100 mm dishes (200,000 cells per dish) or 25 mm coverslips (10,000 cells per coverslip) or 96 well plates (1000 cells per well) in differentiating DMEM (containing 1% horse bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) with 100 ng/ml of NGF (Sigma-Aldrich) for various experiments.

Plasmid preparation

Plasmid DNAs, pCMV6 entry (Empty) vector and pCMV6-Brn-3b (a full length cDNA encoding Brn-3b containing a DDK tag) were purchased from Origene (Rockville, MD). Purified preparations of the plasmids were obtained from maxi-preps of transformed *E. coli* (DH5α strain) cultures harboring the recombinant expression vectors using a plasmid purification kit from Origene. Following transfection of cell lines, positive expression of the encoded proteins was confirmed by Western blot.

Transient transfection

661W cells were transiently transfected with plasmid pCMV6-Empty or pCMV6-Brn-3b using 5 µl of Lipofectamine 2000 (Life Technologies, Inc, Grand Island, NY) and 5 µg of the plasmid in a total volume of 1 ml of transfection mix and maintained overnight in the transfection medium. The cell culture medium was changed to complete medium (DMEM containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)). The pCMV6-Brn-3b or pCMV6-Empty transfected cells were used 24 h post transfection for isolating cytoplasmic and nuclear extracts for immunoblot analysis.

PC12 cells were transiently transfected with the plasmid pCMV6-Empty vector or pCMV6-Brn-3b using the Lipofectamine 2000 reagent. Transfections were carried out using 5 µl of Lipofectamine and 5 µg of the plasmid in a total volume of 1 ml of the transfection mix. After 6 h of transfection, culture medium was changed to the differentiating medium (DMEM containing 1% horse bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml)) with NGF 100 ng/ml and incubated overnight. The pCMV6-Brn-3b or pCMV6-Empty transfected cells were used 24 h post-transfection for isolating cytoplasmic and nuclear extracts for immunoblot analysis. A similar transfection procedure was carried out for PC12 cells seeded on coverslips for immunocytochemistry using 1.5 µg of either pCMV6-Empty vector or pCMV6-Brn-3b followed by maintenance in differentiating medium for 5 days.

Hypoxia Chamber

To examine the effect of hypoxia, PC12 cells overexpressing either Brn-3b or Empty vector were used. Following transfection and incubation in differentiation medium, PC12 cells overexpressing Brn-3b or empty vector were subjected to a hypoxic insult for 2h in glucose-

free DMEM. For the hypoxic insult, cells were incubated for 2 hours in 0.5% O₂ and 5% CO₂ (hypoxia) in an Invivo2 200 hypoxic chamber (Biotrace International, Mid Glamorgan, UK) used in conjunction with Ruskinn gas mixer module. For the normoxia controls, PC12 cells overexpressing Brn-3b or empty vector were incubated for 2h in 5% CO₂ and 95% air in a standard incubator and maintained in differentiating media.

Cell Proliferation assay (MTT assay)

Cell Proliferation assay was performed as described previously (Prasanna et al. 2002). A commercially available one-solution cell proliferation assay with the tetrazolium compound MTS (CellTiter 96Aqueous; Promega, Madison, WI), was used to evaluate the effects of hypoxic conditions on PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b. The MTS compound is bio-reduced to a formazan by reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH) produced by metabolically active dehydrogenase enzymes of cells which can be detected at 490 nm. PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b were maintained either in differentiating medium (containing NGF) or in low glucose DMEM without NGF. After normoxia or hypoxic insults, the culture media were discarded and to each well 100 µl of fresh DMEM along with 20 µl of the MTS solution was added and incubated at 37°C for 30 minutes. The 96 well plate was then placed in a kinetic microplate reader (Infinity M200 TECAN US, Morrisville, NC) and absorbance was read at 490 nm. DMEM with NGF was used as negative control.

Preparation of cytoplasmic and nuclear extracts

Cytoplasmic and nuclear extracts from transfected cells were isolated according to the method described by Krishnamoorthy et al., (1999). Briefly, pCMV6-Brn-3b or pCMV6-Empty vector transfected cells were collected 24 h post-transfection. Cells were suspended in 100 µl of Buffer C (10 mM HEPES buffer pH 7.9, 10 mM KCl, 0.2 mM EDTA, 10% glycerol, 1mM dithiothreitol (DTT), and 0.5mM phenylmethylsulfonyl fluoride (PMSF)). After incubation on ice for 15 min, 3 µl of 10% Igepal CA-630 (Sigma-Aldrich, St. Louis, MO) was added to the suspension and briefly vortexed. Cell nuclei were pelleted by centrifugation at 1,000g for 5 min at 4°C. The post-nuclear supernatant (cytoplasmic extract) was collected and stored at -80°C. The nuclear pellet was resuspended in 80 µl of Buffer D (20 mM HEPES, pH 7.9, 400 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM PMSF). The suspension was incubated for 20 minutes at 4°C followed by a centrifugation at 14,000g for 5 minutes. The supernatant (nuclear protein extract) was transferred to a fresh microcentrifuge tube and stored at -80°C. Protein concentrations of the cytoplasmic and nuclear extracts were measured with a detergent-compatible Protein Assay Kit (Bio-Rad), using bovine serum albumin as a standard.

Total Cellular Lysate

PC12 cells, overexpressing either pCMV6-Empty or pCMV6-Brn-3b subjected to normoxia or hypoxic insults, were homogenized in 300 µl of ice cold buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-630 (Sigma-Aldrich, St. Louis MO), 1% sodium deoxycholate, 1 mM sodium orthovanadate, 20 mM NaF, 10 mM sodium pyrophosphate decahydrate, 1M DTT, 0.25M PMSF and Halt™ Protease inhibitor single-use cocktail

(Thermo-scientific, Rockford, IL). The cellular homogenates were sonicated and protein concentration were measured with a detergent-compatible Protein Assay Kit (Bio-Rad), using bovine serum albumin as a standard.

Western blot analysis

Expression levels of Brn-3b, GAP-43 and ac-TUBA in pCMV6-Brn-3b or pCMV6-Empty vector transfected 661W or PC12 cells were analyzed by immunoblot analysis as described by Krishnamoorthy et al., (1999). Cytoplasmic extracts were used to detect GAP-43, ac-TUBA, GAPDH, calnexin, whereas nuclear extracts were used to detect Brn-3b and TATA binding protein. The following primary antibodies were used: an anti-mouse DDK tag (1:1000 dilution; Origene #TA50011-1, Rockville, MD), anti-rabbit GAP-43 (1:2000 dilution; Sigma-Aldrich #HPA015600, St. Louis, MO), and anti-mouse ac-TUBA (1:20000 dilution; Sigma-Aldrich #T7451, St. Louis, MO). The incubation with primary antibodies was carried out either for 1h at room temperature or overnight at 4°C. For normalization of protein loading, anti-mouse TATA binding protein (1:1000 dilution; Abcam #ab818 Cambridge, MA), anti-rabbit calnexin (1:5000 dilution; Enzo Life sciences # ADI-SPA-860, Farmingdale, NY), and anti-mouse GAPDH antibody (1:1000 dilution; Santa Cruz #sc-32233, Dallas, TX) were used. Incubation with an appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies and subsequent detection using Supersignal West Dura (Femto) Extended Duration substrate (peroxidase labeled appropriate secondary antibodies) were carried out. Serial images were taken in a Bio-Rad gel imager and the ratio of normalized band intensities were compared between different treatment groups.

Immunocytochemical Analysis

PC12 cells were seeded on poly D-lysine coated coverslips, transfected with either pCMV6-Brn-3b or pCMV6-Empty and maintained in differentiating media containing 100 ng/ml NGF for 4 days. The cells were subjected to either normoxia or hypoxia. Immunocytochemical detection of Brn-3b, GAP-43 and ac-TUBA proteins were carried out essentially as described by O'Connor et al., (2009). Briefly, the cells were fixed with 4% paraformaldehyde (PFA) containing 4% sucrose in 1x phosphate buffer (PBS) for 20 min at room temperature. Permeabilization was carried out with 0.1% Triton X-100, 0.1% sodium citrate in 1x PBS for 5 minutes at room temperature. To prevent non-specific binding, blocking was carried out for 1 h with 0.2% gelatin, 0.1% Triton X-100, 5% normal donkey serum and 5% BSA in 1x PBS. Following blocking, cells were treated with one of the following primary antibodies: a custom-made Brn-3b antibody (1:250 dilution of a rabbit polyclonal anti-Brn-3b from Antibody Research corporation, MO, USA); mouse anti-GAP-43 antibody (1:500 dilution; Sigma-Aldrich # G9264), mouse ac-TUBA (1:2000 dilution) and incubated overnight at 4°C. The primary antibody incubations were followed by incubation for 1 h with an appropriate donkey anti-IgG secondary antibody conjugated with Alexa 488, Alexa 633 or Alexa 546 (1:1000 dilution; Molecular Probes, Invitrogen, Eugene, OR). PC12 cells in which primary antibody incubation was excluded served as blank controls and were used to assess non-specific staining by the secondary antibody. Fluorescent images were taken in a Zeiss LSM 510 META confocal microscope.

Neurite growth analysis

Neurite outgrowth from PC12 cells overexpressing Brn-3b or Empty vector in hypoxic or normoxic conditions was counted by using the NIH ImageJ software (Neuron-J plug in, PC version: <http://rsbweb.nih.gov/ij/>) (Pool et al 2008; Torres-Espin et al. 2014; Barnat et al. 2010; Berg et al. 2013). Phase contrast images in 10 fields of view per coverslip per well were taken with an average of 4–5 cells per field. The number of neurites was determined by counting cells with at least one neurite having a length equal to the cell body diameter and expressed as percentage of the total cells in the field. Neurite length was measured by manually tracing the length of the longest neurite per cell with Neuron J software for all cells in a field that had an identifiable neurite and for which the entire neurite arbor could be visualized. Data from the 10 fields in each coverslip were pooled. Experiments were repeated at least five times (n=5) using cultures prepared on separate days.

Statistical analysis

For comparing results between two groups, student's *t*-test was performed. For comparison of results between more than two experimental groups, statistical analysis of data was carried out using one way One-Way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls post-hoc tests. In some experiments, ANOVA on ranks followed by Mann-Whitney Rank Sum test or Kruskal-Wallis post-hoc test was performed. Data was reported as mean $\pm$ SEM. A significant difference was defined as having a probability (p value) less than 0.05.

Results

Upregulation of GAP-43, ac-TUBA in 661W cells overexpressing transcription factor Brn-3b

Previous studies have shown a significant decrease in GAP-43 expression in the E14.5 retina from Brn-3b knockout mice as compared to the wild type mice, suggesting a potential regulatory linkage between Brn-3b and expression of GAP-43, a protein associated with axon elongation (Mu et al. 2001). However, it is unclear if Brn-3b upregulation could increase GAP-43 expression in cultured neurons. To test this, initial experiments were carried out using the cultured transformed 661W mouse photoreceptor cell line.

661W cells were transfected with either the pCMV6-Empty or pCMV6-Brn-3b vector (n=3) following which cytoplasmic and nuclear extracts were prepared and examined by western blot analysis. Overexpression of Brn-3b was confirmed by using the Brn-3b-DDK-tag antibody compared to 661W cells transfected with pCMV6-Empty vector (Figure 1a). Immunoblot analysis of 661W cells showed an appreciable increase in band intensities for GAP-43 and ac-TUBA in the pCMV6-Brn-3b transfected cells as compared to the pCMV6-Empty vector transfected cells (Figure 1a). Densitometric analysis of band intensities followed by statistical analysis indicated a significant increase in both GAP-43 and ac-TUBA in 661W cells over expressing Brn-3b (Figure 1b and 1c). GAPDH expression was used as a loading control in these experiments. These observations suggest that GAP-43 and ac-TUBA expression was markedly increased in 661W cells transfected with pCMV6-Brn-3b, compared to 661W cells transfected with pCMV6-Empty.

Overexpression of transcription factor Brn-3b lead to a significant increase in the expression of GAP-43, and ac-TUBA in PC12 cells

Since an increase in GAP-43 and ac-TUBA were observed by immunoblot analysis in 661W cells transfected with pCMV6-Brn-3b, further confirmation was done by overexpressing Brn-3b in a different cell line namely, the PC12 cells.

We sought to determine whether there are any changes in GAP-43 and ac-TUBA expression in PC12 cells transfected with pCMV6-Brn-3b. Briefly, PC12 cells were transfected with either the pCMV6-Empty or pCMV6-Brn-3b plasmid (n=3). Following transfection, cytoplasmic and nuclear extracts were made and subjected to western blot analysis. Overexpression of Brn-3b in PC12 cells was confirmed by using a Brn-3b-DDK tag antibody (Figure 2a). Similar to data obtained using 661W cells, immunoblot analysis indicated a statistically significant increase in the expression of GAP-43 and ac-TUBA in PC12 cells transfected with pCMV6-Brn-3b, compared to PC12 cells transfected with pCMV6-Empty vector (Figure 2a, 2b and 2c). GAPDH and TATA binding protein used as loading controls for cytoplasmic and nuclear extracts respectively were not appreciably different between the various experimental groups.

Further confirmation of these results was obtained from immunocytochemical analysis of PC12 cells overexpressing Brn-3b. As shown in Figure 2d, PC12 cells transfected with pCMV6-Brn-3b showed increased immunostaining for Brn-3b, confirming overexpression of Brn-3b in these cells, compared to PC12 cells overexpressing the pCMV6-Empty vector (n=3). Moreover, an appreciable increase in expression of growth cone-enriched protein, GAP-43, and ac-TUBA were found (detected by increased immunostaining) following overexpression of Brn-3b in PC12 cells. Furthermore, a marked change in morphology of pCMV6-Brn-3b transfected cells was also observed as compared to the pCMV6-Empty vector transfected cells (Figure 2d). Taken together, these results indicate that expression of GAP-43, ac-TUBA was significantly increased and neurite outgrowth was enhanced in PC12 cells transfected with pCMV6-Brn-3b, compared to PC12 cells transfected with pCMV6-Empty vector.

Overexpression of Brn-3b persisted under hypoxic conditions in PC12 cells transfected with pCMV6-Brn-3b

To determine whether Brn-3b overexpression persists under hypoxic conditions, we performed immunoblot analysis and immunocytochemistry in PC12 cells transfected with either pCMV6-Empty or pCMV6-Brn-3b vector. Following transfection, one set of transfected cells was exposed for 2 h to hypoxia (0.5% O₂, 5% CO₂) in glucose-free DMEM. Another set of similarly transfected cells was maintained in 5% CO₂ and 95% air (normoxia) in a standard incubator in differentiating medium (n=3) (Figure 3a: Experimental Scheme). Immunoblot analysis of pCMV6-Brn-3b transfected PC12 cells in normoxic as well as hypoxic conditions confirmed overexpression of Brn-3b, as compared to the corresponding pCMV6-Empty vector transfected cells (Figure 3b). In addition, increased immunostaining for Brn-3b was observed in pCMV6-Brn-3b overexpressing cells under conditions of both normoxia and hypoxia (n=3). No Brn-3b immunostaining was detected in pCMV6-Empty vector transfected cells under normoxia or hypoxia (Figure 3c). These data

indicate that overexpression of Brn-3b in pCMV6-Brn-3b transfected cells, also persists under conditions of hypoxia.

Upregulation of GAP-43 expression in pCMV6-Brn-3b transfected PC12 cells in normoxic and hypoxic conditions

Since a significant increase in GAP-43 expression was observed in pCMV6-Brn-3b transfected cells, compared to pCMV6-Empty vector transfected PC12 cells (Figure 2), GAP-43 expression was also assessed in PC 12 cells under hypoxic conditions. PC12 cells were transfected either with pCMV6-Empty or pCMV6-Brn-3b vector. One group of transfected cells was subjected to hypoxia (0.5% O₂, 5% CO₂) in glucose-free DMEM for 2 h, while another group of transfected cells was maintained in normoxic conditions (5% CO₂ and 95% air) in standard incubator. Immunoblot analysis of pCMV6-Brn-3b transfected cells demonstrated a marked upregulation in GAP-43 expression compared to those of pCMV6-Empty transfected cells in normoxia. pCMV6-Empty transfected PC12 cells maintained under hypoxic conditions showed a significant increase in GAP-43 expression, compared to PC12 cells overexpressing empty vector in normoxia. (Figure 4a and b) (n=7). PC12 cells maintained under hypoxia after transfection with pCMV6-Brn-3b showed a trend towards upregulation of GAP43 compared to cells transfected with the empty vector and maintained under hypoxia, however this was not statistically significant.

Using immunocytochemical analysis, (Figure 4c) PC12 cells transfected with pCMV6-Brn-3b showed increased immunostaining for GAP-43 in normoxia as well as in hypoxia, as compared to the corresponding pCMV6-Empty vector transfected cells in both conditions (n=3).

PC12 cells transfected with pCMV6-Brn-3b show increased expression of ac-TUBA in normoxia and hypoxia

Our previous experiments demonstrated that under normoxic conditions overexpression of pCMV6-Brn-3b in PC12 leads to increased expression of ac-TUBA. To evaluate the expression of ac-TUBA during hypoxic conditions, PC12 cells were transfected either with pCMV6-Empty vector or pCMV6-Brn-3b. One group of transfected cells was subjected to hypoxia (0.5% O₂, 5% CO₂) in glucose-free DMEM for 2 h, while another group of transfected cells was kept in normoxic conditions. As shown in figure 5a and 5b, immunoblot analysis indicated a significant upregulation of ac-TUBA in pCMV6-Brn-3b transfected cells under both the conditions, as compared to pCMV6-Empty transfected cells in normoxia. The levels of ac-TUBA are maintained in PC12 cells transfected cells with the pCMV6-Brn-3b in normoxia as well as in hypoxia. Under hypoxic conditions, there was an increasing trend (not statistically significant) in the expression of ac-TUBA in PC12 cells overexpressing Brn-3b compared to PC12 cells transfected with empty vector subjected to hypoxia. However, ac-TUBA expression in empty vector transfected PC12 cells subjected to hypoxia, compared to empty vector transfected cells maintained in normoxia was not significantly different. Figure 5c shows a prominent increase in immunostaining of ac-TUBA in PC12 cells overexpressing Brn-3b in normoxia as well as hypoxia, as compared to PC12 cells transfected with pCMV6-Empty vector under both conditions (n=4). There was a readily discernible change in the morphology of PC12 cells overexpressing Brn-3b in

normoxia and hypoxia, compared to the corresponding control cells transfected with the empty vector.

Significant increase in mean neurite length and number of cells with processes in Brn-3b overexpressing PC12 cells in normoxia as well as in hypoxia

Analysis of DIC images of PC12 cells transfected with the pCMV6-Brn-3b vector subjected to both normoxia or hypoxia indicated a substantial increase in neurite outgrowth, compared to the corresponding empty vector transfected cells. Using the Image J (NeuronJ-plugin in) software, the mean neurite length and number of cells having processes were manually counted. The experiment was performed five times (n=5) and the cumulative values of mean neurite length and number of cells with processes were computed. The mean neurite length in PC12 cells transfected with pCMV6-Brn-3b in both normoxia and hypoxia was found to be significantly greater than in PC12 cells transfected with pCMV6-Empty vector under both the conditions (Figure 6a). Moreover, the number of cells having processes was also significantly higher in Brn-3b overexpressing cells in both normoxia and hypoxia, compared to the empty vector overexpressing cells under normoxia (Figure 6b). There was trend towards an increase (not significant) in number cells having processes in Brn-3b transfected PC12 cells, compared to the empty vector transfected PC12 cells under conditions of hypoxia. There was no statistically significant difference in number of cells with processes between Brn-3b overexpression in normoxia and Brn-3b overexpression in hypoxia (Figure 6b).

Effects of hypoxia on PC12 cells overexpressing Brn-3b with or without NGF

To assess cell survival after hypoxia, a cell proliferation assay (MTT assay) was performed with PC12 cells transfected with either the pCMV6-Empty or pCMV6-Brn-3b vector. After transfection, one set of PC12 cells were maintained in differentiated medium with NGF and exposed to either normoxia or 2 h hypoxia (0.5% O₂, 5% CO₂) in glucose-free DMEM (Figure 7a). Another set of similarly transfected cells was maintained in differentiating medium without NGF and subjected to either normoxia or hypoxia in a standard incubator (Figure 7b). The experiment was carried out twice with eight wells for each treatment group (n=16). Our results demonstrate that in the presence of NGF there is a modest, yet significant decrease in viability of PC12 transfected with empty vector maintained under hypoxia, compared to empty vector transfected PC12 maintained under normoxia. This suggests that the 2h hypoxic insult was sufficient to produce a modest reduction in cell survival. Moreover, a significant increase in cell survival was found in cells transfected with the Brn-3b construct under hypoxia compared to corresponding empty vector transfected cells maintained under hypoxia (in the presence of NGF) (Figure 7a).

The other set of PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b in the absence of NGF subjected to either hypoxia or normoxia, did not show any significant changes in viability when the different treatment groups were compared (Figure 7b).

Discussion

The present study suggests the involvement of Brn-3b in neurite outgrowth (Gan et al. 1999; Erkman et al. 2000; Erkman et al. 1996; Gan et al. 1996; Wang et al. 2000; Camp et al. 2011) in PC12 cells under conditions of normoxia, as well as hypoxia, in the presence of NGF, and also delineates some mechanisms underlying Brn-3b-mediated neurite outgrowth.

Brn-3b plays a pivotal role in the development and physiological differentiation of RGCs, which is evident by the loss of almost 70% of these cells in Brn-3b deficient mice. During development, lack of Brn-3b results in abnormal axon projections and defects in axon pathfinding (Xiang et al. 1997; Erkman et al. 1996). The function of Brn-3b in normal physiology in the adult retina, as well as in pathological conditions, is largely unknown. Studies examining the effects of hypoxia and hypoxia-reoxygenation on cultured neurons, simulate some cellular aspects of the clinical and pathological course of several neurodegenerative diseases, such as Alzheimer's disease, Prion diseases, Parkinson's disease, Huntington's disease and glaucoma (Alvarez-Tejado 2001; Czyzyk-Krzeska et al. 1994). The protective role of Brn-3b under hypoxic conditions in neuronal cells overexpressing Brn-3b has not been investigated. This is the first study that demonstrates that Brn-3b has the potential to enhance neurite outgrowth in PC12 cells and this was demonstrated under conditions of both normoxia and hypoxia.

The structural and functional integrity of the brain and retina depends upon a regular oxygen and glucose supply. Any disruption of this supply may result in a severe loss of brain or retina function. Specifically, reduction in oxygen availability (hypoxia) caused by disturbances in blood circulation cannot be tolerated for long durations due to inadequate energy supply to the brain and retina by anaerobic glycolysis. Past studies have shown that hypoxia plays an important role in the pathology of a number of central nervous system disorders, including stroke, brain trauma and neurodegenerative diseases (Acker and Acker 2004). The posterior ciliary artery circulation plays an important role in maintaining perfusion of the various ocular structures including the ciliary body and optic nerve head. Disruption in blood circulation of the retina and optic nerve results in various ocular and optic nerve head disorders, causing varying degrees of visual loss. It includes glaucomatous optic neuropathy, anterior ischemic optic neuropathy and optic ischemic neuropathy (Hayreh 2004; Gupta and Yucel 2007). In these diseases, repeated episodes of hypoxia lead to chronic damage to the optic nerve and eventually apoptosis of RGCs. Therefore, the experimental paradigm of mild hypoxia provides a suitable model to study the changes occurring at cellular and molecular level in these diseases.

Since there is no available retinal cell line, 661W and PC12 cells were used as a model for this study. 661W is a mouse photoreceptor cell line, which is difficult to differentiate. Therefore, we used PC12 cell line which has been shown to differentiate into cells with a neuron-like phenotype after treatment with NGF. PC12 cells differentiated with NGF have been widely used to study molecular mechanisms underlying neurite growth of cultured neuronal cells. NGF was added to PC12 cells in almost all neurological studies so that the cells could differentiate into neurons (Lee et al. 1977; Cowley et al. 1994; Pang et al. 1995; Takebayashi et al. 2002; Das et al. 2004, Alvarez-Tejado et al. 2001; Czyzyk-Krzeska et al.

1994). In the current study, without addition of NGF, we could not overexpress Brn-3b in PC12 cells.

Transcription factor Brn-3b could be a potential candidate for the treatment of various neurodegenerative conditions including glaucoma. Gene therapy with AAV-Brn-3b could have minimal undesirable effects, as Brn-3b is endogenously expressed in adult retinas and different parts of the brain, including superior colliculus, and interpeduncular nucleus of trigeminal ganglion (Turner et al. 1994). Although, Brn-3b is constitutively expressed in adult RGCs, its role in the physiology of mature RGCs remains to be understood. Studies have shown that a decrease in Brn-3b expression occurs prior to RGCs death in optic nerve injury model in rats (Soto et al. 2008; Weishaupt et al. 2005; Naskar and Thanos 2006).

One of the integral components of a growth cone is GAP-43, which plays an important role in regulation of axon growth in the neurons (Benowitz and Routtenberg 1997). Studies have shown a reduction in GAP-43 expression in the E14.5 retina of Brn-3b deficient mice (Mu et al. 2001). The rat GAP-43 promoter has a putative Brn3 binding site, located at 735 bp upstream from the transcription start site. This could account for the ability of transcription factor Brn3b to upregulate GAP-43 expression in PC12 cells. Our data demonstrate that Brn-3b contributes to upregulation of GAP-43, and the levels of GAP-43 are sustained in Brn-3b overexpressing PC12 cells under hypoxia. Increased expression of GAP-43 has been shown to enhance neurite outgrowth in PC12 cells. The current study suggests that Brn-3b has the potential to be a neuroprotective agent in various neurodegenerative diseases and neuronal injury. However, detailed research on Brn-3b and proteins regulated by Brn-3b is needed to understand its potential neuroprotective role in different animal models of neurodegeneration and nerve injury.

It is clear from several studies that the cytoskeleton is a key target for many signaling pathways that affect neurite outgrowth. In the axonal transport machinery, microtubules and actin filaments serve as “rail road” tracks for active transport. Kinesin, dynein and myosin are ATP dependent molecular motors responsible for transport of various molecular cargos. It is known that axonal transport can be affected by variations in the components of the transport machinery. Defects in the axonal transport, such as mitochondrial dysfunction and axonal transport failure induce RGCs death in glaucoma and also contribute to the pathogenesis of a number of neurodegenerative diseases (Qu et al. 2013; Liu et al. 2012; Millecamps et al. 2013; Almasieh et al. 2012). In the current study, we demonstrate that there is a significant increase in the expression of ac-TUBA in PC12 cells overexpressing Brn-3b under conditions of both normoxia and hypoxia. Acetylated α -tubulin (ac-TUBA) plays an important stabilizing role during the protrusion and elongation of axons. Acetylation of the α -tubulin using a histone deacetylase inhibitor has been shown to stimulate anterograde as well as retrograde transport in primary cortical neurons (Gaub et al. 2010). Sengottuvel et al., (2011) have shown that a low concentration (3 nM) of the microtubule stabilizing drug, taxol, promotes neurite elongation of RGCs in culture. Another study demonstrated that total tubulin mRNA levels decrease after injury to the optic nerve but increase in those RGCs that regenerated their axons into a peripheral nerve graft (Fournier and Mckerracher 1995). Hence, the ability of Brn-3b to produce an increase in ac-TUBA has implications for its axonal regenerative effects following axonal injury during

hypoxia in the presence of NGF, which has not been studied earlier. However the precise mechanisms underlying the ability of transcription factor Brn-3b to upregulate ac-TUBA are not completely understood and will be the subject of future studies. In the current study, two different cell lines (661W and PC12 cells) were used to study the effect of Brn-3b overexpression for confirmation of a significant increase in expression of GAP-43 and ac-TUBA in Brn-3b overexpressing cells. Even though PC12 cells and 661W cells have no relation to RGCs, they could provide an insight into basic mechanisms governing cell survival and neurite outgrowth during hypoxia. One caveat to this model is that cellular and molecular processes occurring in cell lines may not be accurately reflective of what occurs *in vivo*.

PC12 cells transfected with the empty vector and maintained under hypoxia show a modest increase in ac-TUBA (not statistically significant), which was not reflected in the neurite outgrowth or number of cells with processes. On the other hand, PC12 cells transfected with empty vector and maintained under hypoxia showed a significant increase in GAP-43 expression, compared to PC12 cells maintained in normoxia, suggesting that upregulation of GAP-43 alone is not sufficient to promote neurite outgrowth under conditions of hypoxia. Overexpression of Brn-3b produce an increase in the mean neurite length as well as number of cells with processes under conditions of both normoxia and hypoxia, in comparison with empty vector transfected PC12 cells. All the experiments (except the viability assay) were performed in the presence of NGF. We tried to carry out initial experiments in the absence of NGF treatment, however the cells were unhealthy and there was no efficient transfection of Brn-3b in the absence of NGF treatment in PC12 cells. Hence, while there may a confounding effect of NGF treatment, this was the case in all the treatment groups. Overall, the data suggests that transcription factor Brn-3b could upregulate expression of several genes contributing to neurite outgrowth in PC12 cells.

In summary, this study demonstrates that overexpression of Brn-3b in PC12 cells under normoxia as well as hypoxia leads to upregulation of GAP-43 and ac-TUBA and results in potentiation of neurite outgrowth. However, Brn-3b mediated effects on gene expression and potential targets need to be studied in greater detail in animal models of neurodegeneration. Additional research is needed to understand the potential role of Brn-3b in RGC survival and neuroprotection in glaucomatous neurodegeneration.

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Figure 1. Brn-3b overexpression leads to a significant increase in expression of GAP-43 and ac-TUBA in 661W cells

a. Immunoblot analysis of Brn-3b-DDK tag, GAP-43, and ac-TUBA in 661W cells transfected either with the pCMV6-Empty or pCMV6-Brn-3b vector, 24 h post-transfection. The blots were probed for GAPDH as a loading control. **b, c.** Densitometric analysis shows a significant increase of GAP-43 and ac-TUBA in Brn-3b transfected 661W cells as compared to the empty vector transfected 661W cells. Fold change in proteins expression were shown as mean $\pm$ SEM, $n=3$. Student's t-test was used for statistical analyses (* $p<0.05$).

Figure 2. Up-regulation of GAP-43, ac-TUBA in PC12 cells transfected with Brn-3b

a. Immunoblot analysis of nuclear extracts for Brn-3b-DDK tag expression and cytoplasmic extracts for expression of GAP-43 and ac-TUBA in PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b vectors, 24 h following transfection. GAPDH was used as the loading control. **b, c.** Overexpression of Brn-3b in PC12 cells induced a significant up-regulation of GAP-43 and ac-TUBA when compared to the empty vector transfected PC12 cells. Student's t-test was used for statistical analyses (* $p < 0.05$). Fold change in the specific protein expression is shown as mean $\pm$ SEM, $n = 3$. **d.** Immunocytochemical analyses of Brn-3b, GAP-43 and ac-TUBA expression after 4 days in differentiating medium with NGF in PC12 cells transfected with Brn-3b. The immunostaining was detected by using corresponding Alexa 488-, Alexa 547- and Alexa 633- conjugated donkey anti-IgG secondary antibodies. Green: Brn-3b; Pink: GAP-43; Red: ac-TUBA; Blue: DAPI. Fluorescence images were taken in a confocal microscope and merged images of the different proteins immunofluorescence with DIC and DAPI was obtained. Scale bar indicates 20 μm ($n = 3$).

Figure 3. Overexpression of Brn-3b persisted under conditions of hypoxia in PC12 cells transfected with pCMV6-Brn-3b

a. Experimental scheme used for assessing protein expressions in PC12 cells under conditions of hypoxia. Cells were seeded on poly-D-lysine coated 100 mm dishes for western blot analyses and transfected with either pCMV6-Empty or pCMV6-Brn-3b (5 μ g). Following transfection, cells were maintained in differentiating medium with NGF (100ng/ml) overnight and were either maintained in normoxia or subjected to oxygen glucose deprivation condition (0.5% O₂ and 5% CO₂) for 2 h. Cytoplasmic and nuclear extracts were prepared and used for various immunoblot analyses. In separate experiments for immunocytochemistry, pCMV6-Empty and pCMV6-Brn-3b transfected cells were maintained in differentiating medium with NGF (100 ng/ml) for 4 days. The transfected cells were subjected to conditions of normoxia or hypoxia for 2 h, as described earlier. PC12 cells were then fixed and expression levels of specific proteins were analyzed by using immunocytochemistry. **b.** Immunoblot analysis of Brn-3b DDK tag in PC12 cells transfected with either pCMV6-Empty or pCMV6-Brn-3b and maintained under either normoxia or hypoxia (n=3). **c.** PC12 cells seeded on coverslips were transfected with either pCMV6-Empty or pCMV6-Brn-3b, subjected to either normoxia or hypoxia and immunostained using specific custom-made antibody to Brn-3b. A marked increase in Brn-3b staining was observed in Brn-3b overexpressing cells in normoxia and in hypoxia when compared to cells overexpressing the empty vector in both the conditions (n=3).

Figure 4. Transcription factor Brn-3b overexpression mediated increase in growth cone marker GAP-43 in PC12 cells under conditions of hypoxia

a. Western blot analysis was carried out to detect the protein level of GAP-43 in PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn-3b vector, and maintained for 2 h in normoxia and hypoxia, 24 h post-transfection. Calnexin, a housekeeping gene, served as a loading control. **b.** Densitometric analysis shows a significant increase in GAP-43 expression in Brn-3b overexpressing PC12 cells when compared to PC12 cells overexpressing empty vector in normoxia. One way ANOVA with Student-Newman-Keuls multiple comparison test was used for statistical analysis (* $p < 0.05$). The fold change in GAP-43 expression was shown as mean $\pm$ SEM, ($n=7$). **c.** PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b maintained in differentiating medium for 4 days. On the fifth day, one set of cells were maintained in normoxia, while another set was subjected to conditions of hypoxia for 2h. Cells were immunostained for GAP-43 expression (pseudocolor: pink) using GAP-43 antibody. A dramatic increase in GAP-43 staining and neurite elongation was observed in PC12 cells overexpressing Brn-3b in normoxia and in hypoxia as compared to PC12 cells overexpressing the empty vector in both conditions. Scale bar indicates 20 μ m ($n=5$).

Figure 5. Brn-3b promoted an increase in expression ac-TUBA in PC12 cells under conditions of normoxia and hypoxia

a. Immunoblot analysis of total cellular extracts of PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn-3b vector, maintained under either normoxia or hypoxia. The blots were probed for calnexin as a loading control. **b.** A significant increase in ac-TUBA expression was observed in PC12 cells overexpressing Brn-3b in normoxia as well as in hypoxia. Mann Whitney Rank Sum test was used for statistical analysis (* $p < 0.05$). Fold change in ac-TUBA was shown as mean $\pm$ SEM, ($n = 5$). **c.** Immunostaining of ac-TUBA (red fluorescence) was carried out in PC12 cells transfected with pCMV6-Empty or pCMV6-Brn-3b vector and maintained in differentiating medium for 4 days, followed by 2h in normoxia or hypoxia. A prominent increase in immunostaining for ac-TUBA was observed in PC12 cells overexpressing Brn-3b in normoxia as well as in hypoxia as compared to PC12 cells overexpressing empty vector in both the conditions. Scale bar indicates 20 μ m ($n = 5$).

Figure 6. Increase in mean neurite length and number of cells having processes in Brn-3b overexpressing PC12 cells in normoxic and hypoxic conditions

a. Mean neurite length was measured at 10 different fields in each coverslip with an average of 10 cells per field using NIH Image J. For statistical analysis, Kruskal –Wallis one way ANOVA was used (* $p < 0.05$). A statistically significant increase in neurite length was observed in PC12 cells overexpressing Brn-3b in both normoxia and hypoxia conditions as compared to PC12 cells overexpressing the empty vector in both the conditions. **b.** Number of cells having processes was measured at 10 different fields in each coverslip with an average of 10 cells per field using NIH Image J. Statistical analysis one way ANOVA followed by Student-Newman-Keuls multiple comparison test was used (* $p < 0.05$). The mean maximum neurite length and mean number of cells having processes values are shown as mean $\pm$ SEM, $n=5$ (total number of cells counted= 612).

Figure 7. Transcription factor Brn-3b overexpression (in the presence of NGF) protects against loss of cell viability under conditions of hypoxia

Cell proliferation assay (MTT assay) was carried out to determine viability of PC12 cells subjected either normoxia or hypoxia after transfection with either the empty vector or pCMV6-Brn-3b in the presence or absence of NGF. The Y axis depicts absorbance values at 490 nm indicative of cell viability. **a.** Cell viability was tested in the presence of NGF: Four experimental groups were tested: PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn-3b vector under conditions of either normoxia or hypoxia. **b.** Cell viability was presented in the absence of NGF. Four experimental groups were tested including: PC12 cells overexpressing pCMV6-Empty or pCMV6-Brn-3b vector under conditions of normoxia and hypoxia. One way ANOVA with Student-Newman-Keuls multiple comparison test was used for statistical analysis (* $p < 0.05$). Absorbance at 490 nm (indicative of cell viability) was shown as mean $\pm$ SEM, (n=16).