Changes in BDNF and MAPK Signaling Pathways in Experimental Glaucoma

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Abstract

Glaucoma is currently recognized to be a multifactorial, progressive, neurodegenerative disorder. It is characterized by the retinal ganglion cells (RGCs) loss of axons as well as optic nerve atrophy, progressive degeneration of RGCs till cell death. In this work, we used DBA/2J mice as a model of spontaneous glaucoma and we investigated the involvement of BDNF and Mitogen-Activated Protein Kinases (MAPK) pathways in correlation with IOP elevation and progression of neurodegenerative processes in the retina of DBA/2J mice. In particular, we performed western blot analysis to study retinal levels of the BDNF and its receptor, TrkB, and to better understand possible modulation of p38 MAPK and ERK1/2 activation at different stages of retinal degeneration in DBA/2J mice. We showed that BDNF starts to decrease already at an early stage in correspondence to IOP elevation (7 months of age). MAPKs, in particular p38 MAPK and ERK1/2, appeared maximally affected at more advanced stages of neurodegeneration (10-12 and 18 months of age) characterized by RGC degeneration and death, optic nerve atrophy. Thus, BDNF signaling and MAPKs are differentially activated at different stages of retinal degeneration in DBA/2J mice, a murine model of glaucoma.

Keywords Retinal degeneration; MAPK; BDNF

Introduction

Glaucoma is a group of eye disorders, currently recognized to be multifactorial, progressive, leading to reduction in vision and eventual blindness. Glaucoma is characterized by progressive degeneration of the retinal ganglion cells (RGCs) and optic nerve fibers; it is one of the leading causes of vision loss. Over 60 million people worldwide were estimated to be affected with open-angle glaucoma (OAG) in 2010, and bilateral blindness from the disease was estimated to be present in 4.5 million people with OAG in 2010, rising to 5.9 million people in 2020 [1]. A generally accepted theory suggests an initial insult to the axons of RGCs in the optic nerve head region, where they exit the eye [2]. Usually, glaucoma develops in adults. Glaucoma is characterized by structural anomalies such as the retinal ganglion cells (RGCs) loss of axons as well as optic nerve (ON) atrophy, progressive degeneration of RGCs till cell death, impairment of visual function with visual field defects and finally loss of neurons in the lateral geniculate nucleus and the visual cortex. In OAG, intraocular pressure (IOP) represents the major risk factor for glaucoma onset and progression. However, in the phase characterized by elevated IOP it is generally accepted that there is no obvious damage to the ON or evidence of visual field change and RGC structural anomalies. It was reported that the rate of untreated ocular hypertension patients developing glaucoma was about 2 percent per year [3,4]. Here we investigated factors/mechanisms involved in the ocular hypertension and glaucoma. To this aim we used DBA/2J mice as a pigment dispersion glaucoma model. The DBA/2J mouse has two mutated genes, tyrosinase-related protein 1 (Tyrp1) and glycoprotein non-metastatic melanoma protein B (Gpnmb); these mice are characterized by anterior chamber pathology consisting of pigment dispersion and development of iris stroma atrophy leading to the blockade of vitreous humor drainage and progressive elevation of IOP

[5]. In DBA/2J mice, elevated IOP is initially detected around 6 months of age in absence of RGC death and ON atrophy and degeneration, followed at a later age (10-12 months of age) by ON atrophy, RGC degeneration and death [5], i.e. the characteristic features of glaucoma. Thus, DBA/2J mouse represents a good model to investigate mechanisms involved in progressive retinal neurodegeneration from a phase characterized by ocular hypertension to glaucoma. We investigated DBA/2J mice at an early stage of degeneration characterized by ocular hypertension with impairment of retinal responses to visual stimuli (7 months of age) without RGC death, at an intermediate stage characterized by ON atrophy and RGC degeneration (10-12 months of age) and at a later stage with massive RGC death and soma loss [6].

We focused our study on Brain-Derived Neurotrophic Factor (BDNF) and Mitogen-Activated Protein Kinases (MAPK) such as the p38 MAPK and ERK1/2 whose state of phosphorylation is modulated by BDNF [7,8]. BDNF is a neurotrophic factor indispensable for the survival and plasticity of several subtypes of neurons in the nervous system, including RGC [9-11]. BDNF is locally produced by cells in the ganglion cell and inner nuclear layers [12]; its TrkB receptor is expressed in RGCs, amacrine and Müller cells [13] that represent the cellular target of BDNF trophic action. TrkB mediates BDNF internalization and its retrograde transport; RGCs take up BDNF and transports it along axons towards target neurons and back to the cell body in the retina.

Recently, it has been reported that BDNF transport is altered in glaucoma with reduction of retinal BDNF level [14,15]; the interruption of BDNF retrograde transport and accumulation of TrkB at the optic nerve head in acute and chronic glaucoma models suggests a role for BDNF retrograde signaling in RGC degeneration in glaucoma. These results are in line with the presence of the BDNF and

its receptor TrkB in RGCs and optic nerve fibers [13], lateral geniculate nucleus (LGN) [16,17] and superior colliculus (SC) [15] of adult mammals. BDNF has been tested in animal models of glaucoma and some studies have shown to reduce RGC death [8,18]. However, the question of whether BDNF and/or its receptor TrkB are affected by ocular hypertension and in the steps involved in progression to glaucoma is largely unanswered. Importantly, supplying BDNF by intravitreal injection or topical eye applications during an early stage in DBA/2J mice (7 months of age) resulted in rescue of visual function and RGC alterations [19]. MAPKs, consisting of three major enzymes-extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK) couple cell-surface receptors to critical regulatory targets and gene transcription [20]. Here we investigated the p38 MAPK and extracellular signal-regulated kinases 1 and 2 (ERK1/2), two intracellular pathways linked to BDNF signaling and involved in optic nerve injury and retinal ischemia [7,8,21-24]. Indeed, ERK1/2 and p38 MAPK can be activated by a variety of different stimuli such as growth factors and stress stimuli. ERK1/2 activation is often associated to pro-survival stimuli also in retinal dysfunction. It has been demonstrated that ERK1/2 phosphorylation decreases in RGCs following optic nerve transection, whereas its induction is linked to neuroprotection in several experimental models [17,25-27]. On the other hand, p38 MAPK isoforms are phosphorylated in neurons, by stress stimuli, including osmotic shock, excitotoxicity, pro-inflammatory cytokines, oxidative injury and beta amyloid [28,29] and act as upstream activators of myriad pathogenic processes; for example, p38 MAPK phosphorylation induces synaptic dysfunction and activates downstream inflammatory signaling cascades [29,30]. We tested the effect of elevated IOP on BDNF signaling and MAPK activation at an early stage of retinal degeneration characterized by ocular hypertension, and at late stages of retinal degeneration with RGC degeneration and ON atrophy in DBA/2J mice.

Materials and Methods

Ethics statement

All experiments were conducted according to the Ministry of Health (the regulatory authority for controlling the use of laboratory animals and ethics on animal experiments in Italy) guidelines (Legislative Decree n. 116/92) and in accordance with the European Community guidelines (European Directive 86/609/EEC). The experimental protocol (IACUC document) was approved by the Ministry of Health (n. 152/2013; IACUC is renewed every three years).

Animal groups and IOP measurement

Animals were adult C57BL/6J (7 months of age) and DBA/2J mice (Charles River Laboratories International Inc., Santangelo Lodigiano, Italy) of different ages: 3, 7, 10-13 (10) and 15-18 (15) months of age, reared on a 12-h light/dark cycle (animals were kept in a 12 h light/12 h dark cycle, with the illumination level below 60 photopic lux). Repeated measurements of IOP were performed using TonoLab tonometer (Icare, Finland) in unanaesthetized mice trained to take position in a partial restrainer without invasive handling; the tonometer was held and maneuvered through the z-axis of a micromanipulator, IOP was measured in the morning 10-12 am to avoid oscillation. We calculated the mean of 6 measurements from each eye. At least twelve eyes per group of mice were analyzed.

Western blot

For western blot analysis the following primary antibodies were used: anti-BDNF 1:50(AB1534SP, Merck Millipore) in TBS with 0.1% Tween-20; anti-TrkB 1:500 (sc-12, Santa Cruz); anti-phospho-p38 1:1000 (09-272, Merck Millipore); anti-p38 1:1000 (506123, Merck Millipore); anti-phospho-ERK1/2 1:1000 (#4377, Cell Signaling); anti-ERK1/2 1:1000 (#4695, Cell Signaling); anti-β-tubulin 1:2000 (#2128, Cell Signaling) all in a solution of Odyssey blocking buffer and PBS 1X with 0.2% Tween-20 (1:1). Secondary antibodies: anti-rabbit IgG antibody HRP-linked 1:20000 (#7074, Cell Signaling) in TBS with 0.2% Tween-20; anti-rabbit IRDye 800CW secondary antibody 1:10000 (926-3221, LI-COR Biosciences) in a solution of Odyssey blocking buffer and PBS 1X with 0.2% Tween-20 (1:1).

Retinas of different mice were dissected from eyes, homogenized in lysis buffer (10 mM Tris HCl, pH 7.6, 100 mM NaCl, 1% Triton X-100, 0.1% SDS) containing protease (S8820, Sigma) and phosphatase (2 mM Na_3VO_4, 1 mM NaF, 20 mM Na_4P_2O7) inhibitors and centrifuged at 13000 rpm for 10 min at 4°C. Retinal homogenates were processed using western blot. Briefly, an equal amount of proteins (50 μg) were resolved electrophoretically on a 4-12% SDS-PAGE Bis-Tris gel (Bio-Rad Criterion XT). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane 0.45 μm, and blocked, for 1 h at room temperature, in Odyssey blocking buffer (LI-COR, Biosciences) or TBS 5% dry skimmed milk and 0.2% Tween-20 depending on the final method of detection.
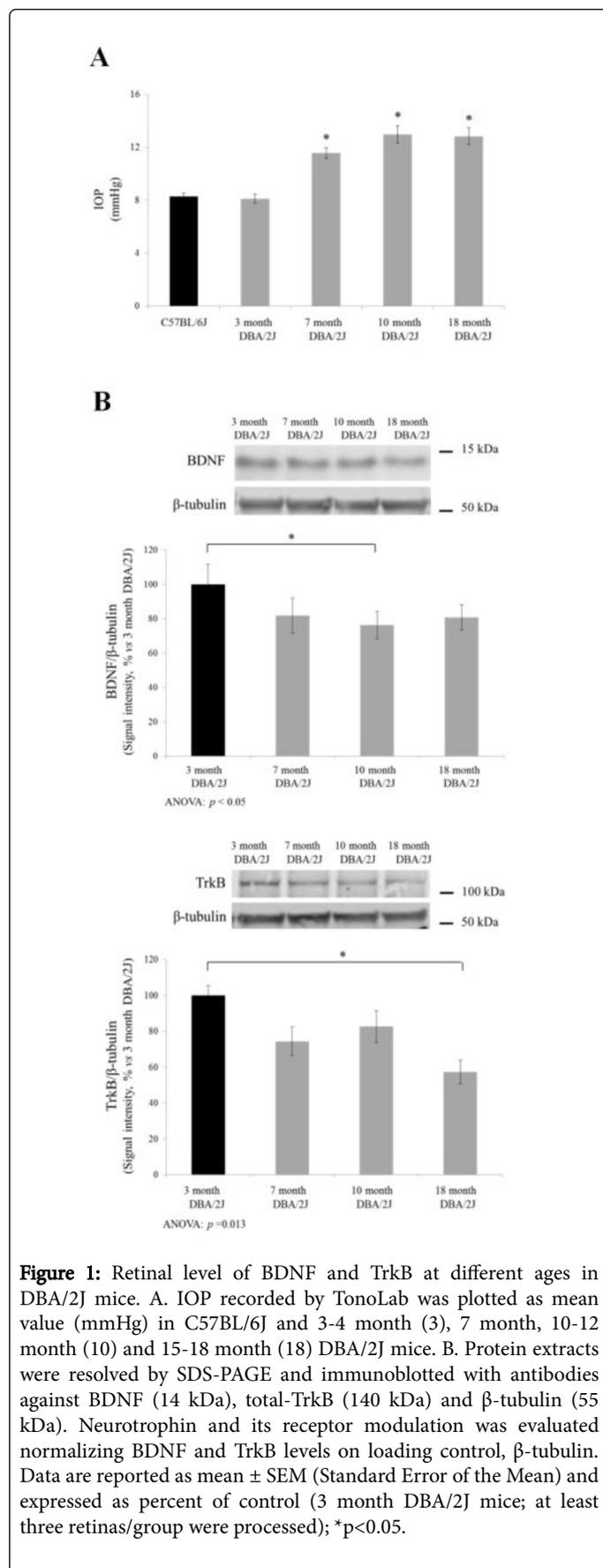
Membranes were incubated with primary antibodies, in appropriate solutions, at 4°C overnight. BDNF and TrkB levels were normalized on the loading control, β-tubulin. To evaluate protein phosphorylation, membranes were probed with primary antibodies against phosphorylated and total form of the specific protein targets. After washing, membranes were incubated for 1 hour at room temperature with secondary antibodies, labeled with HRP or NIR fluorescent dyes, depending on the chosen detection method. As controls in a separate lane we used a known amount of human recombinant BDNF. Proteins were detected through enhanced chemiluminescence (ECL) detection system (Clarity Western ECL Substrate, Biorad) or imaged with a LI-COR infrared imaging system (Odyssey CLx, LI-COR Biosciences), basing on the kind of employed secondary antibody. We processed at least 3 retinas for each group.

Statistical analysis

To analyze IOP value we used one-way analysis of variance (ANOVA)(SigmaStat, Jandell Scientific). For Western blot, all data were reported as percentage (mean ± SEM) of the internal control (DBA/2J mice at the age of 3 months). Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni post hoc test when $P<0.05$ (SigmaStat, Jandell Scientific). Differences were considered significant only when $P<0.05$.

Results

As a model of glaucoma we used the DBA/2J mouse, which develops ocular hypertension in adulthood. IOP was already elevated in 7 month-old-DBA/2J mice [31] (C57BL/6J control mice, n=20 eyes, mean=8.12 mmHg, SEM=0.34; 3 month DBA/2J, n eyes=20, mean=8.10 mmHg, SEM=0.33; 7 month DBA/2J, n eyes=12, mean=11.59 mmHg, SEM=0.40; 10-13 month DBA/2J, n eyes=12, mean=13.00 mmHg, SEM=0.65; 15-18 month DBA/2J, n eyes =12, mean=12.85 mmHg, SEM=0.62) shown in figure 1A.

Figure 1: Retinal level of BDNF and TrkB at different ages in DBA/2J mice. A. IOP recorded by TonoLab was plotted as mean value (mmHg) in C57BL/6J and 3-4 month (3), 7 month, 10-12 month (10) and 15-18 month (18) DBA/2J mice. B. Protein extracts were resolved by SDS-PAGE and immunoblotted with antibodies against BDNF (14 kDa), total-TrkB (140 kDa) and β-tubulin (55 kDa). Neurotrophin and its receptor modulation was evaluated normalizing BDNF and TrkB levels on loading control, β-tubulin. Data are reported as mean ± SEM (Standard Error of the Mean) and expressed as percent of control (3 month DBA/2J mice; at least three retinas/group were processed); *p<0.05.

These data are in agreement to previous results [19]; here we show that IOP elevation in DBA/2J mice persists at the late ages of 13-18 months. We'd like to underline that in the same strain of DBA/2J mice IOP was in the normal range when measured in younger animals, at the age of 3 months (Figure 1A).

We investigated the expression of BDNF and of its receptor TrkB at different ages in DBA/2J mice (Figure 1B). BDNF retinal level was decreased already at 7 months of age in DBA/2J mice with ocular hypertension remaining stably depressed from 7 to 18 months of age (Figure 1B). Concerning the BDNF receptor, the expression level of TrkB receptor is slightly reduced in the retina of 7 month DBA/2J mice; this trend is more pronounced at later ages with the level of TrkB becoming significantly reduced only at 18 months of age. Thus, the retinal level of BDNF is affected by ocular hypertension.

We evaluated MAPK activation, intracellular pathways potentially involved in ocular hypertension and glaucoma. In particular, we decided to study the phosphorylation of two crucial members of the family, p38 MAPK and ERK1/2. Phosphorylation of p38 MAPK started in DBA/2J mice of 10 months of age and raised more in old DBA/2J mice of 18 months of age (Figure 2A). ERK1/2 showed a slight decrease of phosphorylation in 7 month DBA/2J mice with ocular hypertension; at 10 months of age the reduction of p44 (ERK1) and p42 (ERK2) phosphorylation is greater, reaching statistical significance (Figure 2B). Interestingly, the expression of the two ERK isoforms dissociate in the late stage of retinal degeneration at 18 months of age in DBA/2J mice with ERK 1 phosphorylation increasing up to a normal level (Figure 2C). Thus, p38 MAPK and ERK1/2 appear to be strongly affected in intermediate and late stages of retinal degeneration in DBA/2J mice.

Discussion

The DBA/2J mouse is the most widely used model of spontaneously arising elevation of intraocular pressure and glaucoma. In agreement with previous results, reporting IOP increase from an age of 6 months [19,31], we showed that IOP is already elevated in 7 month DBA/2J mice remaining elevated at later ages. We previously demonstrated that IOP elevation plays a critical role in influencing visual response and in causing RGC dysfunction [19]. Here we analyzed the BDNF protein level and its high-affinity receptor, TrkB, in DBA/2J mice of different ages. Accordingly to previous studies on reduced retrograde axonal transport BDNF in experimental glaucoma [14,15], we observed that BDNF starts to decrease already at the early stage, in correspondence to IOP elevation and holds its lower levels in older subjects. Differently from its ligand, TrkB is affected in the later phases of the disease, showing a significant reduction only at the older analyzed. The reduction of BDNF level with TrkB normally expressed in the retina of DBA/2J mice during an early stage with elevated IOP authorizes to boost the retinal level of BDNF trying to arrest the progression to glaucoma; indeed, topical eye treatment with BDNF at 7 months of age in DBA/2J mice with elevated IOP was able to rescue visual function and early anomalies of RGCs [19].

In order to clarify the molecular mechanisms underpinning vision loss and progressive retinal degeneration in experimental glaucoma we investigated MAPK activation in DBA/2J mice of different ages. We started from two crucial members of the family, chosen as indicators of pro-apoptotic and/or pro-survival pathway implication, respectively p38 MAPK and ERK1/2 [22-25,28,32-34]. In our murine model, we confirmed that retinal level of activated p38 MAPK increased in

concomitance with IOP raise, and reached elevated and growing levels at 10 and 18 months of age. P38 MAPK is activated by a variety of stress stimuli, including osmotic shock, oxidative injury, beta amyloid [29,35-36]. We raise the hypothesis that ocular hypertension acts as an upstream activator of p38 MAPK; this may contribute to induce retinal dysfunction and degeneration. BDNF has been demonstrated to be a potent growth factor that is beneficial in neurodegenerative diseases with synaptic dysfunction [29], in RGC functions following optic nerve injury and diseases [10,11] and in murine models of glaucoma [18,19]. We should take into account the possibility that BDNF reduces retinal dysfunction in DBA/2J mice with elevated IOP [19] by attenuating p38 MAPK phosphorylation, similarly to what was previously reported for amyloid toxicity [29].
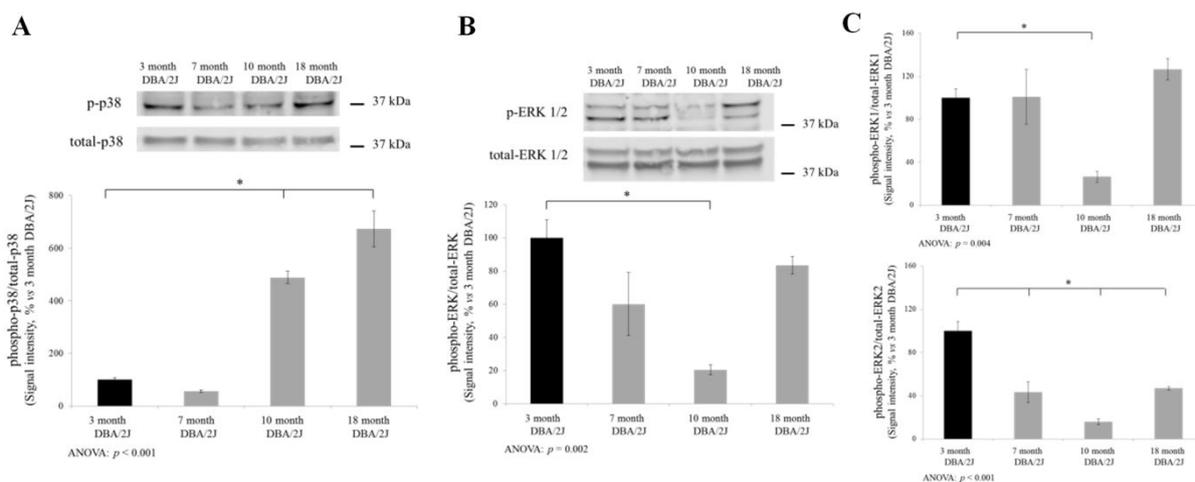

Figure 2: Activation of MAPKs and changes of IOP in DBA/2J mice. Protein extracts were resolved by SDS-PAGE and immunoblotted with antibodies against (A) phospho-p38 MAPK, total-p38 MAPK (38 kDa), (B) phospho-ERK 1/2, total-ERK 1/2 (44 and 42 kDa). (C) ERK 1 and ERK 2 values were separately plotted to underline their different modulation. MAPK activation was evaluated as the ratio between phosphorylated and total protein. Data are reported as mean ± SEM (Standard Error of the Mean) and expressed as percent of control (3 month DBA/2J mice; at least three retinas/group were processed); *$p<0.05$.

TrkB activation leads to an enhanced Erk1/2 phosphorylation in RGCs [7] while pharmacological inhibition of ERK1/2 and MEK appears to be responsible for RGC death [37]. Here we reported that ERK1/2 phosphorylation starts to decline at an early stage characterized by elevated IOP reaching the peak of deactivation at 10 months of age in DBA/2J mice, when optic nerve fibers and RGC start to degenerate [5,6], thus reinforcing the role of ERK1/2 in the control of RGC death [25-27].

Interestingly, in older mice we measured an increase of ERK1/2 activation. We hypothesized that the hyperphosphorylation observed at 18 months of age is mainly linked to ERK 1 contribution, probably as a pro-survival response caused by ERK 2 decrease. Indeed, previous results sustained the pivotal role of ERK 2 in regulating cell survival leaving a marginal part to ERK 1; when a deep and persistent inhibition of ERK 2 activation is present, ERK1 acts as a part of compensatory endogenous machinery, but not always successfully in substituting ERK 2 [38-40].

Conclusions

Our results confirmed the involvement of BDNF and TrkB pathway in glaucoma disease, enforcing our previous outcomes on the crucial neurotrophin role in ameliorating visual function in different models of experimental glaucoma. Furthermore, we demonstrated the involvement of MAPKs at different stages of glaucoma progression, underlying the importance of pharmacological treatments directed to MAPK regulation.

Acknowledgments

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