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Epigenetic intervention with a BET inhibitor ameliorates acute retinal ganglion cell death in mice

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Purpose: The bromo and extraterminal (BET) epigenetic "reader" family is becoming an appealing new therapeutic target for several common diseases, yet little is known of its role in retinal neurodegeneration. We explored the potential of BET inhibition in the protection of retinal ganglion cells (RGCs).

Methods: To test the therapeutic effect of JQ1, an inhibitor highly selective for the BET family of proteins, we used an acute RGC damage model induced by N-methyl-D-aspartic acid (NMDA) excitotoxicity. Adult C57BL/6 mice received an intravitreal injection of NMDA with (or without) JQ1 in one eye and vehicle control in the contralateral eye; RGC loss was assessed on retinal sections and whole mounts. Gene expression and apoptosis were analyzed by quantitative real time (RT)-PCR and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), respectively. For counting RGCs, immunostaining of the marker protein BRN3A was performed on whole mounts.

Results: NMDA treatment eliminated RGCs (day 7 and day 14 post injection) and diminished the expression (mRNAs) of RGC-selective genes, including *Thy1, Nrn1, Sncg*, and *Nfl* (day 3 and day 7). In contrast, co-injection with JQ1 maintained the number and gene expression of RGCs at ~2 fold of the control (NMDA only, no JQ1), and it decreased NMDA-induced TUNEL-positive cells in the RGC layer by 35%. While NMDA treatment dramatically upregulated mRNAs of inflammatory cytokines (TNFα, IL-1β, MCP-1, RANTES) in retinal homogenates, co-injection with JQ1 suppressed their upregulation by ~50%.

Conclusions: Intravitreal injection of a BET inhibitor (JQ1) ameliorates NMDA-induced RGC death, revealing the RGC-protective potential of pharmacological blockage of the BET family. This new strategy of epigenetic intervention may be extended to other retinal degenerative conditions.

Degeneration of retinal ganglion cells (RGCs) is an important cause of visual impairment or loss. Glutamate excitotoxicity triggers RGC death. As a result, N-methyl-D-aspartic acid (NMDA), a synthetic mimetic of glutamate that selectively activates NMDA receptors (a subtype of glutamate receptors), is commonly used to induce an acute RGC death model following intravitreal injection into mice [1,2]. Excessive retinal neuroinflammation has recently been recognized as an important contributor, as well as a potential therapeutic target, in pathologies featuring RGC death [3]. NMDA excitotoxicity elicits retinal inflammatory responses that lead to RGC damage or loss [4,5].

The family of bromo extraterminal domain (BET) proteins represents a novel epigenetic target for anti-inflammatory therapy [6-8]. This family consists of BET2, BET3, BET4 (alternatively abbreviated as BRDs), and a testis-specific member (irrelevant to this study), each containing two tandem bromodomains and an extraterminal domain [9]. BETs promote cellular context-specific transcriptional activation by binding (or reading) chromatin modifications (i.e., histone acetylation) via their bromodomains. As a result, they have been dubbed epigenetic "readers." It was not possible to pharmacologically block BET epigenetic reader activity until the recent and serendipitous discovery of JQ1, the first-in-class BET inhibitor [10]. This designer drug is highly selective for the bromodomains of BET proteins, as shown by the comparative studies using 46 bromodomains, including BET and non-BET proteins [10,11].

While initially found to be effective in mitigating cancer progression [12-14], JQ1 and its derivatives have recently shown prominent inhibitory potency in animal models of inflammatory (e.g., infectious and cardiovascular) diseases [6,8,15-17]. The success of this epigenetic modulation strategy has evoked enormous enthusiasm across different medical research fields; this enthusiasm has been manifested by a

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rapid increase of publications on the BET family. While the role of the BET family in the neuronal system is beginning to be explored, whether a BET blockade could be a viable approach for retinal neuron protection remains unknown.

The current study provides the first in vivo evidence of RGC protection via inhibition of BET epigenetic readers. We administered NMDA in mice with or without JQ1 via intravitreal injection, and we observed partial preservation of RGCs by JQ1. This study may confer a viable template for future development of an optimized BET-targeted epigenetic therapy to mitigate RGC demise.

METHODS

Animals: All animal procedures conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin–Madison. All surgeries were performed under isoflurane anesthesia (through inhaling, flow rate 2 ml/min). Animals were euthanized in a chamber gradually filled with CO_2. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were maintained on a 4% fat diet (8604 M/R, Harkland Teklad, Madison, WI) and subjected to a standard 12 h-12 h light-dark schedule. Both male and female mice in the age range of postnatal 40–60 days were used in the experiments.

Intravitreal injection of NMDA and JQ1: Intravitreal injection was performed as we previously reported [18]. Mice were anesthetized with isoflurane through inhaling. Proparacaine hydrochloride (0.5%; Alcon Laboratories, Inc., Fort Worth, TX) and ofloxacin ophthalmic solution (0.3%; Allergan Inc., Irvine, CA) were applied to the ocular surface before injection for topical anesthesia and infection prevention, respectively. To avoid injuries to the lens, a ~0.5 mm incision posterior to the temporal limbus was first made using a 27-gauge single-use needle (BD, Franklin Lakes, NJ), and then a 30-gauge blunt-end needle (10 mm length; Hamilton, Reno, NV) in a Hamilton 701RN syringe was inserted through the incision. The needle was advanced approximately 1.5 mm deep while avoiding the lens, angled toward the optic nerve until the needle tip was seen in the center of the vitreous; then, 2 µl of the solution was injected. To ensure that no infection would occur, bacitracin ophthalmic ointment (E. Fougera & Co., Melville, NY) was applied immediately after pulling out the needle.

For the experiments, stock solutions of (+)-JQ1 (ApexBio Technology A1910, Boston, MA) and NMDA (Sigma-Aldrich, St. Louis, MO) were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) and were then diluted in PBS buffer for intravitreal injection. Mice were classified into three groups (at least six mice in each): (1) vehicle control (10% DMSO in PBS buffer), (2) NMDA (40 mM in 10% DMSO in PBS buffer), and (3) a mixture of JQ1 and NMDA (0.1 mM JQ1 and 40 mM NMDA dissolved in 10% DMSO in PBS buffer). Each mouse received an injection of NMDA with or without JQ1 (total 2 µl) in the left or right eye (randomly assigned) and vehicle control in the contralateral eye. These sets of experiments were repeated at least two times in 5 months.

Preparation of retinal cryosections and whole mounts: Samples of retinal sections and whole mounts were prepared according to the published methods [19]. Briefly, mice were euthanized by CO_2 asphyxiation at 1, 3, 7, and 14 days after intravitreal injection. Eyeballs were enucleated, fixed in 4% paraformaldehyde overnight at 4 °C, and then cryoprotected by soaking in 30% sucrose for 14 h at 4 °C. The eyeballs were embedded in an optimum cutting temperature (OCT) embedding medium (Sakura Finetek, Torrance, CA), frozen on dry ice, and then used for preparation of 10 µm-thick sections by cutting through the optic nerve head [19,20].

For whole mount preparation [18], eyeballs of euthanized mice were marked on the superior side, enucleated, and fixed in 4% paraformaldehyde for 1 h. After rinsing three times in PBS, eyecups were generated, and retinas were dissected out and placed on a Superfrost Plus slide (ThermoFisher) with the ganglion cell layer (GCL) facing upward. Three additional relaxing cuts were made to allow the retina to lie flat.

Nuclei counting in the RGC layer on retinal cross-sections: The number of neurons was quantified by counting 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei, following a previously published method [21] with minor modifications. Briefly, on each sagittally oriented section, the regions that were 0–1000 µm and 1000–2000 µm from the optic nerve head were designated as central and mid-peripheral, respectively. Neuronal nuclei were manually counted in each (500 µm length of retina) of the four fields chosen in the central and mid-peripheral regions of the RGC layer flanking the optic nerve head. The counts from all 3–4 sections of the same animal were averaged, and the means from 6 to 9 animals were then averaged to calculate the mean and standard error of mean (SEM) for each group of animals.

RGC (BRN3A positive) nuclei counting on retinal whole mounts: BRN3A is considered an RGC-specific nuclear marker protein. Counting of immunostained (see the method

below) BRN3A-positive nuclei was performed following our recent publication [18]. Images were captured at 400× on the four whole mount sections generated by four relaxing cuts; then, counts of BRN3A-positive nuclei were obtained from 12 distinct fields (0.09 mm^2) for each retina and averaged together.

Immunostaining of BRN3A on retinal whole mounts: Whole mount immunostaining for BRN3A was performed as we recently described [18]. Briefly, fixed eyecups were incubated in PBS buffer containing 0.5% Triton-X100 and 2% donkey serum (Jackson ImmunoResearch Lab, MS) for 1.5 h at room temperature. They were then transferred into the same buffer containing mouse anti-BRN3A (Millipore, Cat.#MAB1585, 1:50) and incubated overnight at 4 °C. The eyecups were thoroughly rinsed in PBS buffer with 0.5% Triton-X100; they were then fixed for an additional 10 min in 4% paraformaldehyde and rinsed again. The eyecups were whole-mounted onto Fisher Plus slides and incubated in 2% Triton-X100 and 2% donkey serum with a secondary antibody (Alexa-594-conjugated donkey-anti-mouse, 2 µg/ml, Jackson ImmunoResearch Lab, MS) for 2 h at room temperature. The whole mounts were rinsed in PBS buffer and stained with 300 ng/ml DAPI for 5 min at room temperature. After a final wash with PBS buffer, the slides were coverslipped with Immu-Mount (ThermoFisher) and used for fluorescence microscopy. Images were acquired with a Nikon fluorescence microscope using a 20× objective lens and analyzed by Nikon Elements software.

TUNEL staining on retinal cross-sections: Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed as described in our recent report [18], using an In Situ Cell Death Detection kit (Roche, Indianapolis, IN), following the manufacturer's instructions. Briefly, retinal cryosections were TUNEL-stained and imaged to assess DNA fragmentation. TUNEL positive cells were quantified in four fields flanking the optic nerve head on each of the 3–4 sections per eye. Each field represents a 500 µm retinal length in a central and mid-peripheral region (refer to nuclei counting described above). Cells were scored as either TUNEL positive or negative. The counts from all sections of the same animal were averaged for the mean per animal, and the means from 6 to 9 animals were averaged to generate the mean and SEM for the entire group of animals.

Real-time quantitative PCR (qRT-PCR) for measurement of mRNA levels: RNA was extracted from retinal homogenates using Trizol (Qiagen, Valencia, CA) following the manufacturer's instructions. Purified mRNA (1 µg) was used for the first-strand cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad), and quantitative RT–PCR (qRT-PCR) was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA), as described in our previous report [18]. Each cDNA template was amplified in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The primers used are the following: *Nfl*, F: AGC ACG AAG AGC GAG ATG GC, R: TGC GAG CTC TGA GAG TAG CC; *Nrn1*, F: TTC ACT GAT CCT CGC GGT GC, R: TAC TTT CGC CCC TTC CTG GC; *Sncg*, F: GAC CAA GCA GGG AGT AAC GG, R: TCC AAG TCC TCC TTG CGC AC; *Thy1*, F: GAA GTG TCC AAC CGC CAT GG, R: TTG TCT GGG CAT GGT GCG; TNFα, F: CGC GAC GTG GAA CTG GCA GAA, R: GTG GTT TGC TAC GAC GTG GGC T; MCP1, F: CCC ACT CAC CTG CTG CTA CT, R: TCT GGA CCC ATT CCT TCT TG; IL-1β, F: GCA ACT GTT CCT GAA CTC AAC T, R: ATC TTT TGG GGT CCG TCA ACT; RANTES, F: TGC CCT CAC CAT CAT CCT CAC T, R: GGC GGT TCC TTC GAG TGA CA.

Statistical analyses: The required sample sizes in animal experiments were calculated based on the estimation of mean differences, variances, and power. For statistical comparison of two samples, Student *t* test (OriginLab, Northampton, MA) was used. Data are presented as mean ± SEM; $p<0.05$ was regarded as statistically significant.

RESULTS

JQ1 treatment partially preserves cell numbers in the RGC layer on retinal cross-sections: We used JQ1, a bromodomain inhibitor highly selective for the BET family, to test the potential RGC-protective effect in the NMDA excitotoxicity mouse model. NMDA is reported to induce neurotoxicity specifically in RGCs [22]. The experiments were performed in such a way that NMDA in PBS buffer was co-injected with or without JQ1 into one eye of the mouse, and the vehicle control (10% DMSO in PBS, no NMDA) was injected into the contralateral eye. Mice were euthanized at 1, 3, 7, and 14 days after injection, and eyeballs were enucleated. Retinal cryosections were then prepared in order to count DAPI-stained nuclei in the RGC layer. As shown in Figure 1, NMDA induced a continuous decrease of cells in the RGC layer to 42.1% of vehicle control at day 14. In contrast, JQ1 co-injected with NMDA kept the cell number at 63.3% and 57.6% at day 7 and day 14, respectively. Slight protection by JQ1 was also observed at day 1 and day 3, albeit not statistically significant compared to NMDA treatment without JQ1. Injection of JQ1 alone without NMDA did not affect the density of DAPI-stained nuclei (data not shown).

JQ1 treatment partially preserves RGCs (BRN3A-positive) on retinal whole mounts: Since only 50–60% of cells in the RGC

layer are RGCs [21], DAPI staining of retinal cross-sections could not distinguish RGCs from other cell types in the same layer, including displaced amacrine cells, astrocytes, and microglia. To determine an RGC-specific protective effect of JQ1 in a scale of the entire retina, we used retinal whole mounts to immunostain BRN3A, a specific marker protein localized in the RGC nucleus (Figure 2). The whole mounts were collected at day 7 post injection, which is the time point that revealed the best JQ1 protective effect, as seen in Figure 1. Consistent with the data obtained from cross-sections (Figure 1), while NMDA eliminated ~80% of the BRN3A-positive cells (i.e., RGCs) compared to vehicle control, co-injection with JQ1 significantly preserved the RGC number compared to NMDA alone without JQ1, maintaining the number at ~45% of vehicle control. Thus, the forgoing data (Figure 1 and Figure 2) together demonstrate an in vivo protective effect of JQ1 against NMDA-induced RGC loss.

JQ1 treatment partially preserves RGC-specific gene expression: To further confirm the observed partial protection of RGCs by JQ1 treatment under NMDA-induced neurocytotoxicity, we used another approach, qRT-PCR, to determine expression levels of RGC-selective genes, including *Thy1, Nrn1, Sncg*, and *Nfl,* at day 3 and day 7 post injection. The data (Figure 3) show that, while NMDA (versus vehicle control) diminished mRNAs of all four genes, JQ1 co-injection significantly protected those mRNAs compared to NMDA alone. This result and the nuclei counting data (Figure 2) confirm that RGCs were partially rescued from

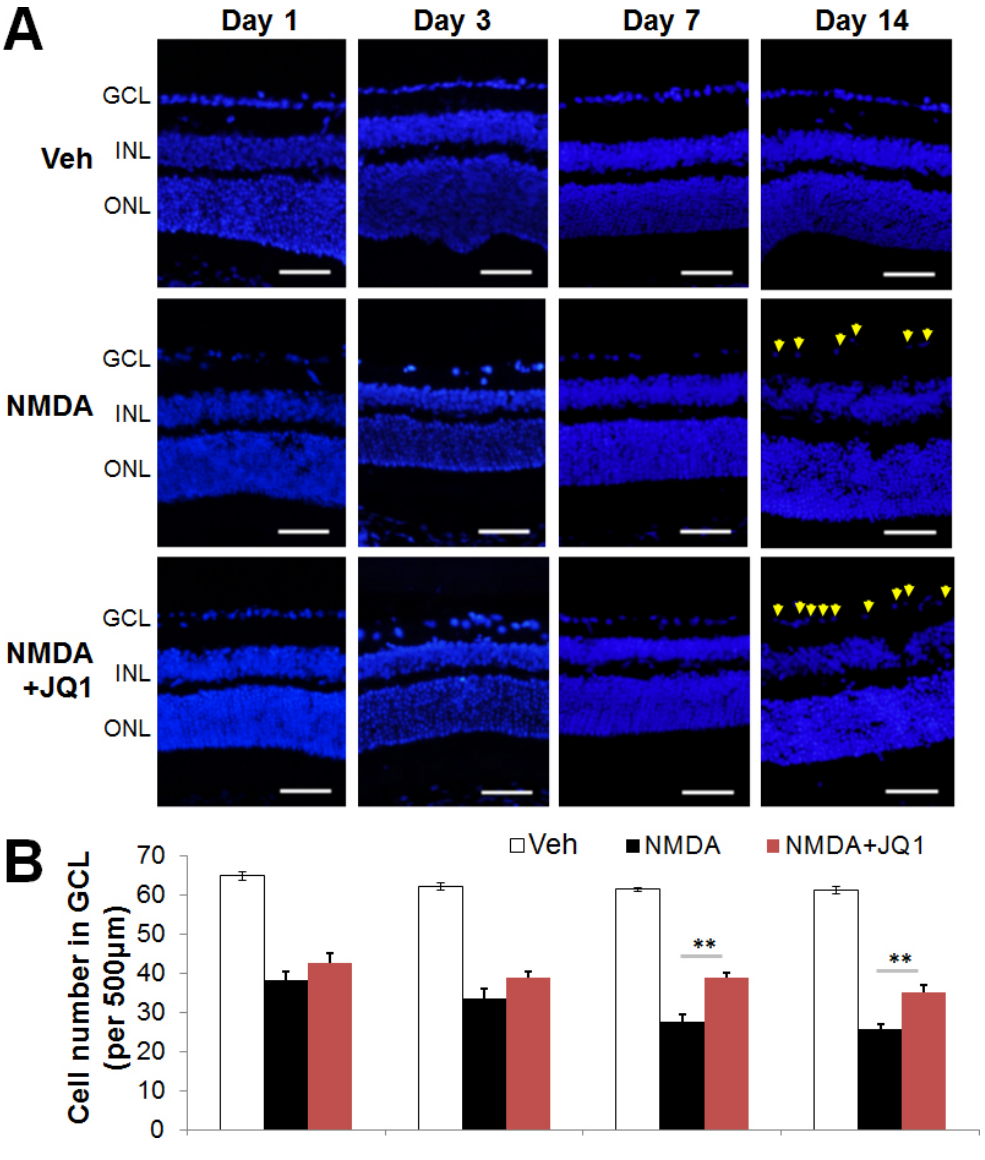

Figure 1. JQ1 ameliorates NMDA-induced cell loss counted in the RGC layer of retinal sections. Mice received NMDA mixed with (or without) JQ1 in one eye and vehicle control (equivalent amount of DMSO, no NMDA) in the contralateral eye; they were euthanized at 1, 3, 7, and 14 days after injection, as described in the Methods section. Retinal cryosections were prepared for counting DAPI-stained nuclei. **A**: Representative fluorescent images. Scale bar: 50 µm. GCL, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Arrows highlight DAPI-stained nuclei in the GCL. **B**: Quantification of nuclei in the GCL (per 500 µm retinal length): mean ± SEM, n>6 mice; **p<0.01 compared to NMDA alone (no JQ1).

NMDA-induced cell loss or damage by a single intravitreal co-injection of JQ1.

JQ1 treatment mitigates NMDA-induced RGC apoptosis: Apoptosis is known to be the ultimate pathway underlying NMDA-induced RGC death [23]. We assessed whether JQ1 protected RGCs by mitigating apoptosis, using TUNEL assay for the determination of DNA fragmentation (Figure 4A,B). In this study, we used a high dose of NMDA, which typically results in a rapid increase of TUNEL-positive nuclei, which peaks within 48 h of intravitreal injection and is then followed by aggressive RGC degeneration at later time points [22]. We thus chose a time point of 24 h post injection for TUNEL assays. NMDA was injected with or without JQ1, eyeballs were collected 24 h after injection, retinal cross-sections were prepared for TUNEL staining, and the number of TUNEL-positive cells in the RGC layer was counted. As quantified in Figure 4C, TUNEL-positive cells markedly increased in NMDA-treated retinas compared to vehicle control. JQ1 co-injection significantly reduced (by 35%) TUNEL-positive cells compared to NMDA treatment without JQ1. Taken together, the foregoing results (Figure 1, Figure 2, Figure 3, and Figure 4) indicate that treatment with BET epigenetic inhibitor JQ1 attenuates RGC apoptosis and partially protects RGC survival when under the stress of NMDA-elicited excitotoxicity.

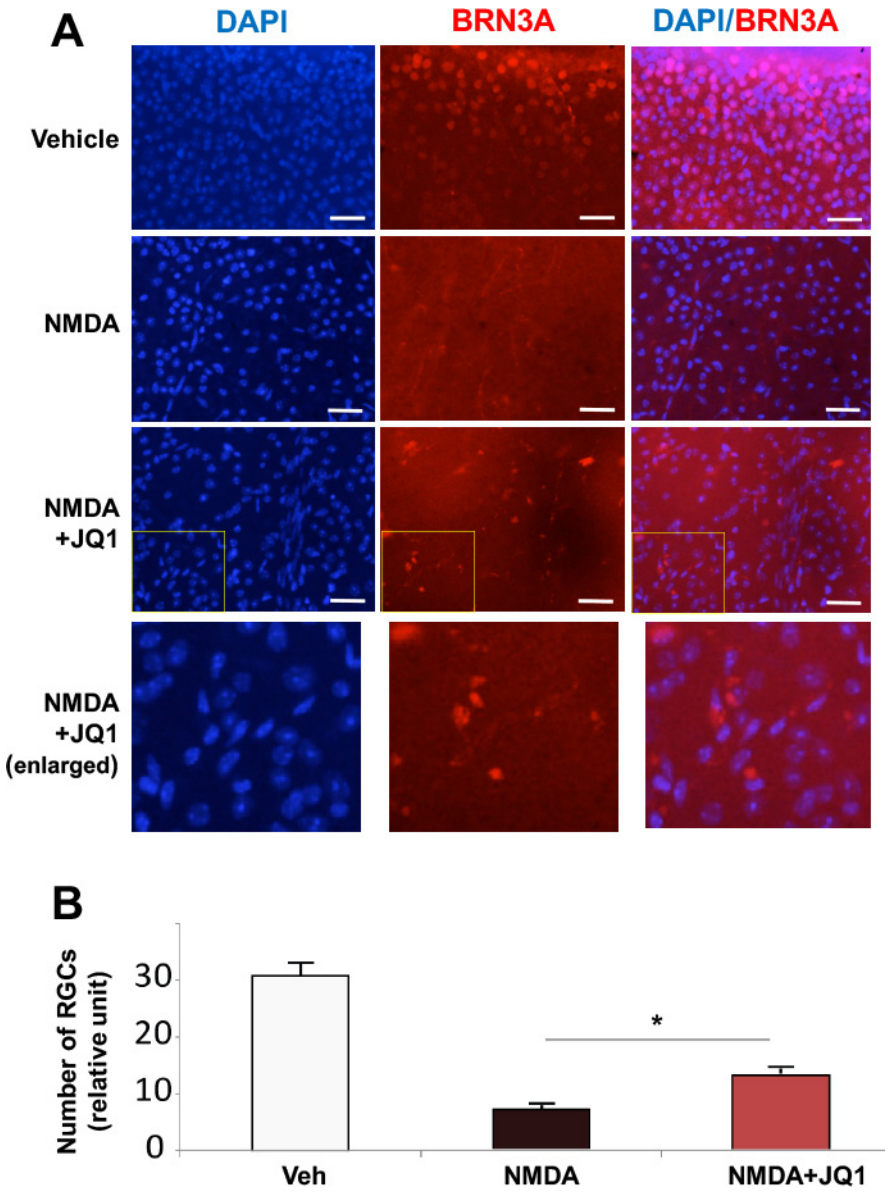

Figure 2. JQ1 ameliorates NMDA-induced RGC loss counted on retinal whole mounts. Intravitreal injections were performed as described in Figure 1. Mice were euthanized at day 7 post injection, and retinal whole mounts were prepared for fluorescence microscopy. **A**: Representative images of BRN3A immunostaining. Scale bar: 50 µm. To highlight BRN3A-positive cells, boxed areas within the dashed line are enlarged below. **B**: Quantification of BRN3A-positive cells: mean ± SEM; n=6–9 animals. *p<0.05, compared to NMDA alone.

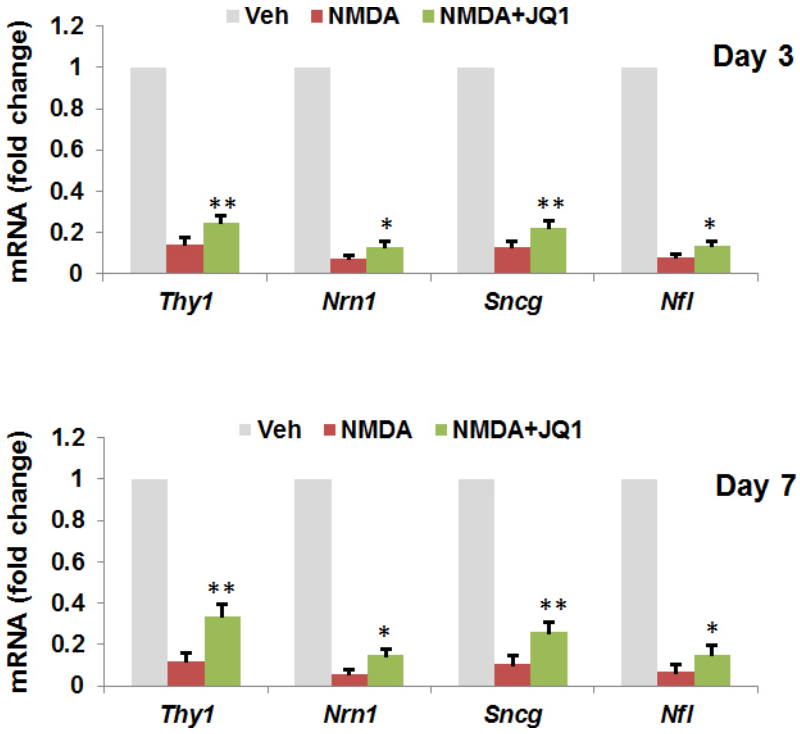

Figure 3. JQ1 rescues NMDA-mitigated RGC-selective gene expression. Intravitreal injections were performed as described in Figure 1. Mice were euthanized at day 3 and day 7 post injection for preparation of retinal homogenates and qRT-PCR. Quantification: mean ± SEM; normalization to vehicle control; n>6 animals; **p<0.01, *p<0.05, compared to NMDA alone.

JQ1 treatment inhibits the expression of inflammatory cytokines in the retina: Recently, neuroinflammation has been increasingly recognized as an important mechanism of RGC neurodegeneration under various stress conditions [24,25]. Moreover, it has been reported that NMDA excitotoxicity stokes neuroinflammation characterized by the elevated expression of inflammatory cytokines, including TNFα, MCP-1, IL-1β, and RANTES [26,27]. We thus determined the effect of JQ1 on mRNA levels of these cytokines by quantitative real time (qRT)-PCR using retinal homogenates collected at day 3 post injection (Figure 5).

We found that, compared to vehicle control, NMDA treatment dramatically stimulated inflammatory cytokine expression (e.g., a 70-fold increase of MCP-1 mRNA). However, this upregulation of inflammatory cytokines was substantially attenuated (by ~50%) in the mice co-injected with NMDA and JQ1. This result indicates a prominent inhibitory effect of JQ1 treatment on retinal neuroinflammation stimulated by NMDA neurotoxicity.

DISCUSSION

Epigenetics refers to reversible and heritable changes in gene function without alteration of the underlying DNA sequence itself. Sensitively reacting to external pathogenic stimuli, epigenetic mechanisms profoundly influence disease processes by regulating the expression of defined groups of downstream genes [9]. Recently, epigenetic modulations have come to attention for holding the potential to identify and develop new therapeutic targets [11]. Since the recent discovery of the first-in-class inhibitor (JQ1), the BET protein family has emerged as a highly attractive epigenetic target. Of particular note is that inhibiting BETs with JQ1 or other BET-selective inhibitors has shown remarkable effects in mitigating inflammation in preclinical disease models [7,11]. While glaucomatous blindness is caused by RGC death, neuroinflammation (acute and chronic) stimulated by various pathogenic factors is now recognized as a prominent contributor to the demise of RGCs [24]. In this context, we were prompted to test whether the blockade of BETs with JQ1 mitigates RGC death under NMDA excitotoxicity. Indeed, we found that one intravitreal co-injection of JQ1 with NMDA effectively attenuated NMDA-stimulated apoptosis (by 35%) and inflammatory cytokine expression (by ~50%), significantly ameliorating RGC loss. These results suggest a promising epigenetic approach for RGC protection that can be optimized in future investigations.

Over the past decade, epigenetic writers and erasers—enzymes that respectively add and remove an acetyl or methyl mark on chromatin—have shown potential as intervention targets [28]. Recently, histone deacetylases were reported to play important roles in mouse nuclear atrophy of apoptotic RGCs induced by optic nerve crush [29] or in

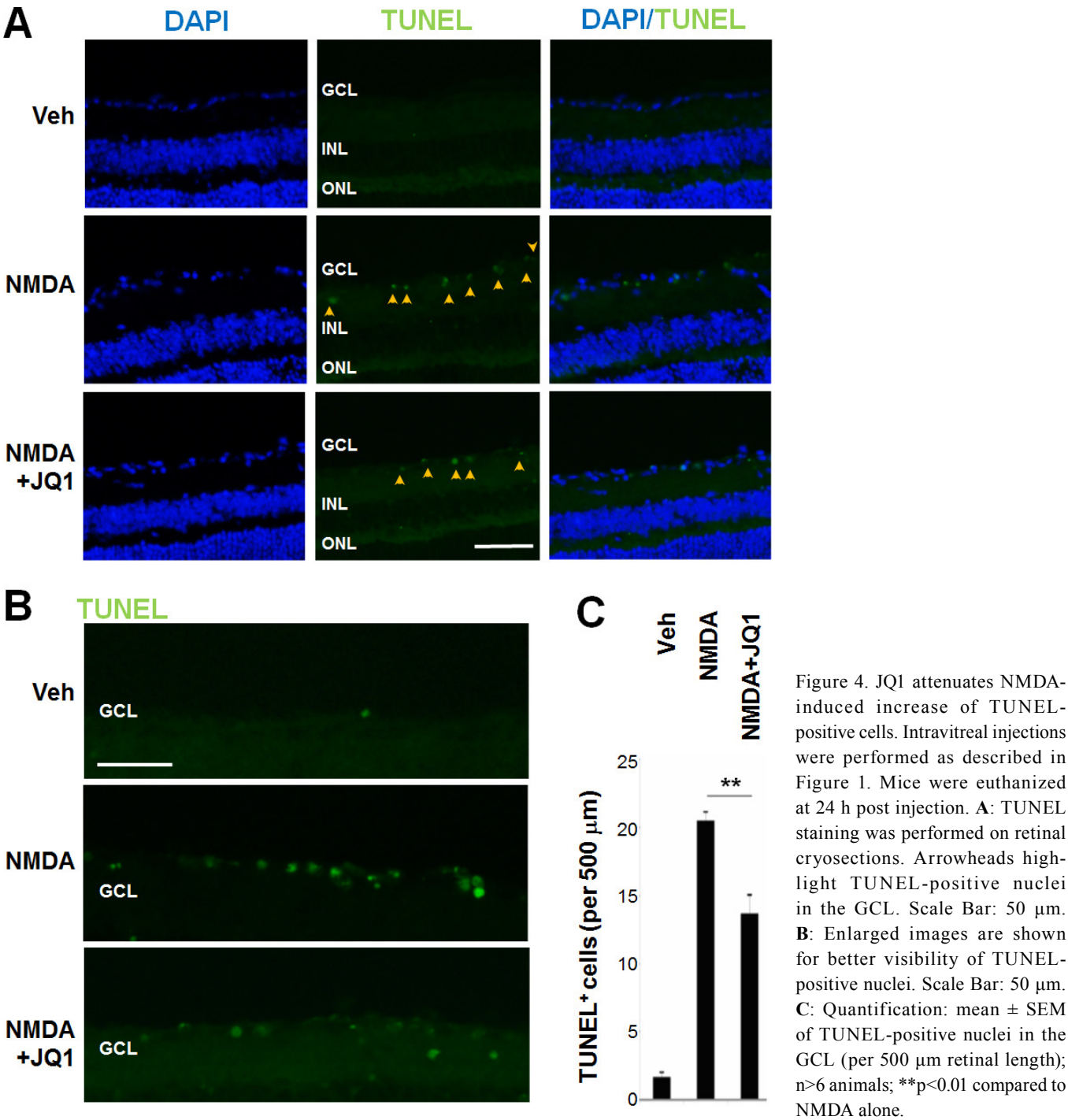

Figure 4. JQ1 attenuates NMDA-induced increase of TUNEL-positive cells. Intravitreal injections were performed as described in Figure 1. Mice were euthanized at 24 h post injection. **A**: TUNEL staining was performed on retinal cryosections. Arrowheads highlight TUNEL-positive nuclei in the GCL. Scale Bar: 50 µm. **B**: Enlarged images are shown for better visibility of TUNEL-positive nuclei. Scale Bar: 50 µm. **C**: Quantification: mean ± SEM of TUNEL-positive nuclei in the GCL (per 500 µm retinal length); n>6 animals; **p<0.01 compared to NMDA alone.

rat RGC death caused by elevated intraocular pressure [30]. However, specifically targeting these epigenetic regulators has been challenging [11,31,32]. Fortunately, the BET epigenetic "readers" (proteins that recognize and bind acetylated histones) have emerged as a new class of intervention targets. Understanding their functions in diseases had been hindered by the lack of small molecule inhibitors. The BET family was widely viewed as undruggable until the recent discovery of JQ1 [10] and the subsequent discovery of its derivatives as BET bromodomain blockers [11]. Importantly, the excellent selectivity of JQ1 for the BET family has been shown in studies screening 46 bromodomains, including BET and non-BET proteins [10,11]. It is thus reasonable to infer that the

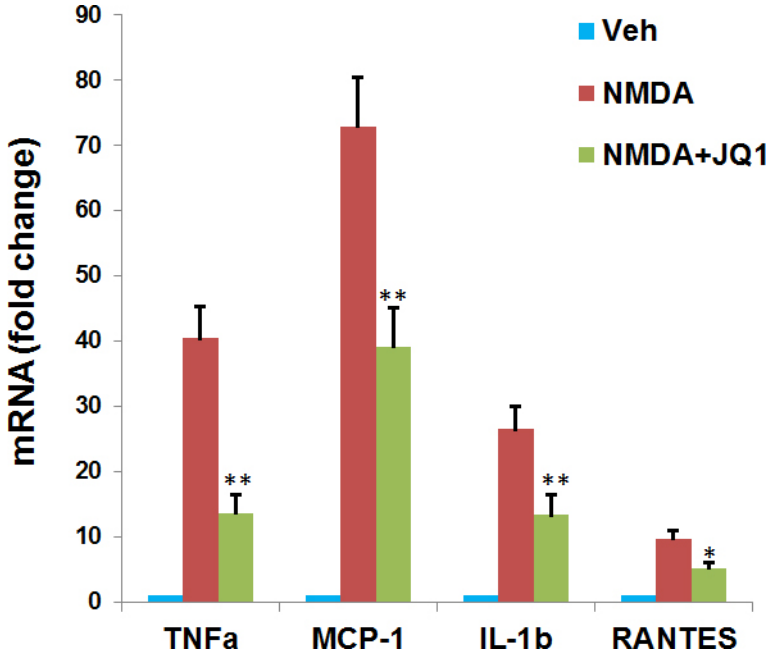

Figure 5. JQ1 inhibits NMDA-induced increase of inflammatory cytokine expression. Intravitreal injections were performed as described in Figure 1. Mice were euthanized at day 3 post injection for preparation of retinal homogenates and qRT-PCR. Quantification: mean ± SEM; normalization to vehicle control; n>6 animals; **p<0.01, *p<0.05, compared to NMDA alone.

RGC-protective effect of JQ1 observed here is likely attributable to its inhibition of the BET family.

The therapeutic potential of BET inhibition was first demonstrated in cancers [12-14] and then quickly extended to other inflammatory and proliferative diseases including sepsis and cardiovascular diseases [6,8,15-17]. Reports concerning the BET family in neuronal systems have also recently emerged. Two recent brain studies identified BET-associated transcriptional activation during memory formation [33] and cocaine-induced neuronal plasticity [34]. Another new study showed an inhibitory effect of JQ1 on neovascularization in an oxygen-induced retinopathy mouse model [35]. However, the effect of BET inhibition on retinal neurons is not known. Therefore, it remains unclear as to whether blocking BET epigenetic functions affects retinal ganglion neuron survival.

Our study provides the first line of in vivo evidence supporting a BET-targeted strategy to protect RGCs from stress-induced damage. The JQ1 protective effect on RGCs was confirmed with four different methods: counting the cell number in the RGC layer of retinal cross-sections, staining an RGC-specific marker (BRN3A) on retinal whole mounts, quantifying RGC-selective mRNAs in retinal homogenates, and TUNEL staining of apoptotic cells in the RGC layer. As evaluated by these assays, NMDA-inflicted RGC damage was significantly attenuated by one injection of JQ1. A lack of complete rescue of RGCs likely stems from the following facts. (1) NMDA neurotoxicity is an aggressive treatment that causes severe damage to RGCs [22], which is also evidenced in our data where 7 days after NMDA injection >80% of RGCs disappeared. (2) The binding of JQ1 to its target, the BET family, is reversible [10]; thus, JQ1 may have quickly dissociated and diffused away. (3) In light of the reported JQ1 half-life in mouse serum (~1 h) after systemic delivery [10], the drug may have been metabolized over time. In future investigations, more robust RGC protection could be achieved by applying a BET inhibitor with improved bioavailability (or half-life), by using nanoparticles to extend drug release time [18], or by using a combination of both.

While chronic neuroinflammation is thought to play a key role in glaucomas [36], excessive inflammatory responses are known to also cause RGC death in acute RGC damage models [3,25]. These responses are characterized by a dramatic increase of inflammatory cytokines (e.g., TNFα, IL-1β, and MCP-1, and RANTES). We found that JQ1 effectively suppressed an NMDA-induced surge of these cytokines. This JQ1 inhibitory effect on neuroinflammation is also supported by a recent study indicating that JQ1 treatment dampens neuroinflammation in an Alzheimer disease mouse model [37]. In our study, while JQ1 may have protected RGCs indirectly via its prominent inhibitory effect on retinal neuroinflammation, we cannot exclude the possibility that JQ1 may also inhibit apoptosis directly in RGCs. Future investigations using purified primary RGCs or RGC-specific BET knockout mice are needed to definitively determine whether BET regulates apoptotic programs directly in RGCs.

Conclusions: To the best of our knowledge, this is the first in vivo investigation revealing an RGC-protective effect of a BET inhibitor. This pilot study opens a new avenue for future development of BET-targeted epigenetic interventions to mitigate RGC loss. Our finding is significant—especially considering the urgent clinical need of effective RGC-protective therapeutic methods.

Given its confined scope, this study is associated with several limitations that warrant more in-depth research in the future. First, a more profound therapeutic effect requires a BET inhibitor with improved bioavailability combined with optimized delivery methods or regimens. Second, it remains to be elucidated whether JQ1 protects RGC survival via direct inhibition of RGC apoptotic pathways. Third, there is no direct evidence to link the RGC-protective effect of JQ1 to its attenuation of NMDA-stimulated retinal inflammation. Lastly, since JQ1 is a pan-specific inhibitor that binds bromodomains in all BET family proteins, our study was not able to distinguish which BET protein was the primary functional site of JQ1. Nonetheless, on the basis of our findings, continued investigations may lead to new therapeutic methods suitable for treating RGC-degenerative conditions or other neurodegenerative pathologies. The potential of BET-targeted therapies is also evidenced by a rapidly growing list of BET inhibitors, some being used in clinical trials [11,38,39].

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