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Persistence of intact retinal ganglion cell terminals after axonal transport loss in the DBA/2J mouse model of glaucoma

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Abstract

Axonal transport defects are an early pathology occurring within the retinofugal projection of the DBA/2J mouse model of glaucoma. Retinal ganglion cell (RGC) axons and terminals are detectable after transport is affected, yet little is known about the condition of these structures. We examined the ultrastructure of the glaucomatous superior colliculus (SC) using three-dimensional serial block-face scanning electron microscopy (3-D EM) to determine the distribution and morphology of retinal terminals in aged mice exhibiting varying levels of axonal transport integrity. After initial axonal transport failure, retinal terminal densities did not vary when compared to either transport-intact or control tissue. While retinal terminals lacked overt signs of neurodegeneration, transport-intact areas of glaucomatous SC exhibited larger retinal terminals and associated mitochondria. This likely indicates increased oxidative capacity and may be a compensatory response to the stressors this projection is experiencing. Areas devoid of transported tracer label showed reduced mitochondrial volumes as well as decreased active zone number and surface area, suggesting oxidative capacity and synapse strength are reduced as disease progresses, but before degeneration of the synapse. Mitochondrial volume was a strong predictor of bouton size independent of pathology. These findings indicate that RGC axons retain connectivity after losing function early in the disease process—creating an important therapeutic opportunity for protection or restoration of vision in glaucoma.

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Author Contributions

M.A.S., S.D.C., B.R.S., D.M.I., and C.M.D-C were involved in the conception, design, acquisition, analysis and interpretation of data, and the writing/revision of the manuscript. C.Z.X and K.M.F were involved in the acquisition of the data.

Conflicts of Interest

None to disclose

Keywords

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Introduction

Dysferopathy and synaptopathy (i.e., defects in axonal transport and synaptic structure/function, respectively) have been implicated as early pathologies in chronic neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's diseases well before neuronal cell body loss (Yoshiyama et al., 2007; Adalbert et al., 2009; Brady and Morfini, 2010; Lin and Koleske, 2010; Südhof, 2012). Dysferopathy has also been implicated in glaucoma (see Crish and Calkins, 2011 for a review), a group of chronic neurodegenerations that make up the leading cause of irreversible blindness worldwide (Quigley and Broman, 2006).

Most glaucomas are age-related, sharing mechanistic and epidemiological similarities with Alzheimer's and Parkinson's diseases (McKinnon et al., 2002; Conforti et al., 2007; Crish and Calkins, 2011). It has been postulated since the mid-19th century that glaucoma produces atrophy of the optic nerve that leads to irreversible vision loss (Mueller, 1858; Schnabel, 1905), a view that is supported by more recent pathological evidence and animal models of the disease (Libby et al., 2005; Casson et al., 2012). What is unclear however, is the cause and progression of optic nerve degeneration. Common hypotheses include apoptotic retinal ganglion cell loss due to neurotrophin deprivation or Wallerian degeneration beginning at the optic nerve head where retinal ganglion cell (RGC) axons exit the eye (Kerrigan et al., 1997; Quigley, 1999; Quigley et al., 2000; Burgoyne et al., 2005). Recently, a more progressive course of pathology in glaucoma has been shown—including early axonal transport deficits (Crish et al., 2010; Dengler-Crish et al., 2014), distal axonopathy (Schlamp et al., 2006; Crish et al., 2010), synapse loss within the retina (Stevens et al., 2007), and retinal ganglion cell dysfunction (Della Santina et al., 2013; Chen et al., 2015).

Early synaptopathy predicts that deficiencies in neuronal signaling could produce clinical deficits in the absence of cell body loss (Chapman et al., 1999; Mizuno et al., 2000; Sergezenko et al., 2013). Most of the efforts exploring synaptopathy in glaucoma have focused on synapse and dendrite loss in the retina (Stevens et al., 2007; Fu et al., 2009; Liu et al., 2011; Park et al., 2014) but, as in other neurodegenerations (Terry et al., 1991; Spires and Hyman, 2004; Day et al., 2006), some of the earliest changes occur in the axon and its terminals (Buckingham et al., 2008; Baltan et al., 2010; Crish et al., 2010; Dengler-Crish et al., 2014). Previously, we reported structural persistence of a semi-functional axon prior to eventual degeneration in a common model of hereditary glaucoma, the DBA/2J mouse (Crish et al., 2010, 2013; Dengler-Crish et al., 2014).

Despite these investigations, very little is known of the physiology and synaptic connectivity of retinal axons during early pathology. Here, we describe early synaptic changes in the glaucomatous brain using anterograde tract tracing with fluorescent cholera toxin-B (CTB)

injection into the eye followed by 3-D reconstruction of serial block-face scanning electron microscopy (3-D EM) of the retinal ganglion cell (RGC) terminals in the SC of DBA/2J mice exhibiting various levels of transport pathology. Our findings showed that retinal terminals not only persist, but remain connected to their postsynaptic targets. These terminals undergo an initial hypertrophy followed by reductions in indicators of synaptic activity and strength before eventual degeneration.

Experimental Procedures

Animals

We used mixed-sex 9–14 month old DBA/2J (n=6; RRID:IMSR_JAX:000671) mice as our experimental animals and 11 month old DBA/2J *Gpnmb*⁺ (n=3; RRID:IMSR_JAX:007048) mice as controls. DBA/2J *Gpnmb*⁺ (D2G) mice have the same background as the DBA/2J; however, they express a wild type *Gpnmb* allele that prevents them from developing glaucomatous pathology (Howell et al., 2007). Ages of the DBA/2J's were chosen where little if any RGC loss is evident in this model (Filippopoulos et al., 2006; Buckingham et al., 2008; Crish et al., 2010; Dengler-Crish et al., 2014). From these nine animals, 10 collicular tissue samples were collected, three D2G, three DBA/2J with intact CTB label (CTB+), and four DBA/2J without detectable CTB label (CTB–) (see Figure 1). All mice were maintained in a 12 hour light/dark cycle with standard rodent chow available *ad libitum* and housed in the same room under the same environmental conditions. The Northeast Ohio Medical University Institutional Animal Care and Use Committee approved all experimental procedures.

Anterograde transport labeling (intravitreal injections)

Mice were placed prone in a stereotaxic device (Stoelting, Wood Dale, IL) equipped with a nose cone that delivered 2.5% isoflurane at 0.8 ml/min. Injections (1.5 µl) of 0.1% cholera toxin subunit B (CTB) conjugated to Alexa Fluor-488 (Molecular Probes, C-34775) in sterile phosphate buffered saline (PBS) were administered into the vitreal chamber of each eye using a 33-ga needle attached to a 25 µl Hamilton syringe. CTB was used because it is selectively taken up by RGCs (except in the cases of advanced retinal pathology; Crish et al., 2010; Inman et al., 2012) and is visible in the Golgi apparatus and axons—allowing us to determine uptake and transport in the retina. Furthermore, CTB has significant advantages over other commonly used tracers for anterograde transport in that it travels along all axons of the retinofugal projection and is visible in each component (Angelucci et al., 1996).

Tissue collection and preparation

Seventy-two hours after injections, subjects received a single 0.01 ml intraperitoneal injection of Beuthanasia-D Special (390 mg sodium pentobarbital, 50 mg phenytoin) and were transcardially perfused with 4% paraformaldehyde (PFA). This time frame was selected to allow sufficient CTB transport from the retina to the superior colliculus (Kahn and Krubitzer, 2002). Before histological preparation, the cortex overlying SC was removed and the superior colliculus was photographed under a Zeiss Axiozoom V16 microscope (Jena, Germany) to determine extent of CTB coverage. Additionally, retinas were flat-mounted and examined to determine success of injection and RGC uptake of CTB (see

Wilson et al., 2015). Retina was also imaged; failed CTB injections were identified by lack of tracer in the retina and these projections were excluded from analysis. Collicular areas showing intact CTB label were microdissected away from collicular areas devoid of tracer label (with confirmed tracer in corresponding retinal areas) prior to final processing. Collected tissue samples then were post-fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for at least 48 hours.

Serial block-face scanning electron microscopy

Tissue preparation and imaging were performed by Renovo Neural, Inc. (Cleveland, OH). Samples were stained with heavy-metal solutions and embedded in EPON as described previously (see Salloum et al., 2014). Tissue was imaged with serial block-face electron microscopy at 70 nm/slice at 6.2 nm/pixel. We imaged 125,000 μm^3 regions (tissue cubes with 50 μm dimensions) exhibiting intact or deficient anterograde transport (3 control samples, 3 transport-intact DBA/2J samples; 4 transport-deficient DBA/2J samples).

Tracing & 3-D reconstruction

Two 125 μm^3 ($5 \times 5 \times 5 \mu\text{m}$) subregions in each retinorecipient neuropil (Valverde, 1973; Wiener, 1986) exhibiting intact or deficient anterograde transport were randomly selected for reconstruction. All synaptic boutons, presynaptic active zones, and associated mitochondria within these blocks were traced using Reconstruct software (Synapse Web Reconstruct, <http://synapses.clm.utexas.edu/synapseweb/software-0>, RRID:SCR_002716; Fiala, 2005) and characterized as one of the following bouton types: retinocollicular (retinal), non-retinal excitatory presynaptic boutons, inhibitory boutons, and dendritic (D). Using established morphological criteria (e.g. Famiglietti, 1970; Lund, 1972; Valverde, 1973; Ranney Mize, 1983; Vidal-Sanz et al., 1987; Calkins et al., 2005; Boka et al., 2006), synaptic boutons were characterized into one of these types by three trained observers who were blind to strain and transport condition of samples. Briefly, retinal boutons were identified based on their large, round, sparsely-cristated mitochondria and relatively sparse, round synaptic vesicle distribution. As a result of the loosely packed cristae, retinal projection mitochondria appear pale compared to mitochondria of other bouton types. Non-retinal boutons were classified with elongated, dark mitochondria (densely packed cristae) and dense concentrations of synaptic vesicles. Non-retinal boutons encompass excitatory and inhibitory inputs. Excitatory and inhibitory synapses are typically distinguished by synaptic vesicle shape [spherical (excitatory) vs. flattened (inhibitory)] and junctional symmetry [asymmetric (excitatory) vs. symmetric (inhibitory)], but due to the resolution inherent to this preparation it was difficult to confidently distinguish these subtle attributes in some boutons so they were combined into one group for analysis. D boutons were presynaptic dendrites of collicular neurons exhibiting pre-synaptic active zones within dendrites that were apposed by post-synaptic densities. X boutons were small boutons (relative to other bouton types) that contained synaptic vesicles and at least one active zone. These did not have mitochondria and could not be confidently assigned to one of the above groups.

For every object, all serial sections were traced until the object was no longer visible in either direction. Tracings were completed by three different trained observers and

representative tracings were cross-checked between observers for consistency and validity. To ensure that the axon shaft was not included in measurements of bouton size, the transition from axon shaft to bouton was identified by the disappearance of microtubules and the change from a constant diameter of the axon to the changing diameter of the bouton. Any bouton that resided within or came into contact with the outside boundary of the cube was traced. Active zones were determined by the presence of both synaptic vesicle clustering at the membrane and apposed by a post-synaptic density. Densities that were absent of clear synaptic vesicle clustering and could not be followed through multiple sections were assumed to be puncta adherentia and were not traced (Gray, 1963; Rowland et al., 2000).

Mitochondrial health scores

Mitochondria were graded by using a five-point scale, determined by observing cristae, inner and outer membrane, and overall appearance (Coughlin et al., 2015). At the extremes, health scores were coded as 1 for the most degenerated or absent- appearing cristae with/without presence of black inclusions, and 5 for the most intact and uniform (scale is illustrated in Figure 5A). Multiple observers were trained to use the scale and ratings were compared per sample to ensure conformity.

Statistical analysis

The total number of boutons characterized for each synapse type per D2G, transport-intact DBA/2J, and transport-deficient DBA/2J groups are provided in the legend of Figure 2. Bouton surface area and volume, active zone size and quantity, and mitochondria volumes and quantity were analyzed as dependent variables in one-way analyses of variance (ANOVA) and were contrasted between the three tissue conditions of the study: D2G control SC, transport-intact DBA/2J SC, and transport-deficient DBA/2J SC. Planned or post-hoc comparisons using Bonferroni corrections were used to identify significant differences between each of these conditions following a significant omnibus ANOVA result. Analyses were conducted separately for all terminal classes.

Prior to analysis, data for individual animals used in the study were examined for outliers, and in one case of CTB– DBA/2J SC (D11-7), outlying counts ($n=178$) of X boutons were identified. Image analysis and X bouton quantification was reconfirmed in this case by multiple observers. Retinal terminal measurements (bouton density, size, mitochondria number, size, etc.) for this particular animal were analyzed separately and compared with scores averaged from the remaining animals in the transport-deficient DBA/2J group. Comparison of means between the outlier and remaining cases showed that the outlying case did not yield significantly different values from the group means on these variables. Therefore, this case was retained for further analyses of both retinal and non-retinal boutons despite its extremely large number of X boutons. It is possible that this case was representative of the wide variation in late-stage disease progression in this model that is typically seen beyond 12 months in DBA/2J mice, hence we deemed it valid for inclusion.

For multiple regression analyses used to predict bouton size from active zone quantity, size, and mitochondrial volume in retinal synapses, preliminary data screening was performed and data were shown to be normal, linear, and homoscedastic. Initial examination of these

variables for all cases indicated positive correlations between all pairs of variables, but the correlation between predictor variables of active zone size and mitochondria size ($r = .62$) did not indicate extremely high multicollinearity (active zone size and quantity were highly correlated at $r = .86$, but this was anticipated). All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22 (IBM Corp; Armonk, NY; RRID:SCR_002865).

Results

At the ages examined in the current study (9–14 months), elements of the RGC projection such as axons and somata persist in the DBA/2J mouse model of glaucoma despite substantial deficits in axonal transport function (Danas et al., 2003; Buckingham et al., 2008; Crish et al., 2010; Dengler-Crish et al., 2014). Pathology increases as a function of age in this model but in a highly variable fashion, so age is not a reliable predictor of pathological severity in aging DBA/2J mice. Therefore, consistent with previous studies (Crish et al., 2013), we used transport outcome as a more specific predictor of pathological state in our aged DBA/2J mice.

We were able to microdissect collicular areas showing intact CTB label (CTB+) away from collicular areas devoid of tracer label (CTB–; Figure 1A&B) in DBA/2J mice along with SC from D2G control mice for visualization and reconstruction of the 3-D ultrastructural composition of all synaptic boutons including mitochondria, membranes, and active zones (Figure 1C & D). Size, shape, and density of these elements were measured and used as metrics for determining RGC synapse integrity with respect to transport integrity, as such metrics are strongly correlated with important physiological functions including synaptic strength, efficacy, and oxidative capacity (Propst and Ko, 1987; Yeow and Peterson, 1991; Pierce and Lewin, 1994; Holderith et al., 2012).

Bouton characterization

The neuropil of the superficial SC exhibited well characterized terminal types: retinocollicular (retinal), non-retinal (excitatory and inhibitory), and dendritic. A small subset of terminals were unclassifiable (labelled X). Figure 2 illustrates examples of each terminal type, including identification criteria (panel A) and three-dimensional reconstruction (panel B). While we cannot eliminate the possibility that predegenerative changes shift bouton appearance to more resemble other classes, we are confident in these characterizations because retinal boutons are known to retain their identifying characteristics across wide morphometric variation in experimental degenerations (Xiong and Finlay, 1993).

Bouton density

Retinal terminals predominated in the retinorecipient SC (Figure 2C; Valverde, 1973). Density of retinal boutons did not differ between glaucomatous DBA/2J mice and the controls, nor were there differences in the number of retinal terminals in transport-intact versus transport-deficient areas ($F_{2,7} = .094$, $p = .912$, ns ; Figure 2C). These boutons appeared normal, and usually did not display any classic degenerative signs (Wang et al.,

2002; Raisman and Matthews, 2012). Specifically, most terminals in transport-deficient areas maintained at least one mitochondrion and active zone, lacked intracellular electron dense inclusions, and lacked the classic darkened cytoplasm with swelling of the extracellular membrane characteristic of degenerating boutons. These findings are consistent with previous reports of the persistence of a semi-functional retinal projection in DBA/2J mice (Crish et al., 2010, 2013; Dengler-Crish et al., 2014). This further supports a significant separation between functional and structural loss in axons undergoing progressive degeneration.

Bouton density of non-retinal ($F_{2,7} = 1.573$, $p = .273$, *ns*) or dendritic terminals ($F_{2,7} = .952$, $p = .431$, *ns*) did not differ between transport-deficient and transport-intact DBA/2J SC or the control strain (Figure 2C). We observed increased X bouton numbers in the SC of one transport-deficient DBA/2J mouse (Figure 2C; red circle) and based on overall counts, DBA/2J mice had more X boutons than controls. However, these differences were not statistically significant.

Bouton morphometry

While we found that retinal boutons persist and are mostly devoid of overt degenerative characteristics, further analysis of boutons and mitochondria yielded substantial variations in the glaucomatous DBA/2J mouse SC. Transport-intact DBA/2J SC showed 47% greater total bouton volume than transport-deficient SC, and 29% greater bouton volumes than D2G controls ($F_{2,781} = 19.738$, $p < 0.01$, Figure 3A, left panel). In terms of mitochondrial volume, transport-intact DBA/2J SC were 60% larger than transport deficient DBA/2J SC and 29% larger than D2G controls ($F_{2,781} = 19.074$, $p < 0.01$; Figure 3A, right panel). In transport-deficient DBA/2J SC, retinal bouton volume was significantly reduced compared to bouton volume in transport-intact tissue ($p < 0.01$), but it did not differ from D2G control tissue bouton volume ($p = 0.190$, *ns*). This finding was in contrast to results obtained for mitochondrial volume, which was significantly decreased in transport-deficient boutons compared to both transport-intact ($p < 0.01$) and control SC ($p < 0.05$). Representative images and 3-D reconstructions of boutons (panels B & C) and mitochondria (panels D & E) are shown in Figure 3.

Non-retinal bouton volumes did not vary across transport-deficient and transport-intact glaucomatous DBA/2J SC or control strain tissue ($F_{2,477} = .185$, $p = .449$, *ns*; Figure 4A, left panel). When examining mitochondria, we found that non-retinal terminal mitochondria in transport-deficient areas were significantly smaller than those in transport-intact areas ($F_{2,476} = 8.73$; $p < 0.01$ in all comparisons; Figure 4A, right panel). Micrographs (panel B) and 3-D reconstructions (panel C) of non-retinal boutons and mitochondria are illustrated in Figure 4. No obvious pathological characteristics accompanied X boutons.

Mitochondria health

We assessed the health of mitochondria associated with boutons using previously established morphological criteria (Coughlin et al., 2015), classifying mitochondria on a 1–5 scale (5 = no flaws and 1 = undergoing mitophagy) based on cristae and membrane appearance (Figure 5A). Despite the volume variation in DBA/2J mitochondria compared to D2G controls in

retinal boutons, 90.4% of transport-intact and 71.1% of transport-deficient DBA/2J retinal bouton mitochondria were scored as healthy (ratings of 3 or above; Figure 5B).

Bouton active zone number and size are reduced in transport deficient areas of glaucomatous SC

Active zones are the final conduit of communication between the retina and brain, and their number and composition vary in both normal and disease states. Despite persistence of retinal terminals in the SC, the number of active zones per retinal bouton was significantly reduced in transport-deficient DBA/2J tissue (by 67% and 74% respectively), compared to transport-intact and control strain tissue ($F_{2,781} = 14.973$, $p < 0.01$; Bonferroni-corrected post-hoc comparisons $p < 0.03$), with the latter two groups showing no significant differences (Figure 6A, left panel). Furthermore, the remaining active zones in transport-deficient areas were significantly smaller than both transport-intact DBA/2J and control strain regions ($F_{2,751} = 14.456$, $p < 0.01$; Bonferroni-corrected post-hoc $p < 0.01$; Figure 6A, right panel). This is indicative of reduced synaptic contacts, and therefore, these findings suggest there may be reduced information flow between the presynaptic RGC and recipient neurons in the brain (Pierce and Lewin, 1994; Murthy et al., 2001; Matz et al., 2010; Chen et al., 2015). Representative micrographs (panel B) and 3-D reconstructions (panels C & D) illustrating active zones and their relative surface area are shown in Figure 6.

Relationships between components of synaptic compartment morphometry in retinal terminals

Increased synaptic strength is due to a large degree to increases in active zone size and number. More active zones require more support and machinery, which in turn require more energy (i.e. more oxidative capacity of mitochondria). Using standard multiple regression, we found that for each experimental group, mitochondrial volume, active zone surface area, and active zone quantity were significant predictors of retinal bouton volume in the SC (Table 1). For the D2G control group, the overall regression model was significant ($R = .89$, $R^2 = .75$, adjusted $R^2 = .75$, $F_{3,245} = 248.9$, $p < 0.001$). Bouton volume could be predicted quite well from these variables, accounting for 75% of the variance in bouton volume. To assess the respective strength of each of these predictors, t-ratios for individual regression slopes (beta values) were analyzed and yielded significant values for all three predictors ($p < 0.05$ in all cases). Mitochondria volume was the strongest predictor of bouton size in this set of variables ($\beta = .62$), followed by active zone quantity ($\beta = .217$) and active zone surface area ($\beta = .156$).

For transport-intact and transport-deficient retinal terminals, the overall regression models were also significant (transport-intact model $R = .83$, $R^2 = .70$, adjusted $R^2 = .69$, $F_{3,235} = 179.36$, $p < 0.01$; transport-deficient model $R = .90$, $R^2 = .81$, adjusted $R^2 = .80$, $F_{3,292} = 405.38$, $p < 0.01$). Again, mitochondrial volume was the strongest predictor of bouton size in transport-intact ($\beta = .678$, $p < 0.01$) and transport-deficient ($\beta = .718$, $p < 0.01$) groups, followed by active zone surface area (transport-intact $\beta = .280$, $p < 0.01$; transport-deficient $\beta = .263$, $p < 0.01$). However, active zone quantity was not a significant predictor of bouton volume in either transport-intact or transport-deficient projections.

Discussion

There is increasing evidence that functional deficits in axonal transport and neuronal signaling characterize early stages of glaucoma (Crish et al., 2010; Takihara et al., 2013; Dengler-Crish et al., 2014; Fahy et al., 2015; Chen et al., 2015), similar to early pathologies of many chronic neurodegenerative conditions (Stokin, 2005; Conforti et al., 2007; Yoshiyama et al., 2007; Adalbert et al., 2009; Brady and Morfini, 2010). Given the slow progression of glaucomatous vision loss in humans, identification of early steps in the pathology represent potential targets for stopping or reversing the subsequent visual deficits. Blindness may begin with nonfunctioning axons that either do not generate or transmit electrical signals to the brain and/or have impaired neurotransmitter release at the synapse—both of which can elicit a slow degenerative process that eventually results in cell loss (Schonfeld-Dado et al., 2009; Zhao et al., 2011). The results of the current study support the idea of early synaptopathy in the retinal projection in glaucoma. The overarching implication of this study is that the earliest pathological changes in glaucoma may not be overt somata or axon loss, but functional changes in neuronal communication. Identification of these disturbances in axonal function present an advantage in researching treatments for this disease because a) they signal the onset of a longer window of time in which to intervene and slow the pathological process, and b) they would be amenable to neuroprotective therapies that seek to restore such function, which is a far more tractable problem to solve than trying to replace lost cells and connections (Bechtold et al., 2004, 2005; Chang and Goldberg, 2012).

Synapses are an obvious point of vulnerability in disease because of their energetic demands and complexity (see Engel, 2014 for a discussion). Our data illustrate ultrastructural changes in the synapse before and during axonal transport failure in the DBA/2J glaucoma mouse model, while the density of retinal terminals in this region remained largely unchanged. In the SC of DBA/2J mice with intact axonal transport, retinal boutons and their mitochondria were larger than normal, and these boutons showed few or no signs of degeneration. In contrast, retinal boutons in SC transport-deficient areas exhibited smaller mitochondria and fewer, smaller presynaptic active zones than both control and transport-intact collicular areas; these boutons likewise lacked signs of overt degeneration. Our 3-D EM studies also showed additional changes in non-retinal boutons within the SC—however, these changes were likely secondary to those occurring in retinal axons.

Changes in retinal boutons in the glaucomatous SC

Our data show that an intact retinal projection persists for a significant window of time after axon transport deficits manifest. It remains to be determined if the persistent synapses retain normal function. Global metabolic and electrical signaling defects have been measured in the optic nerves of DBA/2J mice (Baltan et al., 2010), however, these changes were not resolved at the level of individual optic nerve axons. As glaucoma is not a diffuse degenerative process but incurs focal pathology (i.e. pathology begins and then spreads from sector to sector in the retina and its projection), it is important to distinguish functional changes in individual subregions. Neighboring axons or other neural elements in the visual central nervous system can exhibit massive variation in pathology—some mechanisms and

injuries may not be evident except when examining these structures with respect to these differences, as was done with our use of neuronal tracing here.

Changes in non-retinal boutons in the glaucomatous SC

As expected, we did not find a reduction in the density of non-retinal synapses in the SC, but we did observe changes in both mitochondrial volume and active zones in non-retinal boutons. Given that many of these synapses carry visual information from extra-retinal sources or mediate neural communication within the SC, the decreased mitochondrial volume in transport-deficient areas may represent a reduction in activity and metabolic need of these structures in response to attenuated retinal synaptic activity. Whether these changes are adaptive or maladaptive (e.g., preliminary to subsequent degeneration) were not determined in the current experiment. However, the observation that the SC circuitry can respond to changes in the retinal projections may provide hope for intervention strategies that aim to rescue retinal axons even after the initial stages of glaucomatous changes.

Synaptic changes and progression of pathology

The initial increase in bouton volume in transport-intact DBA/2J SC parallels increased volume reported for RGC somata in other glaucoma models (Steward and Vinsant, 1978; Weber and Harman, 2005) and increased axon caliber in the DBA/2J mouse optic nerve (Cooper et al., 2015). These changes may influence action potential propagation and neurotransmitter release (Meyer et al., 2014). Although bouton swelling can initiate or indicate degenerative pathology in other disease models (Masliah et al., 1991; Schroer et al., 1992; Hernández-Fonseca et al., 2009; Kujawa and Liberman, 2015; Nordström et al., 2015; Sumi-Akamaru et al., 2015), it is not likely to be the case here. Changes in overall bouton volume were accompanied by similar changes in mitochondrial volume and active zone areas, suggesting these are coordinated changes linked to each other rather than runaway pathology (Meyer et al., 2014). Increased size of transport-intact DBA/2J boutons and mitochondria relative to controls may coincide with early RGC swelling in the retina that precedes degeneration, as has been shown in other glaucoma models (Urcola et al., 2006; Pinar-Sueiro et al., 2011; Kalesnykas et al., 2012), although it is often indicative of increased synaptic strength and neurotransmitter release (Propst and Ko, 1987; Yeow and Peterson, 1991; Pierce and Lewin, 1994). In transport-deficient areas, reduction in bouton volume in the absence of evidence of degeneration such as electron-dense inclusions, membrane separation, etc., suggests plasticity rather than overt neurodegeneration at early stages after transport loss. An intriguing possibility related to this finding is that we see a trend of more X boutons in DBA/2J mice. While we could not confirm this statistically, this pattern may be indicative of retinal boutons undergoing plasticity (e.g. retraction or sprouting) in response to stressors. Such “reactive plasticity” has been previously reported in glaucoma and other neurodegenerative disorders (Lam et al., 2003; Rubio, 2006; Georgiou et al., 2010; Ly et al., 2011).

Our data show an initial increase in mitochondrial volume in transport-intact retinal terminals in our disease model. We hypothesized that RGC mitochondria in the DBA/2J SC where transport remains intact would have increased oxidative capacity since more regularly packed, intact cristae can accommodate more proteins of the electron transport chain and

oxidative phosphorylation. Increased size of healthy mitochondria in transport-intact areas could be a compensatory response to stressors influencing this projection as, with the increased bouton size, has been reported in aging and other degenerative conditions (Bertoni-Freddari et al., 1990; Sisková et al., 2010; Soghomonian et al., 2010). This contrasts sharply with our findings that retinal terminals in transport-deficient areas had smaller and less healthy mitochondria (even though most mitochondria in these terminals are still considered healthy). Aside from a lack of electron dense inclusions, these reductions in mitochondrial morphometry are opposite to the prototypical characterization of pathological, predegenerative mitochondria. Degenerating mitochondria often swell rather than shrink and, while the cristae can become malformed, the overall density of the matrix remains unchanged (McMahan, 1967).

Reduced oxidative capacity in the transport-deficient DBA/2J colliculi suggest a metabolic deficiency, as has been reported in other parts of this projection (e.g. optic nerve; Baltan et al., 2010). These mitochondrial changes, as well as the reduction in active zone size and number in these terminals, strongly suggest that neuronal signaling is reduced between RGCs and their postsynaptic targets (Murthy et al., 2001; Tyler and Pozzo-Miller, 2001; Stanic et al., 2003; Matz et al., 2010; Soghomonian et al., 2010; Holderith et al., 2012). Changes in mitochondrial morphology and function have been implicated as an early hallmark of various neurodegenerative disorders (Wallace, 2005; Lin and Beal, 2006; Sisková et al., 2010; Zamboni et al., 2011) including glaucoma (Abu-Amero et al., 2006; Baltan et al., 2010; Coughlin et al., 2015). Similarly, we have previously reported reduced trace metal levels (e.g. magnesium, zinc) in the DBA/2J SC (DeToma et al., 2014). Considering the importance of these elements at the synapse, a reduction in these metals may be linked to the decreased number and size of active zones reported in the current study. Such reduction in neuronal signaling may be a major contributor to the eventual neurodegeneration characteristic of glaucoma (Ward et al., 2014; Weitlauf et al., 2014).

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Figure 1.

Serial block face electron microscopy preparation and imaging to create 3-D reconstructions of superior colliculus (SC) boutons. (A) Whole mount example of the exposed SC with overlying cortex removed of an 11-month old D2G control mouse with complete presence of cholera toxin-beta (CTB) label (green) across entire breadth of SC, indicative of intact anterograde axonal transport. Rostral is right; scale bar = 1000 μm for A and B. (B) Whole mount example of the exposed SC of an 11-month old DBA/2J mouse. The green fluorescent areas of this mouse were microdissected out for the transport-intact (CTB+) tissue group, while areas absent of green label were microdissected out for the transport-deficient (CTB-) tissue group. (C) An image stack of electron micrographs illustrating the random selection of a 125 μm^3 block (area shown by gray-shaded box) in the neuropil of layer II of the SC within the greater 125,000 μm^3 tissue block. All SC boutons along with their corresponding mitochondria and active zones within and/or touching the borders of the 125 μm^3 box were traced from every section as long as the element clearly persisted in the field of view. After tracing was complete all boutons were classified (see text and Figure 2 for details). (D) 3-D reconstructions of all the traced and classified boutons within one 125 μm^3 region of interest. Orange = RGC terminals, blue = non-retinal excitatory, green = inhibitory, pink = dendritic, Red = x

Figure 2.

Density of bouton types across tissue groups. (A) Representative electron micrographs from each of the four bouton types within the SC neuropil: retinal, non-retinal, D, and X (orange, yellow, pink, and teal; respectively; mitochondrial inset and outlined in purple). Retinal boutons were characterized with large, sparsely cristated mitochondria and relatively sparse, round vesicle distribution. Non-retinal boutons were classified with elongated, dark mitochondria and dense concentrations of smaller synaptic vesicles. D boutons were associated with dendrites of collicular neurons and generally had both pre-synaptic (#) and post-synaptic (&) active zones. X boutons were small, synapsing boutons that did not have mitochondria to allow for further classification. Scale bar = 1 μm. (B) 3-D reconstructions of serial electron micrograph tracings of retinal, non-retinal, D, and X boutons (left to right) represented in (panel A). (C) Average number of retinal, non-retinal, D (dendritic), and X boutons (RGC synapses) found in control (blue; D2G SC areas), CTB+ (green; transport-intact DBA/2J SC), and CTB- (red; transport-deficient DBA/2J SC) tissues. Average retinal, non-retinal, and D bouton count did not differ significantly between control, CTB+, and CTB- groups ($p > 0.05$, ns, in all cases; analysis of variance (ANOVA)). Although statistical results were not significant, distribution of X boutons in one particular CTB- DBA/2J was exceptionally high (red dot). Sample $n = 249, 239$, and 296 for control, CTB+, and CTB- retinal boutons; $n = 132, 112$, and 236 for control, CTB+, and CTB- non-retinal boutons; $n = 59, 39$, and 56 for control, CTB+, and CTB- D boutons; $n = 34, 59$, and 250 for control, CTB+, and CTB- X boutons respectively. Data plotted in bar graph of panel C are mean $\pm$ SEM

Figure 3.

Differences in retinal bouton volume and mitochondrial volume between tissue groups. (A) (Left) Average volume of retinal synapses found in control (blue; D2G SC areas), CTB+ (green; intact transport in DBA/2J SC areas), and CTB- (red; transport compromised in DBA/2J SC areas) tissue. CTB+ average bouton volume was significantly larger than both control and CTB- average synapse volume (** $p < 0.01$, one-way ANOVA with Bonferroni corrected comparisons). $n = 249, 239$, and 296 for control, CTB+, and CTB- retinal boutons respectively. (Right) Average mitochondrial volume of retinal synapses found in control, CTB+, and CTB- tissue. Average volume was computed as the mean of the total sum of mitochondrial volume per bouton. All three tissue groups significantly differed in average mitochondrial volume ($p < 0.01$, one-way ANOVA). Bonferroni-corrected post hoc

comparisons showed that CTB– retinal boutons had smaller average mitochondrial volume compared to controls (* $p < 0.05$) and CTB+ samples (** $p < 0.01$); and that CTB+ samples showed greater mitochondria volumes than controls (** $p < 0.01$). $n = 386, 456$, and 478 for control, CTB+, and CTB– retinal mitochondria respectively. Data in panel A are represented as mean $\pm$ SEM. (B) Representative electron micrograph-traced examples of retinal bouton volume size differences found in control, CTB+, and CTB– tissue (left to right). Scale bar = $1 \mu\text{m}$. (C) 3-D reconstructions of serial electron micrograph tracings of retinal boutons represented in (B). CTB+ retinal bouton volume was visually larger than control and CTB– bouton volumes. (D) Representative electron micrograph traced examples of retinal bouton and its mitochondria (purple) in control, CTB+, and CTB– tissue (left to right). Scale bar = $1 \mu\text{m}$. (E) 3-D reconstruction of serial electron micrograph tracings of retinal bouton and its mitochondria represented in (D). CTB– retinal mitochondria was visually the smallest, the control mitochondria was larger than the CTB–, and the CTB+ mitochondria was visually the largest of the three tissue groups

Figure 4.

Non-retinal bouton volumes are similar across tissue types but mitochondrial volume is reduced in CTB- SC. (A) (Left) Average non-retinal bouton volume in control (blue; D2G SC areas), CTB+ (green; intact transport in DBA/2J SC areas), and CTB- (red; transport compromised in DBA/2J SC areas) tissue. There were no significant differences between the average non-retinal bouton volumes in these three groups ($p > 0.05$, ns: one-way ANOVA). $n = 132$, 112 , and 236 for control, CTB+, and CTB- non-retinal boutons respectively. (Right) Average mitochondrial volume of non-retinal synapses found in control, CTB+, and CTB- tissue, where average mitochondrial volume was an average of the total sum of mitochondrial volume per bouton. Average mitochondrial volume was significantly smaller in CTB- tissue compared to both control and CTB+ tissue (** $p < 0.01$, one-way ANOVA with Bonferroni-corrected comparisons). $n = 196$, 197 , and 329 for control, CTB+, and CTB- non-retinal mitochondria respectively. Data in bar graphs are represented as mean $\pm$ SEM. (B) Representative electron micrograph traced example of non-retinal bouton and its mitochondria (purple) found in control, CTB+, and CTB- tissue groups (left to right). Scale bar = $1 \mu\text{m}$. (C) 3-D reconstruction of serial electron micrograph tracings of non-retinal bouton and its mitochondria represented in (B) to detail the decreased total mitochondrial volume in CTB- non-retinal bouton

Figure 5.

Mitochondria health of retinal boutons across tissue groups. (A) Representative electron micrograph sections of mitochondria chosen for a standard scale to grade retinal mitochondria health. Grade 5 was the optimal score for mitochondrial health, and the grade number decreased as degenerative characteristics found in the retinal mitochondria increased. (B) A single representative 125 μm^3 cube from each of the tissue groups (control, CTB+, and CTB-; n = 88, 146, and 97 respectively) was selected and each cube's retinal mitochondria were graded according to the scale in (A). Overall, most mitochondria were scored as 3 or higher, representing a healthy state. While we saw more defective (scoring 1 or 2) mitochondria in CTB- areas, most of the mitochondria were healthy

Figure 6.

Retinal boutons from transport deficient areas had fewer and smaller active zones. (A) (Left) Active zone number per retinal bouton in control (blue; D2G SC areas), CTB+ (green; intact transport in DBA/2J SC areas), and CTB- (red; transport compromised in DBA/2J SC areas) tissue. CTB- retinal active zone number per bouton was significantly decreased compared to control and CTB+ tissue groups (** $p < 0.01$: one-way ANOVA with Bonferroni-corrected comparisons). $n = 249, 239$, and 296 for control, CTB+, and CTB- retinal boutons respectively. (Right) Average active zone surface area found in control, CTB+, and CTB- tissue, where average active zone surface area was the average of the total sum of active zone surface area per bouton. Average CTB- retinal active zone surface area was significantly smaller compared to control and CTB+ groups (** $p < 0.01$ in both cases: one-way ANOVA with Bonferroni-corrected comparisons). $n = 570, 603$, and 501 for control, CTB+, and CTB- retinal active zones respectively. (B) Representative electron micrograph traced example of retinal bouton and its active zone(s) (yellow dotted line) found in control,

CTB+, and CTB- tissue groups (left to right). Scale bar = 0.5 μ m. (C) 3-D reconstruction of serial electron micrograph tracings of retinal bouton examples and its active zone(s) represented in (B). The number of active zones per bouton was greater in the control and CTB+ than in the CTB- retinal bouton. (D) Magnified 3-D reconstruction of one of the active zones in (B, C) for visual representation of the decreased active zone surface area in the CTB- retinal bouton. Data are represented as mean $\pm$ SEM

Table 1

Beta values for variables predicting bouton volume

	Bouton volume		
	D2G	^a CTB+	^a CTB–
Mitochondrial volume	0.620 ^b	0.678 ^b	0.718 ^b
Active zone surface area	0.156 ^b	0.280 ^b	0.263 ^b
Active zone #	0.217 ^b	–.069	–.001

^aCTB+ is abbreviation for transport-intact DBA/2J SC; CTB– indicates transport-deficient SC

^bSignificant effect at $p < 0.01$