When is a control not a control? Reactive microglia occur throughout the control contralateral visual pathway in experimental glaucoma

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Abstract

Background: Glaucoma remains a leading cause of irreversible blindness worldwide. Multiple animal models show retinal ganglion cell injuries that replicate features of glaucoma. In these experiments the contralateral eye is commonly used as an internal control. As in humans, in rodents and non-human primates there is significant cross-over of retinal ganglion cell axons from the ipsilateral to the contralateral side at the level of the optic chiasm which may confound findings when damage is restricted to one eye. While neuroinflammation may be a critical event that damages retinal ganglion cells and their axons during glaucoma progression the effect of unilateral damage on the contralateral visual pathway has largely been unexplored.

Methods: Ocular hypertensive glaucoma was induced unilaterally or bilaterally by intracameral injection of paramagnetic beads in adult Brown Norway rats. Retinal ganglion cell neurodegeneration and dysfunction were assessed. Neuroinflammation was quantified in the retina, optic nerve head, optic nerve, lateral geniculate nucleus, and superior colliculus by high resolution imaging, and in the retina by flow cytometry and protein arrays.

Results: Following ocular hypertensive stress the retinal vasculature remodels, peripheral monocytes enter the retina, and microglia become highly reactive. This effect is more marked in animals with bilateral induction of ocular hypertensive glaucoma. In rats where glaucoma was induced unilaterally there was significant microglia activation in the contralateral (control) eye. Microglial activation extended into the optic nerve and terminal visual thalami, where it was similar across hemispheres irrespective of whether ocular hypertension was unilateral or bilateral.

Conclusions: Ocular hypertensive glaucoma in the rat recapitulates many of the features of human glaucoma. In this model microglia become reactive throughout the visual pathway during glaucoma pathogenesis. These effects are not isolated to the experimental eye and its pathway and significant neuroinflammation occurs in the contralateral 'control' eye and pathway. These data suggest that caution is warranted when using the contralateral eye as control in unilateral models of glaucomatous damage.

Key words: microglia, monocyte, neuroinflammation, glaucoma, retina, optic nerve, optic nerve head, lateral geniculate nucleus, superior colliculus, dendrite, synapse

Background

Glaucoma is the leading cause of irreversible blindness, affecting ~80 million people worldwide (1). The underlying pathology is complex and characterised by the progressive dysfunction and loss of retinal ganglion cells and their axons. Major risk factors are increasing age, elevated intraocular pressure, and genetic predisposition. Despite strategies to lower intraocular pressure (the only treatable risk factor for glaucoma), over 40% of treated glaucoma patients will go blind in at least one eye (2) suggesting that factors other than high intraocular pressure drive retinal ganglion cell degeneration.

Neuroinflammation is a shared feature of glaucoma pathogenesis in human glaucoma patients (3) and animal models of glaucoma (genetic and inducible (4)). In times of stress, retinal ganglion cells are reliant on a supportive network of glial cells to provide neurotrophic and metabolic support, especially to their long, unmyelinated axons in the retina and optic nerve head. Emerging evidence supports a paradigm in which retinal ganglion cell axons are insulted throughout their trajectory to retinorecipient areas; the dorsal lateral geniculate nucleus and the superior colliculus. Microglia and/or other immune-derived cells are likely to be key players in these pathogenic events.

The eye is commonly used as a tool to explore neurodegenerative events. However, a common feature of these experiments is the use of the contralateral eye as an internal control (which is often used to normalise data to the experimental eye). These data should be qualified because: (i) retinal ganglion cell numbers differ between eyes even within individual animals (5), (ii) significant numbers of retinal ganglion cell axons do not cross at the optic chiasm and terminate in ipsilateral thalami (ranging from ~3% in mouse and ~3-10% in rat to ~45% in primates and ~50% in human (6-8)), (iii) albino rodents are commonly used (*e.g.* Wistar and Sprague-Dawley rats) which have reduced ipsilateral projections (9-12), and, (iv) most human primary open angle glaucoma is bilateral. Microglial activation is an early and persistent feature across glaucoma models and species with evidence of immune-cell activation in human tissue from advanced glaucoma (13-17). To date, there is a paucity of data regarding microglial activation in the contralateral eye of mice with unilateral ocular hypertension (largely qualitative with quantification only through cell counting (18-20)). Activation has been reported in the optic nerve and in terminal brain thalami (15, 21, 22), but contralateral activation has yet to be fully explored in these tissues.

To address these issues and explore any neuroinflammation throughout the visual pathway in glaucoma, we fully characterise inflammatory changes in an inducible rat model of ocular hypertensive glaucoma in which intraocular pressure is elevated by intracameral (anterior chamber) injection of paramagnetic beads to occlude the drainage structures of the eye (23, 24). This results in sustained ocular hypertension driving a reproducible and significant loss of retinal ganglion cell numbers as well as neuroinflammation (without uveitis). Using this model, we

assessed the effects of unilateral or bilateral induced ocular hypertension on glaucoma pathology throughout the retinal ganglion cell projections. These experiments were performed in the Brown Norway rat as it is the genetic standard for rats, is inbred, pigmented, and avoids retinothalamic pathway issues present in albino animals. We demonstrate that neuroinflammation persists throughout retinal ganglion cell pathways with monocyte infiltration and pro-inflammatory cytokine release in the retina. Critically, these changes are mirrored in the contralateral visual pathway warranting caution when using the contralateral eye as a 'control' in ophthalmic research.

Methods

Rat strain and husbandry

Adult, male Brown Norway rats (aged 12-16 weeks, weighing 300-375 g, SCANBUR) were housed and fed in a 12 h light / 12 h dark cycle with food and water available *ad libitum*. All experimental procedures were undertaken in accordance with the Association for Research for Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Research. Individual study protocols were approved by Stockholm's Committee for Ethical Animal Research (10389-2018).

Induction of ocular hypertension

Ocular hypertension (OHT) was induced bilaterally (OHT/OHT; n = 19 rats, 38 eyes) or unilaterally (OHT/NT; n = 12 rats, 12 eyes). For unilateral OHT rats, the contralateral eye was an un-operated, normotensive (NT) eye (NT/OHT; n = 12 rats, 12 eyes). Bilateral NT rats served as controls (NT/NT; n = 17 rats, 34 eyes). OHT was induced using a magnetic microbead injection model (23, 24) with modifications detailed below. Microbeads (4.5 µm diameter) (Dynabead Epoxy M-450, Thermo Fisher) were prepared for injection by washing with 1x Hank's balanced salt solution (HBSS -$CaCl_2$ -$MgCl_2$ -phenol red, Gibco) and re-suspended in 0.25x volume of HBSS (~2.7 x 10^6 beads/µl). These beads were selected as the coating of glycidyl ether groups confers a hydrophobic nature that allow for easy dispersion within the anterior chamber while retaining blockage of the iridocorneal angle through binding amine and sulphydryl groups on cell surfaces. Rats were anaesthetised with an intraperitoneal injection of ketamine (37.5 mg/kg) and medetomidine hydrochloride (1.25 mg/kg). Using a nanofil syringe (NanoFil; WPI) with 33 G tribevelled needle (WPI), 6-8 µl of bead solution was injected into the anterior chamber of the eye using a tunnel incision through the cornea. Beads were distributed to block the iridocorneal angle using a handheld rare earth magnet (cylindrical with a 4 mm diameter). Intraocular pressure (IOP) was measured using a Tonolab rebound tonometer (Icare) in awake and unrestrained rats. Rats were habituated to the tonometry procedure the week before surgery. Baseline IOP was recorded on the day of surgery (day 0) and recorded every 2-4 days afterwards until the endpoint (postoperative day 14), with IOP recordings always taken between 9 and 10am to avoid the effects of the circadian rhythm on IOP (2-3 hours from lights on). IOP was taken as the average of 5 tonometer readings. NT/NT rats followed the same 14 day time course (without surgery). The presence of optic nerve head cupping was determined by optical coherence tomography. In brief, rats (n = 4 NT/NT, n = 6 OHT/OHT) were anesthetised as above and optical coherence tomography was performed using a Phoenix MICRON IV (Phoenix technology group). Images were reconstructed using ImageJ and the presence of cupping was confirmed in eyes from OHT/OHT animals.

General histopathology

Rats were heavily anaesthetized at day 14 by intraperitoneal injection of pentobarbital (75 mg/kg), and euthanised by cervical dislocation. Eyes were immediately enucleated (with 1 mm of optic nerve post globe preserved) and immersed in 3.7% PFA in 1x PBS. Brains (with attached optic nerves) were removed and immersed in 3.7% PFA in 1x PBS. Eyes, optic nerves, and brains for cryo-sectioning were maintained in fixative for 24 hours before cryo-protecting in 30% sucrose in 1x PBS for 24 hours, freezing in optimal cutting temperature medium (Sakura) on dry ice, and stored at -80 ˚C. Cryo-sections were cut on a cryostat (Cryostar NX70, Thermo Scientific) and stored at -20 ˚C. Eyes were sectioned at 20 µm thickness (anterior to dorsal plane), with sections maintained from ~200 µm nasal to ~200 µm temporal of the optic nerve head (n = 3 eyes for NT/NT, OHT/OHT, NT/OHT, and OHT/NT). Optic nerves for cryo-sectioning were maintained as pairs attached at the optic chiasm, and sectioned at 20 µm to generate longitudinal nerve sections (n = 4 optic nerves for NT/NT, OHT/OHT, NT/OHT, and OHT/NT). Brains were serial sectioned (coronal plane) at 50 µm from -4 mm caudal of cranial landmark Bregma, to -8 mm caudal of Bregma in order to capture the entire dLGN and SC (n = 3 brains [6 independent hemispheres] for NT/NT, OHT/OHT, NT/OHT, and OHT/NT). Eyes for paraffin sectioning (n = 6 NT/NT eyes, 10 OHT/OHT eyes) were prepared in paraffin and sectioned (anterior to dorsal plane) to give optic nerve head sections (3 µm thickness) on a microtome (Microm HM 355S, Thermo Scientific). Optic nerves for paraffin sectioning (n = 4 NT/NT nerves, 5 OHT/OHT nerves) were dissected free from the brain 2 mm post optic chiasm. Optic nerves were separated at the chiasm with a diagonal cut used to maintain orientation and prepared in paraffin for sectioning. Transverse sections were cut (3 µm thickness) from the end proximal to the eye.

Immunohistochemistry and immunofluorescence

Antibodies and stains used for immunofluorescence, immunohistochemistry, and histological labelling are detailed in **Table 1**. The same immunofluorescent labelling protocol was followed for cryo-sections, paraffin sections, and flat mount retina with exceptions noted where relevant. Tissue was isolated using a hydrophobic barrier pen (VWR), permeabilised in 0.5% Triton X-100 in 1x PBS for 1 h, blocked in 5% bovine serum albumin in 1x PBS for 1 h, and primary antibody applied overnight at 4 ˚C (see **Table 1**). Tissue was then washed for 5 x 5 min in 1x PBS, secondary antibody applied (1:500 in 1x PBS) for 4 h at room temperature, and washed for 5 x 5 min in 1x PBS. DAPI nuclear stain (500 µg/ml stock; used 1:500 in 1x PBS) was applied for 10 min, the tissue washed for 5 min in 1x PBS, dried and mounted using Fluoromount-G and glass coverslips (Invitrogen). For cryo-sections, tissue was air dried for 15 min and rehydrated in 1x PBS for 15 min before following the protocol above. For paraffin sections, sections were deparaffinised, rehydrated through xylene and an alcohol gradient before performing antigen retrieval using EDTA buffer (pH 9, 20 min, 100 °C). Immunohistochemistry was performed in a Bond III robotic system (Leica Biosystems, Newcastle, UK).

Table 1. Antibody details

Antibody	Target	Host	Dilution	Details	Use
βIII-tubulin	Microtubules specific to RGCs	Chicken	1:250	Novussbio cat # NB100-1612	IF
PE CD11b/c	CD11b (clone OX-42)	Mouse	1:400	Nordic BioSite cat # 201807	FC
AF 647 CD45	CD45 (clone OX-1)	Mouse	1:2000	Nordic BioSite cat # 202211	FC
BV 421 CD90.1	Thy1.1 (clone OX-7)	Mouse	1:200	Nordic BioSite cat # 202529	FC
GS	Glutamine synthethase	Rabbit	1:5000	Sigma-Aldrich cat # G2781	IF
GFAP	Glial fibrillary acidic protein	Rabbit	1:1000	Abcam cat # ab 207165	IF
Isolectin GS-IB_4 (IsoB4)	Poly-*N*-acetyllactosamine, found on microglia, endothelial cells, monocytes/macrophages	Lectin from *Griffonia simplicifolia* conjugated to biotin	0.1mg/ml	Invitrogen cat # I21414	IF
NeuroTrace™ 530/615 red fluorescent Nissl stain	Nissl material	NA	1:100	Molecular Probes cat # N-21482	IF
NF-M	Neurofilament medium chain	Chicken	1:500	Novusbio cat # NB300-222	IF
MBP	Myelin basic protein	Rabbit	1:4000	Abcam cat # ab210811	IF
MAP2	Neuronal dendrites	Chicken	1:250	Novusbio cat # NB300-213	IF

RBPMS	RNA-binding protein, RGC specific in the retina	Rabbit	1:500	Novusbio cat # NBP2-20112	IF, IHC
Synaptophysin 1	Synaptophysin 1, pre-synaptic protein	Rabbit	1:500	Synaptic Systems cat # 101 002	IF
Goat-anti Rabbit AF 488	Rabbit primary antibody	Goat	1:500	Abcam cat # A11008	IF
Goat-anti Rabbit AF 568	Rabbit primary antibody	Goat	1:500	Abcam cat # A11011	IF
Goat-anti Chick AF 488	Chick primary antibody	Goat	1:500	Abcam cat # A11039	IF
Goat-anti Chick AF 568	Chick primary antibody	Goat	1:500	Abcam cat # A11041	IF
Streptavidin AF 488 conjugate	Biotin	NA	4 µg/ml	Invitrogen cat # S11223	IF

FC (flow cytometry), IHC (immunohistochemistry), IF (immunofluorescence)

Assessment of neurodegeneration following ocular hypertension.

Eyes for flat mount retina preparations (*n* = 6 NT/NT eyes, 6 OHT/OHT eyes, 5 NT/OHT eyes, 5 OHT/NT eyes) were maintained in fixative as globes for 2 h before the retina was dissected free at the optic nerve head, the vitreous removed, and the retina flat mounted ganglion cell layer up on a coated glass slide (Superfrost+, Thermo Fisher). Retinas were labelled with antibodies against RBPMS and Isolectin B4, and counterstained with DAPI. Flat mounted retinas were imaged on a Zeiss Axioskop 2 plus epifluorescence microscope for quantification of retinal ganglion cell densities. Six images per retina (40X magnification, 0.25 µm/pixel) were taken equidistant to the optic nerve head (1000 µm eccentricity). Images were cropped to 150 μm^2 and RBPMS+ cells and DAPI nuclei counted using the cell counter plugin for Fiji (25). Cell counts were averaged across the 6 images, and expressed as a density per 100 μm^2. On cryo-sections (*n* = 4 NT/NT eyes, 4 OHT/OHT eyes), neurofilament labelling at the optic nerve head was imaged by epifluorescence microscopy (as above). On paraffin sections (*n* = 6 NT/NT eyes, 10 OHT/OHT eyes), H&E and Nissl staining were performed and imaged on an Olympus IX73 (20X, tiled colour image, 0.51 µm/pixel). The optic nerve head qualitatively assessed as either normal or cupped. Optic nerve axon loss was assessed in 3 µm transverse paraffin sections (*n* = 4 NT/NT nerves, 5 OHT/OHT nerves). Sections were labelled with antibodies targeting NF-M and MBP and airyscan images acquired on a Zeiss LSM800-Airy (63X, 1.3X optical zoom, 35 nm / pixel). Five 66.76 μm^2 images were collected per nerve. Axons were counted using ImageJ's cell counter plugin (in Fiji) and averaged. Dendrite and synapse loss were quantified by MAP2 and Synaptophysin1 staining in the inner plexiform layer (*n* = 3 NT/NT eyes, 3 OHT/OHT eyes) and images were acquired on a Zeiss

LSM800-Airy (20X, 319.45 μm^2, 0.312 µm/pixel, *z*-stack). Image parameters were kept constant. Images were *z*-compressed (sum slices) and the inner plexiform layer cropped. Images were thresholded to a constant range, binarised, and a watershed separation performed before analysing particles using the ImageJ particle analyser (in Fiji). The number of particles/100 μm^2, average size of particles (μm^2), and fractal analysis (fractal box count) were performed to determine dendrite and synapse integrity and space filling.

Vasculature analysis

For retinal blood vessel analysis (*n* = 10 NT/NT eyes, 8 OHT/OHT eyes), images were captured on a Zeiss Axioskop 2 plus epifluorescence microscope (5X magnification, 2861.86 x 2144.33 µm area, 2.05 µm/pixel). Images were stitched in Fiji using the pairwise stitching plugin (26). The Isolectin B4 / blood vessel channel was isolated from the image in Fiji and background from the image removed by thresholding. Retinas were cropped to 3000 um^2 to maintain consistency (representing >80% of retinal explant area). AngioTool (27) was used to measure blood vessel morphologies (total blood vessel length, junction density, lacunarity) with the following parameters: blood vessel diameter (8-30 um) and pixel intensity (0-255).

Retinal stretch

Ocular globe measurements were recorded using digital callipers (VWR; resolution 0.01mm). Eye diameter at the limbus was measured before anterior chamber bead injections in anaesthetised animals (*n* = 20 OHT eyes, and following enucleation after euthanasia (*n* = 31 NT eyes, 35 OHT eyes). Axial length (diameter from cornea to optic nerve) was also measured post-enucleation (*n* = 31 NT eyes, 35 OHT eyes). To determine whether retinal stretch also occurred in OHT, eyes were processed for paraffin sectioning as above (*n* = 6 NT eyes, 8 OHT eyes). Sections through the optic nerve head (3 µm thickness; *n* = 6 NT/NT eyes, 10 OHT/OHT eyes) were labelled for RBPMS, counter stained with X, and imaged on an Olympus IX73 (20X, tiled colour image, 0.51 µm/pixel). Retinal length was measured in Fiji and RBPMS counts across the whole retina performed using the cell counter plugin and expressed as a density. *Spearman's* correlations were performed in R for comparisons of eye size, IOP measures, RBPMS density, retinal length and animal weight. Multiple regression analysis was performed in R using the *lm* function and relative importance calculated using the *calc.relimp* function in the relaimpo package (28).

Cholera toxin tracing

Intravitreal injection of cholera toxin was used as a neuronal tracer to visualise retinal ganglion cell projections and measure axon transport. At day 11, rats were anaesthetised as above and 3 µl of Alexa Fluor 488 or 594 conjugated cholera toxin, subunit β, (CT-β; Invitrogen) was injected intravitreally using a NanoFil syringe with 33 G tribevelled needle. CT-β was injected rapidly and the syringe withdrawn after 30 seconds. CT-β AF488 was injected into the left eye and CT-β AF594 into the right eye (*n* = 6 eyes for NT/NT and OHT/OHT). Rats were euthanised on day 14 as above. Flat mounted optic nerves and optic chiasms, and flat mounted SC were imaged on Zeiss

Axioskop 2 plus epifluorescence microscope (5X and 2.5X magnification respectively). Eyes, optic nerves, and brains (n = 6 per group) were cryo-sectioned (20 µm thickness) and stored as above. CT-β labelling at the optic nerve head was imaged on a Zeiss LSM800-Airy (10X, 0.62 µm/pixel) and qualitatively assessed to have either normal or incomplete axon transport. Transverse optic nerve sections, brain sections, flat mounted optic nerves and optic chiasms, and flat mounted SC were imaged on Zeiss Axioskop 2 plus epifluorescence microscope (optic nerves, 10X, 1.03 µm/pixel; dLGN, 5X, 2.05 µm/pixel; SC, 2.5X, 4.25 µm/pixel;) and qualitatively assessed to have/not have axonal transport to the brain (for both dLGN and SC).

Microglia imaging and quantification

All microglia images were acquired on a Zeiss LSM800-Airy (20X, 319.45 μm^2, 0.312 µm/pixel, *z*-stack). Imaging parameters were kept constant to allow comparison of microglial volume. For flat mounted retinas labelled with Isolectin B4, RBPMS, and DAPI, 4 images were taken at 1500 µm superior, nasal, inferior, and temporal to the optic nerve head. In longitudinal optic nerve sections, Isolectin B4 was imaged proximal to the eye and proximal to the chiasm. Images of Isolectin B4, Nissl, and DAPI labelled brain sections were acquired at the midpoint of the SC and dLGN respectively. Microglia from retina and brain regions were reconstructed manually in Imaris (Bitplane) using the Filaments tool with automatic volume filling and *z*-depth. Parameters were kept constant for direct comparison of volumes. In the retina, microglia were classified to either the nerve fibre layer/ganglion cell layer (NFL/GCL) or inner plexiform layer based on *z*-depth, using the lower boundary of RBPMS nuclei as the limit of the ganglion cell layer. In the retina, ~9 microglia per image were reconstructed in the NFL/GCL on average (n = 137 NT/NT, 185 NT/OHT, 197 OHT/NT, 222 OHT/OHT) and ~7 microglia per image in the inner plexiform layer on average (n = 92 NT/NT, 134 NT/OHT, 160 OHT/NT, 170 OHT/OHT). In the brain, ~5 (SC) and ~6 (dLGN) microglia were reconstructed per image on average (SC, n = 103 NT/NT, 114 NT/OHT, 115 OHT/NT, 121 OHT/OHT; dLGN, n = 123 NT/NT, 116 NT/OHT, 138 OHT/NT, 130 OHT/OHT). For individual microglia, total number of branch points, total process length, total process volume, and field area were exported. Total process volume was normalised to total process length in order to distinguish microglia with large but thin processes from those with short but thick processes. Sholl analysis was performed with the soma centre as the origin point and an intersection distance of 3 µm. The area under the Sholl curve (Sholl AUC) was calculated. Individual microglia were grouped by unsupervised hierarchical clustering (HC) in order to define populations of increasing reactivity. HC was performed using Morpheus (https://software.broadinstitute.org/morpheus) where every microglia from the GCL was classified based on the 5 morphological measurements described above. For HC, microglia were clustered using Euclidean distance (linkage method = average). In longitudinal optic nerve sections an area of 312(x) x 624(y) x 12.25(z) μm^3 was cropped (to avoid including the optic nerve sheath). The whole channel corresponding to Isolectin B4 was reconstructed as a volume using the Surfaces automatic volume analysis tool in Imaris. Image thresholding was kept constant. Because adjacent

cells were sometimes considered as one object by the Surfaces algorithm, the number of microglia within the same area was counted manually using the Imaris Spots tool.

Quantification of infiltrating and reactive immune cells

Microglia and monocyte density was calculated by counting all microglia and monocytes in the image volume, taking the average of the 4 regions imaged, and expressing as $n/100\ \mu m^3$ (n = 6 NT/NT eyes, 6 OHT/OHT eyes, 5 NT/OHT eyes, 5 OHT/NT eyes; n = 3 brains [6 independent hemispheres] for NT/NT, OHT/OHT, NT/OHT, and OHT/NT)). Microglia in the retina were classified to the NFL/GCL or inner plexiform layer as above. Microglia and monocyte soma centre positions were recorded and related to RBPMS+ nuclei centres in the retina. For each microglia, nearest neighbour distances (NND) to the nearest microglia and neuron (RBPMS+ in the retina) were calculated in R using the *nndist* function in the spatstat package (29). Monocyte NND was calculated in the same way. Gliosis was determined by glutamine synthetase (GS) staining in the retina (cryo-sections; n = 3 eyes for NT/NT and OHT/OHT) and GFAP staining in optic nerve (transverse paraffin sections; n = 4 NT/NT nerves, 5 OHT/OHT nerves). GS and GFAP labelling were quantified by average pixel intensity and space filling assessed by fractal measurement (Fractal box count). GFAP fibre alignment was measured using OrientationJ (30).

Flow cytometry analysis of microglia and monocyte populations

To assess retinal microglia and monocytes numbers, at day 3 rats were euthanized as above (n = 7 NT/NT eyes, 8 OHT/OHT eyes), whole retinas dissected free from eyes cups under ice-cold HBSS, and dissociated in dispase in 1x HBSS (Corning) at 37 ˚C and 350 RPM on an Eppendorf ThermoMixer C (Eppendorf). Cell were blocked with 1% BSA in 1x HBSS for 1 h and stained with antibodies against CD11b/c, CD45, and CD90.1. Cell numbers were assessed on a BD Influx equipped with 488, 561, 641 and 405 nm lasers. Gates were set based on FS *vs* SS and viability dye exclusion (LIVE/DEAD Aqua, Invitrogen). Microglia were identified as $CD11b/c^{+}/CD45^{lo}/CD90.1^{-}$ cells and monocytes identified as $CD11b/c^{hi}/CD45^{hi}/CD90.1^{-}$ cells. Singlets were discriminated based on FS area *vs* pulse width. Cell samples were run for 600 s while mixing (accounting for approx. 25% of total tissue homogenate) and data analysed using FlowJo (FlowJo LLC).

Cytokine Assay

Cytokine array analysis was performed in order to identify retinal or circulating factors that might influence immune activation in OHT and NT/OHT eyes. Rats were euthanized at day 14 and eyes enucleated (n = 4 eyes for NT/NT, OHT/OHT, NT/OHT, and OHT/NT). Whole retina (without optic nerve head) were dissected in HBSS and lysed in HBSS with protease inhibitors by ultrasonication (Vibra-Cell; Sonics & Materials). Samples were frozen at -80 ˚C overnight, thawed, centrifuged, and protein quantification performed by Bradford assay. The array (Proteome profiler rat XL cytokine array kit; R&D systems) was performed according to manufacturer's instructions. 100 µg of protein was used for each sample and final membranes exposed to *x*-ray

film for 2 s, 5 s, 10 s, 30 s, 1 min, 2 min, 5 min, 10 min, 20 min, and overnight (over-exposure). Developed films were digitised and analysed by densitometry after background subtraction (Fiji). Spots were analysed in duplicates and normalised to the average of the 3 reference spot duplicates. The best exposure was determined by the strength of signal and lack of spot blurring.

Statistical analysis

All statistical analysis was performed in R. Data were tested for normality with a *Shapiro Wilk* test. Normally distributed data were analysed by *Student's t*-test or *ANOVA* (with *Tukey's HSD*). Non-normally distributed data were assessed using a *Kruskal Wallis* test followed by multiple pairwise *Wilcoxon* tests with *Benjamini and Hochberg* correction. Unless otherwise stated, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Results

Unilateral glaucoma does not cause increased intraocular pressure or retinal ganglion cell death in the contralateral eye

Following intracameral injection (**Figure 1A**) paramagnetic beads were directed to the iridocorneal angle (during anaesthesia; **Figure 1B**). Clinically-relevant histopathology confirmed beads were maintained in the angle for the duration of the experiment with beads evident within the drainage structures of the trabecular meshwork and Schlemm's canal (**Figure 1B**). This resulted in a significant and robust IOP increase that was sustained until euthanasia (**Figure 1C**). There was no significant difference between unilateral and bilateral normotensive eyes (NT), or between unilateral and bilateral ocular hypertensive eyes (OHT) in terms of magnitude or duration of IOP increases. Fourteen days of sustained OHT resulted in clinical features of the human disease, including cupping of the optic nerve head and a thinning of the nerve fibre layer (**Figure 1D-F**). These features were also observed *in vivo* by optical coherence tomography imaging (**Figure 1G**). RBPMS labelling demonstrated significant loss of RGCs at 14 days post injection with RGC density being reduced in both OHT/NT and OHT/OHT eyes compared to NT/NT and NT/OHT eyes, with no significant unilateral or bilateral effect in RGC death (**Figure 1H**). There was a small increase in the nearest neighbour distance (NND) between RGCs in OHT eyes and although this increase was small (30% increase in OHT/OHT compared to NT/NT), the range of values for OHT was increased (doubling of the interquartile range in OHT/OHT). Collectively, these data suggest diffuse and random RGC death (*i.e.* not regional cell loss) (**Figure 1H**).

We have previously demonstrated RGC dendritic atrophy at the single RGC-level by DiOlistic labelling (31, 32). Corroborating this, RGC dendrite loss in the inner plexiform layer (IPL) in OHT eyes was observed by a reduction in MAP2 labelling (**Figure 1I**). A reduction in average particle size and a reduction in fractal indicates increased fragmentation and reduced space filling of dendrites in the IPL, which is suggestive of dendritic atrophy and retraction. There was no statistically significant change in synapse number (as assessed by synaptophysin labelling in the IPL) in OHT eyes (**Figure 1I**), with labelling highly variable across groups and measures, suggesting no gross loss of synaptic structure (pre-synaptic to RGCs).

Ourselves and others have previously identified vascular dysfunction in the retina and ONH as a feature of human and animal models glaucoma (33-36). Analysis of Isolectin B4 labelled vessels demonstrated a significant reduction in total vessel length, junction density, and an increase in lacunarity indicting reduced vessel coverage of the inner retina (**Figure 2**). Overall, these data demonstrate that OHT induced in Brown Norway rats by paramagnetic bead injection recapitulate many of the clinical and neurodegenerative features of glaucoma, with no gross unilateral or bilateral effect on RGC survival and degeneration.

Retinal ganglion cell dysfunction extends into terminal brain regions

Retinal ganglion cell neurodegeneration during glaucoma is a compartmentalised event, with OHT driving dendritic and synaptic retraction, soma degeneration, and deafferentation (37). We confirmed loss of RGC axons in the optic nerve by counting individual nerve fibres assessed in neurofilament labelled sections imaged at ultra-high resolution (35 nm / pixel). The estimated total number of axons in NT optic nerves (average 125,000) matched those reported in the literature for adult Brown Norway rats (5) and was significantly reduced in OHT eyes (70% reduction, **Figure 3A**). The reduction is likely an overestimate due to an increased number of dystrophic axons in OHT optic nerves that may mask individual small calibre axons. Although we observed a dramatic decrease in axon number there was no significant change in optic nerve area between NT and OHT eyes when measured from the dura mater or pia mater. GFAP labelling in optic nerves demonstrated astrocyte remodelling, with a significant reduction in fibre coherency following periods of OHT, supporting evidence from human glaucoma and those generated from animal models of glaucoma (38, 39) (**Figure 3B**). These data support a model in which a critical insult damages RGCs resulting in complete atrophy (*i.e.* no uncoupling of axon and soma neurodegenerative events) including damaging insults at the level of the optic nerve head.

We next assessed axonal function across the whole RGC, including projections to the dLGN and SC (**Figure 3C**). Cholera toxin, subunit beta (CT-β; conjugated to AF488 or AF594) was injected intravitreally 72 hours prior to euthanasia. In NT animals, robust labelling was evident throughout the optic nerve (with crossover at the chiasm) and persisted to the SC and dLGN (**Figure 3D**). Labelling was visibly reduced in OHT eyes in the optic nerve, further reducing pre-chiasm with reduced penetrance to the SC indicating markedly reduced axonal transport (**Figure 3D**). Although sectoral regions of functional axonal transport remained, this was not uniform across optic nerves or brain regions and was further evidenced in cryo-sections of eyes, optic nerves, and brain regions (**Figure 3E**). In OHT eyes, axonal transport was largely intact within the retina, stalling at the optic nerve head myelin transition zone. Optic nerve proximal to the optic nerve head demonstrated this lack of functional transport and sectoral dysfunction that was further reduced distally. Labelling in the dLGN was significantly reduced in OHT animals with evidence of some persisting ipsilateral projections. Labelling in the SC was also significantly reduced (**Figure 3E**). These data demonstrate that RGC dysfunction persists throughout the RGC axonal projections, with significant axonal loss at the level of the optic nerve and axonal transport dysfunction throughout these pathways.

Ocular stretch may confound measurement of retinal ganglion cell densities in ocular hypertensive eyes

Following 14 days of OHT, we grossly observed an increase in the size of OHT eyes and hypothesised that chronic high IOP may lead to scleral remodelling and enlargement of the eye. We therefore recorded calliper measurements of the eyes pre-surgery (dimeter at the limbus) and post-euthanasia at day 14 post OHT induction (diameter at the limbus and axial length from cornea

to optic nerve) and compared to NT controls. OHT eyes demonstrated a significant increase in diameter by 14 days compared to both pre-surgery size (30% increase, $P < 0.001$) and NT controls (19% increase, $P < 0.001$; **Figure 4A**). The difference in the magnitude of increase is likely due to the difference in measurement pre-surgery and post-euthanasia, where venous pressure and ease of measurement may affect recordings. This is reflected in the significant difference between pre-surgery OHT eyes and NT eyes (9% increase, $P < 0.001$; **Figure 4A**). Eye diameter at the limbus and axial length at day 14 demonstrated reasonable correlation to measures of IOP, indicating a relationship between the magnitudes of IOP increase and increase in the size of the eye (**Figure 4B-C)**. There was no correlation between eye size and animal weight, confirming that animal age or size was not driving the observed difference (**Figure 4D**).

We reasoned that an increase in eye size may cause the retina to stretch, and as such, measurements of RGC density could be confounded, thus overestimating cell loss. RGCs were identified by immunohistochemistry in paraffin sections through the optic nerve head and RBPMS+ RGCs were counted across the whole retinal length (**Figure 4E**). Retinal length was variable in both NT (8.5 ± 0.8 mm) and OHT eyes (9.5 ± 1.0 mm), and no significant increase in retinal length was observed. RGC density (as an average across the whole retina) was significantly reduced in OHT eyes compared to NT eyes (57% decrease, $P < 0.001$; **Figure 4E**). RGC density was highly correlated with IOP measures and eye size (**Figure 4F**), whereas retinal length demonstrated more modest correlations (**Figure 4G**). We performed a multiple linear regression analysis in order to estimate the degree of RGC density change that could be explained by increased retinal length (summarised in **Table 2**). The regression model generated (adjusted $R^2 = 0.78$, $P < 0.01$) demonstrated that retinal length had the smallest contribution to estimation of RGC density (9.5% of variance explained). Overall, these data demonstrate that globe expansion is a feature of the rat magnetic bead model. Although this does not result in significant retinal stretch, any analysis of RGC density (reported as percentage reduction relative to the contralateral eye) should take account of the variability in the retinal length and RGC numbers seen in both OHT and NT eyes.

Table 2. Multiple linear regression analysis of IOP and ocular measurements

Independent variables	**Variable coefficient**	***t* value**	***P* value**	**Contribution (%)**
Peak IOP	-0.870	-1.080	0.3158	18.6
Mean IOP	-0.775	-0.125	0.9039	17.7
IOP AUC	1.573	0.251	0.8093	17.8
Eye diameter	-0.456	-0.682	0.5171	18.2
Eye axial length	-0.419	-1.059	0.3249	18.0

Retinal length	-0.019	-0.070	0.9463	9.7

Retinal microglia demonstrate increasingly reactive morphologies in unilateral normotensive, unilateral ocular hypertensive, and bilateral ocular hypertensive eyes

We performed volume reconstructions of individual microglia (*n* = 1297 microglia; **Figure 5A-D**) and assessed branching complexity, field area, and volume. Individual microglia were binned by their position within the retinal layers to either nerve fibre layer/ganglion cell layer (NFL/GCL) or IPL microglia. Sholl analysis revealed a reduction in branching density (Sholl AUC) from NT/NT for NT/OHT, OHT/NT, and OHT/NT and with increasing magnitude of reduction through these groups (**Figure 5B**). This pattern of decrease was also observed in the number of branches, total process length, and field area of microglia (**Figure 5B**). These data demonstrate a retraction of microglial processes. Microglial volume increased in OHT eyes compared to NT eyes but there was no significant change in volume between NT/NT and NT/OHT eyes (**Figure 5B**). The data quantitatively demonstrate morphological changes consistent with activation (retraction and increased volume (40, 41)) occurring in microglia in OHT eyes. Microglia in NT eyes contralateral to OHT eyes demonstrate early reactive morphologies (retraction). In the IPL, microglial morphology largely followed the same trend (**Figure 5C-D**). Microglia from OHT eyes demonstrated significantly decreased Sholl AUC, number of branch points, total process length, and field area (**Figure 5D**). Microglial volume was increased in OHT eyes compared to NT eyes (**Figure 5D**). The contralateral response was more muted in IPL microglia with NT/NT and NT/OHT showing no significant difference in Sholl AUC, number of branch points, and total process length but significantly reduced field area and significantly increased volume (**Figure 5D**). OHT/OHT showed significant process retraction compared to OHT/NT but no significant increase in volume (**Figure 5D**), indicating that microglia in the IPL also display activated morphologies, that follow a similar pattern in magnitude of change (OHT/OHT > OHT/NT > NT/OHT > NT/NT), but to a lesser degree than microglia in the NFL/GCL.

We next performed hierarchical clustering (HC) of individual microglia in the NFL/GCL to determine whether these morphological measures alone could group microglia by degree of activation (**Figure 5E**). HC produced 4 clusters: Cluster 1; a small cluster, *n* =16, of NT/NT and NT/OHT microglia, Cluster 2; *n* = 210, predominantly consisting of NT/NT and NT/OHT microglia reflecting microglia with resting morphology (63.5% and 51.4 % of total NT/NT and NT/OHT respectively), Cluster 3; *n* = 156, a mix of microglia from all 4 groups, representing morphologies related to early activation, and Cluster 4; *n* = 359, predominantly OHT/NT and OHT/OHT microglia (67.5% and 85.6 % of total OHT/NT and OHT/OHT respectively), representing retracted and condensed morphologies consistent with activation (**Figure 5E**). This clustering demonstrates that microglial morphological measurements can distinguish biologically meaningful groups of microglia independently of disease grouping.

Monocyte infiltration occurs early following ocular hypertension

Microglial proliferation is a feature of neurodegenerative and neuroinflammatory disease, and has been reported in human and animal models of glaucoma. Increased numbers of microglia in the retina have been reported following OHT, as have mild increases in microglia number in NT eyes contralateral to OHT eyes. All microglia in confocal images of flat mounted retina were counted within the NFL/GCL and IPL. Microglia density was increased in the NFL/GCL in both OHT/NT and OHT/OHT eyes compared to NT/NT (**Figure 6A**). There was no significantly detectable contralateral effect for either unilateral NT or OHT eyes in comparison to bilateral NT or OHT eyes (**Figure 6A**). Microglia density in the IPL was less variable between groups, with only OHT/NT eyes showing a significant increase over other groups (**Figure 6A**). NND analysis revealed that the distance between microglia in the NFL/GCL was smaller on average in OHT eyes than NT eyes, with no change in NT/OHT eyes compared to NT/NT eyes (**Figure 6B**). In the IPL, NND was also smaller between microglia in OHT eyes and in NT/OHT eyes compared to NT/NT eyes (**Figure 6B**). However, the interquartile range indicated that in the IPL the majority of NNDs for OHT eyes remained overlapping with those of NT eyes, whereas in the NFL/GCL these ranges were non-overlapping (**Figure 6B**). These data suggest that changes are modest in the IPL and that microglia here are largely distributed normally, but changes are more pronounced in the NFL/GCL. Given that microglial density was substantially increased (2.8 to 3.5-fold increase), NND was not increased to the same degree (1.6 to 2.4-fold increase) demonstrating that microglia remain well distributed (*i.e.* avoid clumping/clustering). The NND between microglia and RGCs significantly increased in microglia from both the NFL/GCL and IPL under OHT in comparison to NT (**Figure 6B**). Since the increase in microglial density was concurrent with the decrease in RGC density, these data suggest that microglia are not clustering around surviving RGCs.

Extravasation and increased monocyte infiltration has been demonstrated as an early and persistent feature of glaucoma in animal models (35) and in post-mortem human tissue (3), and $CD45^{hi}$ monocytes are present in the retina in low numbers under normal physiological conditions (42, 43). We observed Isolectin B4+ ameboid cells in the majority of retinas in all experimental groups but no significant change in average monocyte density across experimental groups (**Figure 6C**). NND analysis demonstrated a significant decrease in distance between monocytes in OHT eyes compared to NT eyes (all $P < 0.001$) and a substantial shift in the interquartile range, suggesting a clumping of monocytes that may reflect recruitment rather than random transient migration into the retina (**Figure 6C**). As histology was performed at 14 days post-OHT induction, and monocyte infiltration has been shown to be an early event in glaucoma (35), we counted myeloid-derived cells (MDCs) from whole retinal homogenate by flow cytometry at an early time-point (3 days post-OHT induction, representing peak IOP in our experimental groups; **Figure 6D**). Monocytes, but not microglia, were significantly increased at this early time-point in OHT compared to NT as assessed by either raw counts, as a percentage of all viable cells, or as a percentage of all MDCs ($P < 0.01$; **Figure 6D**). This demonstrates early monocyte infiltration in the absence of microglial proliferation. Taken together, this early increase in monocytes and a late increase in microglia

supports a hypothesis in which OHT (and/or OHT-induced vascular dysfunction) drives monocyte entry, and these infiltrating monocytes become monocyte derived microglia-like macrophages.

No detectable Müller glia activation following ocular hypertensive glaucoma

Müller glia provide trophic and metabolic support to RGCs during times of stress, including glaucoma related stresses (44, 45). Müller glia activation, identified by increased glutamine synthetase (GS) labelling, has been demonstrated in different animal models of glaucoma. To determine whether Müller glia activation was a component of glaucomatous neurodegeneration in this model, cryo-sections were labelled with antibodies targeting GS (**Figure 7A**). Analysis of GS labelling in the NFL/GCL/IPL (representing Müller glia association to RGC axon, soma, and dendrites) did not indicate significant Müller glial activation at this time point (**Figure 7B**).

Microglial activation extends into the optic nerve

Since RGC axonal loss and dysfunction extended into the optic nerve in this model, we sought to determine whether this was accompanied by microglial activation. Microglia were assessed in longitudinal optic nerve sections (**Figure 8A**). Microglial counts demonstrated a significant increase in microglial density both proximal to the eye and proximal to the chiasm in OHT optic nerves compared to NT optic nerves (**Figure 8B**). No contralateral change was observed in microglial density in optic nerves (**Figure 8B**). Volume reconstructions of whole Isolectin B4 content demonstrated the same trend, with significant increases in OHT optic nerves compared to NT nerves, which was more pronounced proximal to the chiasm (**Figure 8B**). Microglial activation was most evidently demonstrated at the chiasm where RGC axons from unilateral OHT eyes decussate, with the appearance of activated microglia following both optic tracts post-chiasm (**Figure 8C**).

Microglia are activated in the dorsal lateral geniculate nucleus and superior colliculus across both hemispheres, irrespective of unilateral or bilateral ocular hypertension

Microglial activation in retinothalamic projections has been reported in animal models of glaucoma (21, 22). Brains were serial sectioned and microglia in central dLGN and SC were labelled (**Figure 9A-C**). Microglial activation was evident in the dLGN across both hemispheres in all conditions compared to brains from NT/NT animals. Microglia demonstrated reduced branching complexity, number of branch points, total process length, and field area, and an increased volume (**Figure 9D**). The dLGN contralateral to NT/OHT eyes (majority RGC input from NT eye) demonstrated significant changes in these measures compared to NT/NT dLGN, but not to its fellow dLGN (contralateral to OHT/NT eyes; **Figure 9D**), indicating that inflammation was not restricted, nor proportional, to the degree of RGC decussation. Microglia counts demonstrated no significant increase in microglia density in the dLGN, suggesting activation in the absence of proliferation or infiltration (**Figure 9D**). In the SC, microglial morphology was also indicative of activation, with microglia demonstrating a significant reduction in branching complexity, number of branch points, total process length, and field area, and an increased volume

in both hemispheres from all conditions compared to NT/NT (**Figure 9E**). In the SC, there was no significant difference in these measurements in microglia between hemispheres in unilateral animals, consistent with the findings in the dLGN (**Figure 9E**). Microglia from OHT/OHT animals and both hemispheres of the unilateral animals were more similar in morphology than in the retina (**Figure 9D-E**), suggesting that the degree of microglial activation in the brain is not contingent on the degree of dysfunctional and/or absent RGC input. Microglial density was increased in the SC in OHT/NT eyes, and highly variable in OHT/OHT eyes, suggesting proliferation or recruitment of microglia in the SC (**Figure 9E**). Since ameboid monocytes were not observed in either the SC or dLGN, and that the SC and dLGN are not directly insulted by elevated IOP, microglial proliferation is the most likely scenario. Both unilateral and bilateral OHT result in consistent microglial activation across both hemispheres of the visual thalami (*i.e.* neuroinflammatory signatures propagate even when only a small percentage of axons are damaged or dystrophic).

Upregulation of cytokines in ocular hypertension and in contralateral normotensive eyes

RGC dysfunction or neurodegeneration in the NT/OHT eyes, but with substantial microglial inflammation in the optic nerve and in terminal brain thalami, whole retinal lysate was probed for cytokine signals that could contribute to microglial activation (**Figure 10A**). Of the 79 analytes assessed by cytokine array, 6 were identified as significantly altered across groups by *MANOVA* (**Figure 10B-C**). These analytes were CNTF, Fetuin A, FGF-1, Galectin-3, ICAM-1 (CD54), and Lipocalin-2. OHT eyes demonstrated a significant upregulation of CNTF compared to NT eyes. Fetuin-A and ICAM-1 were significantly increased in OHT/NT eyes compared to NT/NT eyes (**Figure 10C**). Galectin-3 and Lipocalin-2 were significantly increased in OHT/OHT eyes compared to NT/NT eyes (**Figure 10C**). FGF-1 was significantly increased in NT/OHT eyes compared to NT/NT eyes (**Figure 10C**). These inflammatory molecules may represent ideal candidates for further exploratory biomarker analysis or functional testing through targeted inhibition or depletion.

Discussion

In this study we define the neurodegenerative and neuroinflammatory components in an inducible OHT model of glaucoma and have identified microglial activation throughout the retina, optic nerve, and terminal visual thalami. Induction of OHT by blocking aqueous humour outflow with beads has been used in a number of species (46-48). Use of paramagnetic beads, which allows for control of distribution within the eye, was initially described by our group but with limited characterisation beyond cell death in the ganglion cell layer, and retinal ganglion cell dendritic atrophy (23, 31, 32). Here we demonstrate that this model produces robust retinal ganglion cell soma and axon loss, axon transport dysfunction, vascular disruption, and glial activation. We confirm the presence of dendritic atrophy and demonstrate that the model shares a number of clinical features with human glaucoma. In cases of unilateral induction of OHT, inflammatory responses including microglial activation have been reported (18, 19). We confirmed this using a quantitative approach and identified that this activation extended into the optic nerve and terminal visual thalami in the brain.

Retinal ganglion cell degeneration is described as a compartmentalised process, where the soma, axon, and dendritic atrophy and loss differs temporally based on the insult context (49-51). Retinal ganglion cell soma and axon loss is a feature of human glaucoma that is replicated across different animal models in multiple species (50, 52-55). Dendritic atrophy in the inner plexiform layer is also well established, and can be substantial even with mild retinal ganglion cell loss (56-58). In this model we demonstrate robust retinal ganglion cell soma and axon loss in response to sustained OHT within range of human glaucoma IOPs and below ischemic thresholds (59). Retinal ganglion cell axon dysfunction has been well described and disruption of axoplasmic transport, in particular, has long been a focus as one of the earliest features of glaucoma, but is also disrupted by OHT in the absence of neurodegeneration with rapid recovery following pressure normalisation (60-66). We see evidence of axoplasmic transport disruption at the level of the optic nerve head, with the majority of CT-β labelling stalling at the myelin transition zone, with limited penetrance to the optic nerve and brain. These data corroborate clinically available human glaucoma data which suggests damage at the site of the optic nerve head as one of the earliest key damaging events of glaucoma (50, 67).

Retinal ganglion cells are reliant on supportive glia throughout their long projections to terminal visual thalami (68). Following periods of elevated IOP these glia can become reactive, likely as an initial protective response that actually further compromises retinal ganglion cell survival (4, 69). Supporting this, complete ablation of microglia or monocytes only provides short-term neuroprotection in glaucoma (35, 70). In a recent study, almost complete depletion of resident microglia and infiltrating monocytes (using a PLX5622 and clodronate liposome combination treatment) failed to provide benefit in a more acute model (controlled optic nerve crush) and actually delayed axon regeneration following lens injury-induced RGC axon regeneration (71). In the present study, quantification of microglial morphology demonstrates retracted morphologies

and increased process volume consistent with activation from a resting state (40, 41). Activated morphology persisted throughout the retina, optic nerve, and brain mirroring retinal ganglion cell dysfunction that extended along the trajectory of the axon. Reciprocal signalling between microglia and neurons plays an important role in defining microglia phenotypes (72). The resting, non-activated state, is in part controlled by CD200 and fractalkine (CX3CL1) signalling. CD200 is expressed by neurons and through binding CD200R on microglia, maintains a resting state (73, 74). CX3CL1 is expressed in neurons and can be membrane bound and in a secreted form; it signals through its receptor on microglia to maintain a resting state (75, 76). CD200 and CD200R expression changes early in glaucoma while loss of CX3CL1 signalling causes earlier axon dysfunction and in a DBA/2J mouse model of glaucoma (77, 78). Neurons also express markers of injury (*e.g.* surface expression of CCL21 which activates CXCR3 on microglia (74, 79, 80)) or of health (*e.g.* surface expression of CD47, the 'don't eat me' signal (81, 82)). Activated microglia exhibit increased expression of toll-like receptors, complement receptor and components, and pro-inflammatory cytokines and chemokines. Microglia and complement involvement in synapse elimination during the critical period and in neurodegenerative disease is well established (83-85). Complement components are upregulated in glaucoma in animal models and in human tissue, which play a critical involvement in synaptic pruning in glaucoma, and altered retinal ganglion cell survival through its manipulation (31, 33, 86-91). This reciprocal relationship between microglial activation and neuronal dysfunction may underlie why microglial activation is so robust in the SC and dLGN even in the absence of direct insult from OHT (*e.g.* direct IOP and vascular compromise).

The degree to which unilateral OHT affects the contralateral eye requires further elucidation. We confirmed quantitatively the observation of activated microglia in NT eyes contralateral to OHT eyes. In these eyes, as in the brain, activation occurs in the absence of direct OHT insult. The source of microglial insult is therefore either systemic or neuron-derived. IOP is defined by both aqueous dynamics and systolic pressure, and so in unilateral OHT, the contralateral NT eye may experience systemic effects in balancing IOP. We observed no significant change in IOP in NT/OHT eyes over NT/NT eyes (although NT/OHT eyes trended to have higher pressures but this did not reach significance). We also demonstrated no detectable (by gross histology) neurodegenerative effects in the retina in NT/OHT eyes. In the rat, decussation at the chiasm of ~90% of axons to the SC and dLGN contralateral to the eye of origin leaves ~10% of RGC axons projecting ipsilaterally. Significant microglial activation therefore persists along a substantial portion of these retinal ganglion cell axons and presents a pro-neuroinflammatory environment to otherwise healthy RGC terminals. It is possible that this interaction translates into activation at the somal end in the retina, although this remains to be experimentally validated. Of note is the contralateral effect observed as the difference in activation between unilateral and bilateral OHT in the retina. This furthers the hypothesis that cumulative neuronal insult or systemic effects may drive activation to a greater degree in the retina. This graded effect in the retina was not observed in the brain, where microglial activation was consistent across hemispheres irrespective of whether

the majority of input was from an OHT or NT eye in unilateral animals. The degree of microglial activation, and its implications towards neuro-glial signalling, neuroinflammatory environment, and RGC health should caution against the use of the contralateral eye as a control in unilateral OHT animals.

Microglial proliferation and migration and monocyte infiltration have been reported in a number of glaucoma models and in human glaucoma (3, 13, 35, 70, 78, 92-95). We demonstrate that an early increase in monocytes, but not microglia, and a late increase in microglia, but not monocytes occurs following sustained periods of OHT. These data suggest that monocytes infiltrate the retina and become microglia-like monocyte derived macrophages, as has been reported in Alzheimer's disease and other neurodegenerative diseases and in retinal photoreceptor degeneration (96-100). Definitively testing this hypothesis would require complex reporter alleles that are currently unavailable in the rat and therefore outside the scope of this study. We have previously shown that vascular permeability is compromised in glaucoma, leading to increased extravasation of monocytes in addition to recruitment through vascular endothelial changes (35, 95).

In our model, increased microglial activation and monocyte infiltration results in a significant pro-inflammatory environment. Cytokine and chemokine profiling in the retina identified a number of changes in OHT and in contralateral NT eyes. CNTF, Fetuin A, FGF-1, Galectin-3, ICAM-1 (CD54), and Lipocalin-2 were all differentially elevated. CNTF, which can be released from microglia as a response to central nervous system injury, has demonstrated neurotrophic properties and increases retinal ganglion cell survival in glaucoma models (101, 102). CNTF also induces gliosis in Müller cells and leads to upregulation of pro-inflammatory cytokines and chemokines (103-105), and as such its action is likely highly context dependant. Fetuin-A, a serum carrier protein, has been proposed as a biomarker of systemic inflammation, vascular disease, and neurodegenerative disease (106-108). Reduced expression of Fetuin-A is correlates with the severity of cognitive impairment in Alzheimer's disease and Fetuin-A deficient mice demonstrate protection in experimental models of multiple sclerosis (109, 110). Galectin-3 mediates cell migration and adhesion, inflammatory cytokine release and apoptosis and has been shown to activate microglia, astrocytes, monocytes, and other immune cells (111). Plasma levels of Galectin-3 correlate with Huntington's disease severity in patients and animals, and are associated with adverse outcomes in stroke and cerebral infarction (112, 113). Expression of Galectin-3 was increased in Huntington's disease and genetic ablation of *Lgals3* is protective in animal models (112). Microglial released Galectin-3 can act as a ligand for TLR4, sustaining inflammatory responses (114). Increased expression of Galectin-3 has been detected in human glaucomatous donor tissue in the trabecular meshwork and optic nerve head in association with increased fibrosis (115). Icam-1 is a glycoprotein that is expressed on leukocytes, macrophages and vascular endothelium. Its expression is upregulated in response to inflammatory stimuli (IL-1, TNF-α) where it functions as a ligand for integrins to facilitate endothelial binding and transmigration of leukocytes (116). *ICAM1* expression is increased in glaucoma patient leukocytes but not blood

plasma (117, 118). The upregulation of *Icam1* is early and persistent in the optic nerve head and retina of DBA2/J mice, and is reduced by radiation treatment which is highly protective for retinal ganglion cells (70). Lipocalin-2 is an antibacterial protein (acting through iron-sequestration) that elicits pro-inflammatory responses and is implicated in a number of inflammatory and neurodegenerative diseases, including Stargardt disease and age-related macular degeneration in the retina (119, 120). *Lcn2* expression is also highly upregulated in DBA2/J glaucoma and in the rat hypertonic saline glaucoma model (70, 121). A recent GEO screen identified *LCN2* involvement in glaucoma pathogenesis (122). Increased FGF-1 expression was identified in NT/OHT eyes. FGF-1 has demonstrated neuroprotective responses in neurodegenerative disease, but also activates astrocytes in the spinal cord, leading to pro-inflammatory responses (123-126). Whether this represents a neuroprotective or inflammatory response related to microglial activation warrants further exploration. These pro-inflammatory molecules represent ideal candidates for further exploration using targeted knockouts, gene therapies, or antibody therapies in glaucoma.

Following periods of OHT, we identified global eye expansion as a feature of the paramagnetic bead model. While this is not a feature of adult glaucoma, it occurs in congenital glaucoma where the sclera is less rigid and more elastic (127, 128). Increased eye diameter and axial length was highly correlated to the magnitude and cumulative degree of IOP elevation. As the retina is fixed at the optic nerve and ora serrata, we reasoned that it may stretch with the eye globe. Retinal length was highly variable following OHT, and trended towards increase, but no significant change was detected. Multiple regression analysis revealed that a component of retinal ganglion cell density variation could be described by retinal length, though this effect was small. Even so, global expansion should be considered when comparing to contralateral eyes, particularly if reporting percentage reductions to fellow eyes. Our data suggest caution is warranted when attributing findings comparatively to the contralateral 'control' eye, and these findings may, in part, help explain why many glaucoma therapies have failed in translation between rodents and humans.

Conclusions

In a rat glaucoma model we identify substantial microglial activation extending from the retina to visual thalami in combination with retinal ganglion cell dysfunction. In unilateral OHT, activation of microglia occurs in the contralateral control eye and is substantial and comparable throughout the visual thalami. Unilateral OHT animal models should be analysed with caution, particularly given the reciprocal signalling between neurons and immune cells in resting and pathogenic states.

List of abbreviations

AUC (area under the curve), FC (flow cytometry), GCL (ganglion cell layer), H&E (haematoxylin and eosin), HC (hierarchical clustering), IF (immunofluorescence), IHC (immunohistochemistry), IOP (intraocular pressure), IPL (inner plexiform layer), MDC (myeloid-derived cell), NND (nearest neighbour distance), NT (normotensive), NFL (nerve fibre layer), OHT (ocular hypertension).

Declarations

Ethics approval

All experimental procedures were undertaken in accordance with the Association for Research for Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Research. Individual study protocols were approved by Stockholm's Committee for Ethical Animal Research (10389-2018).

Consent for publication

N/A

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The Authors report no competing interests.

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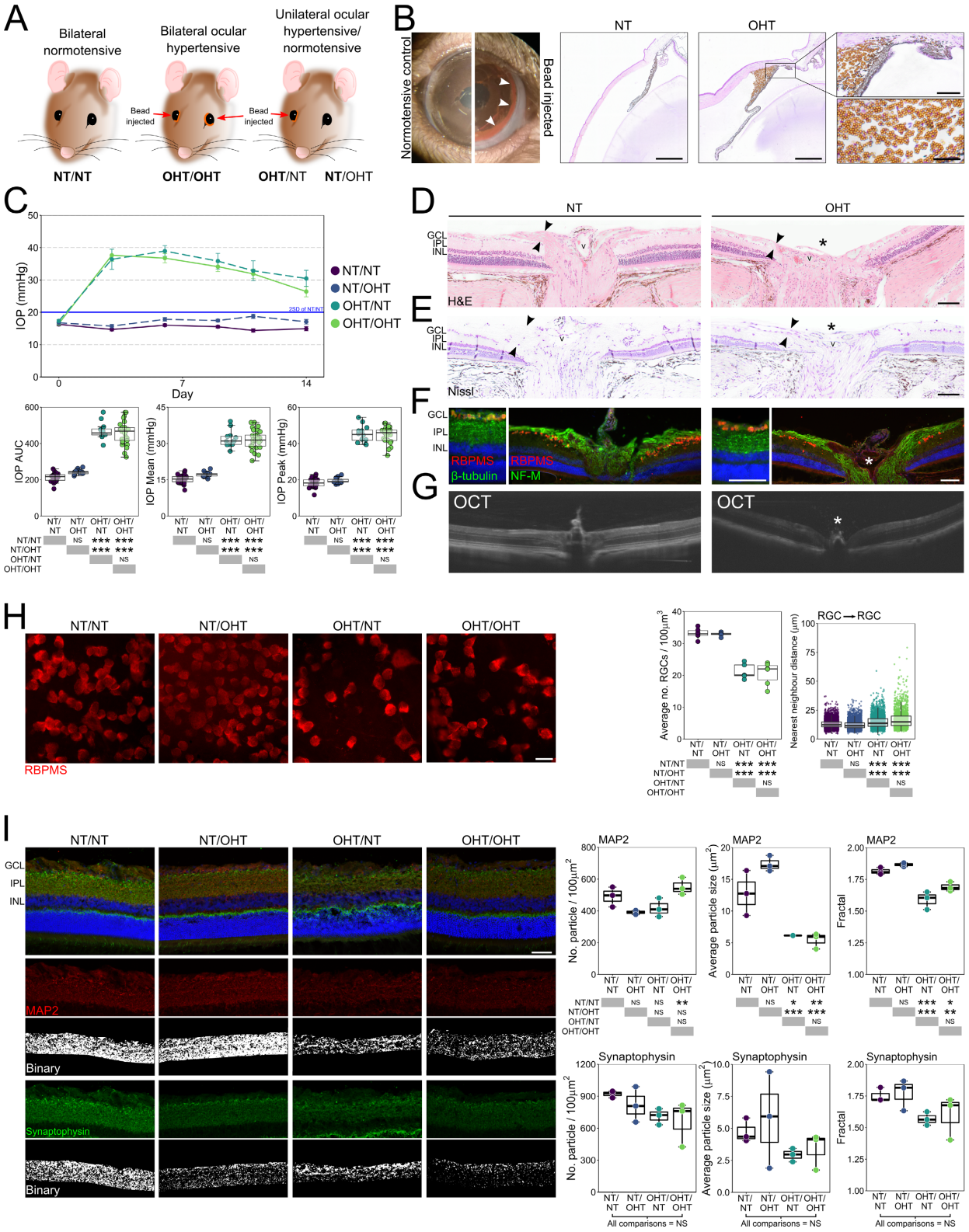
Author contributions

JRT – designed and performed experiments, analysed data, wrote the manuscript; *EK* – designed and performed experiments, analysed data, wrote the manuscript; *AO* – performed experiments, analysed data; *FP* – performed experiments, analysed data; *EL* - performed experiments; *RV* – performed experiments; *MK* - provided resources, wrote the manuscript; *HA* - provided resources, designed experiments, wrote the manuscript; *JEM* – provided resources, designed experiments, wrote the manuscript; *PAW* – conceived, designed, performed experiments, analysed data, wrote the manuscript.

Figure 1


A
Bilateral normotensive
Bilateral ocular hypertensive
Unilateral ocular hypertensive/ normotensive
Bead injected
Bead injected
NT/NT
OHT/OHT
OHT/NT
NT/OHT
B
Normotensive control
Bead injected
NT
OHT
C
IOP (mmHg)
Day
2SD of NT/NT
NT/NT
NT/OHT
OHT/NT
OHT/OHT
IOP AUC
IOP Mean (mmHg)
IOP Peak (mmHg)
D
NT
OHT
GCL
IPL
INL
H&E
E
Nissl
F
RBPMS
β-tubulin
NF-M
G
OCT
H
NT/NT
NT/OHT
OHT/NT
OHT/OHT
RBPMS
Average no. RGCs / 100μm³
Nearest neighbour distance (μm)
RGC → RGC
I
MAP2
Binary
Synaptophysin
No. particle / 100μm²
Average particle size (μm²)
Fractal
All comparisons = NS

(Previous page) **Figure 1: Induction of OHT via paramagnetic bead induction.** (**A**) Brown Norway rats underwent intracameral injections of paramagnetic beads either bilaterally or unilaterally. The contralateral eye remained un-operated. (**B**) Beads were pulled into the drainage structures of the eye via a rare earth magnet (5 mm tip) (*left panel*). Histology (*right panels and insets*) demonstrate that beads remained within the drainage structures of the trabecular meshwork and Schlemm's canal. This resulted in significant and robust IOP increase that was sustained until euthanasia (**C**). 14 days of OHT results in significant RGC atrophy, axon degeneration and nerve fibre layer thinning (black arrows at the optic nerve head), and optic nerve cupping and excavation (*). Inner retinal degeneration is shown through 3 modalities; H&E staining (**D**), Nissl staining (**E**), immunofluorescence for antibodies against RGC specific markers (RBPMS, β-tubulin, NF-M) (**F**), and *in vivo* imaging via OCT (**G**). (**H**) RGC populations were assessed in flat-mount retinas confirming a significant loss of RGCs and increased NND of RGCs following 14 days OHT. (**I**) OHT also resulted in significant dendritic atrophy (MAP2 labelling) but without a gross loss of synapses (synaptophysin labelling). For complete *n* please see **Methods**. Scale bars = 500 µm in **B** (*right panels*), 100 µm in **B** (*right inset, upper*), 50 µm in **B** (*right inset, lower*), 100 µm in **D**, **E** and **F**, 20 µm in **H**, 50 µm in **I** * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 2

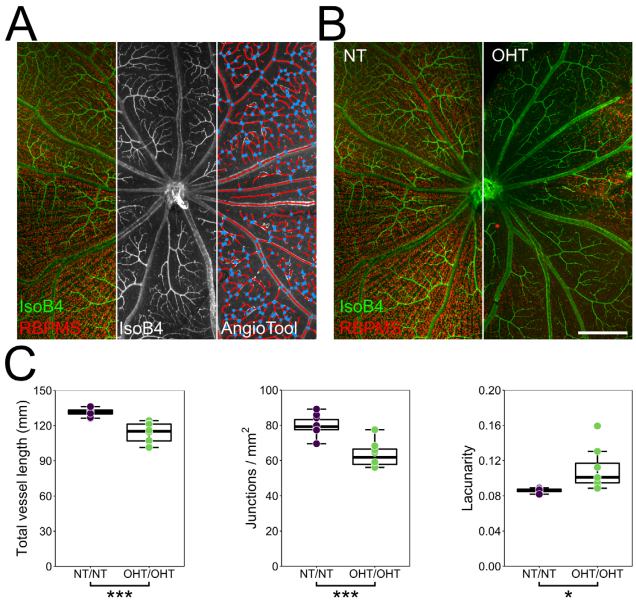


Figure 2: Vascular remodelling following OHT. (**A**) Flat-mounted retinas were labelled with Isolectin B4 and the inner retina imaged to assess blood vessel morphology. Blood vessels were reconstructed with AngioTool (**A**, right panel; vessels = red, junctions = blue). (**B-C**) Following OHT there was significant vascular remodelling assessed by a decrease in total vessel length and number of vessel junctions and an increase in lacunarity indicting reduced vessel coverage of the inner retina. Scale bar = 500 µm in **B**, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

(Following page) **Figure 3: OHT results in axon degeneration and deficits in axoplasmic transport.** (**A**) Total axon number was assessed in optic nerve sections by high resolution imaging of NF-M+ axon fibres and myelin bundles (myelin basic protein; MBP). There were no gross changes to optic nerve total size (as measured from the dura or pia mater) but a significant loss of RGC axons. (**B**) OHT results in significant remodelling of optic nerve astrocytes (a decrease in fibre alignment and coherency as assessed by OrientationJ). Axoplasmic transport was assessed by 2-color CT-β tracing from the retina to the terminal visual thalami (dLGN and SC). A schematic showing RGC trajectories and visual thalami is shown in (**C**). OHT results in an absence of axoplasmic transport to terminal regions assessed by both low power flat-mount SCs (**D**) and in cryo-sections (**E**) as assessed by mean pixel intensity and fractal analysis. Axoplasmic transport was primarily stalled within the optic nerve head (* in **E**) resulting in partial transport through the optic nerve to the dLGN and SC. Optic nerves were assessed both proximal to the eye and proximal to the chiasm. The dLGN and SC were assessed in 3 sections representing rostral, medial, and caudal dLGN or SC respectively. ONH = optic nerve head, ON = optic nerve. Scale bars = 100 µm in **A** (*left panels*), 10 µm in **A** (*right insets*), 100 µm in **B** (*left panels*), 50 µm in **B** (*right insets*), 100 µm in **E** (ONH), 500 µm in **E** (ON, dLGN, SC), * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 3

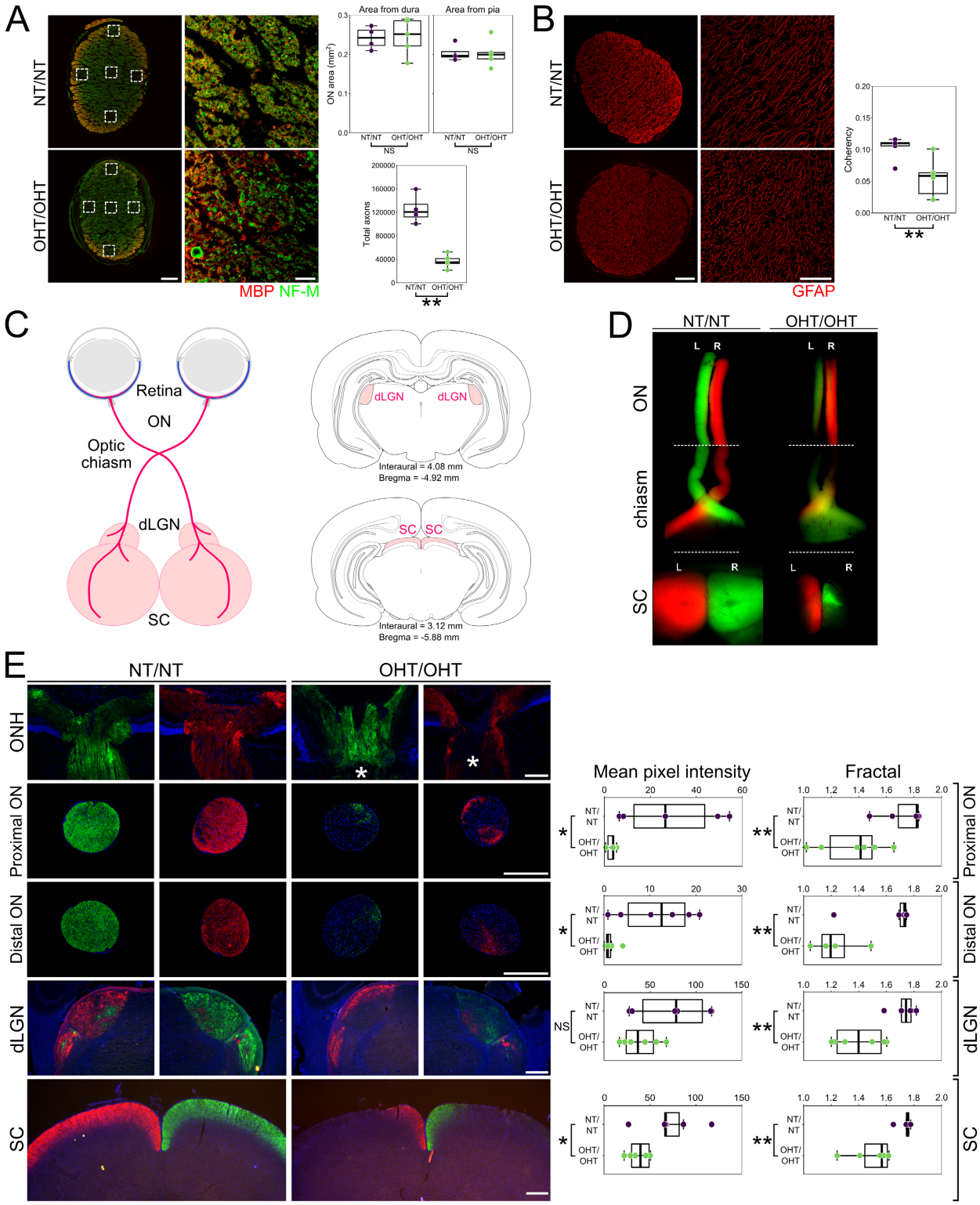
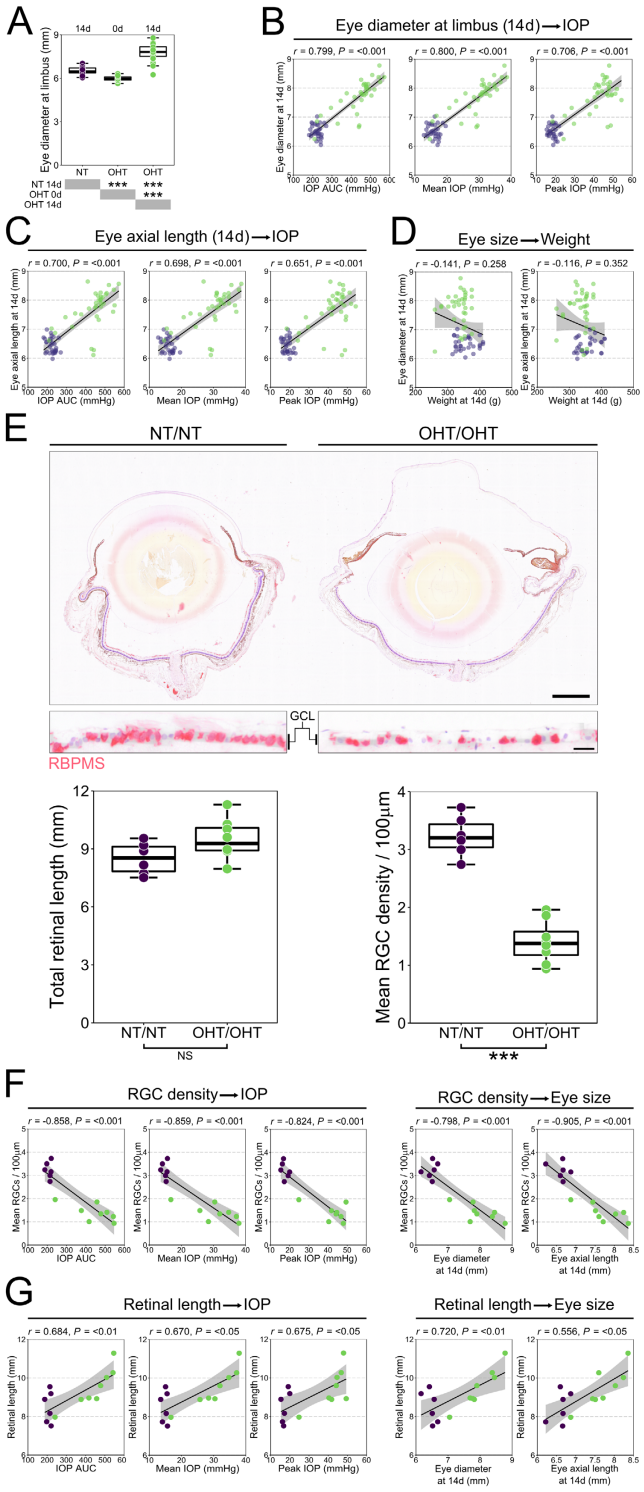

A
NT/NT
OHT/OHT
MBP NF-M
Area from dura
Area from pia
ON area (mm²)
NT/NT OHT/OHT
NS
Total axons
**
B
GFAP
Coherency
C
Retina
ON
Optic chiasm
dLGN
SC
Interaural = 4.08 mm
Bregma = -4.92 mm
Interaural = 3.12 mm
Bregma = -5.88 mm
D
L R
chiasm
E
ONH
Proximal ON
Distal ON
Mean pixel intensity
Fractal
NT/ NT
OHT/ OHT

Figure 4


A
14d
0d
14d
Eye diameter at limbus (mm)
NT
OHT
OHT
NT 14d
OHT 0d
OHT 14d
***
***
***
B
Eye diameter at limbus (14d)→IOP
r = 0.799, P = <0.001
r = 0.800, P = <0.001
r = 0.706, P = <0.001
Eye diameter at 14d (mm)
IOP AUC (mmHg)
Mean IOP (mmHg)
Peak IOP (mmHg)
C
Eye axial length (14d)→IOP
r = 0.700, P = <0.001
r = 0.698, P = <0.001
r = 0.651, P = <0.001
Eye axial length at 14d (mm)
IOP AUC (mmHg)
Mean IOP (mmHg)
Peak IOP (mmHg)
D
Eye size→Weight
r = -0.141, P = 0.258
r = -0.116, P = 0.352
Eye diameter at 14d (mm)
Eye axial length at 14d (mm)
Weight at 14d (g)
Weight at 14d (g)
E
NT/NT
OHT/OHT
GCL
RBPMS
Total retinal length (mm)
NT/NT
OHT/OHT
NS
Mean RGC density / 100μm
NT/NT
OHT/OHT
***
F
RGC density→IOP
r = -0.858, P = <0.001
r = -0.859, P = <0.001
r = -0.824, P = <0.001
Mean RGCs / 100μm
IOP AUC
Mean IOP (mmHg)
Peak IOP (mmHg)
RGC density→Eye size
r = -0.798, P = <0.001
r = -0.905, P = <0.001
Mean RGCs / 100μm
Eye diameter at 14d (mm)
Eye axial length at 14d (mm)
G
Retinal length→IOP
r = 0.684, P = <0.01
r = 0.670, P = <0.05
r = 0.675, P = <0.05
Retinal length (mm)
IOP AUC
Mean IOP (mmHg)
Peak IOP (mmHg)
Retinal length→Eye size
r = 0.720, P = <0.01
r = 0.556, P = <0.05
Retinal length (mm)
Eye diameter at 14d (mm)
Eye axial length at 14d (mm)

(Previous page) **Figure 4: Sustained OHT results in ocular stretch.** (**A**) OHT eyes demonstrated a significant increase in diameter by 14 days compared to both pre-surgery size and NT controls. (**B-C**) Eye diameter at the limbus and axial length at day 14 demonstrated reasonable correlation to measures of IOP (AUC, mean, peak), indicating a relationship between the magnitudes of IOP increase and increase in the size of the eye. (**D**) These correlations were not driven by animal age or weight. (**E**) To assess retinal stretch following OHT whole eyes were paraffin embedded and sectioned, and RGCs labelled with antibodies against RBPMS. There was no significant increase in total retinal length following OHT, which was highly variable even across NT eyes. RGC counts across the retina confirmed gross RGC loss. (**F**) RGC density was highly correlated with all measured of IOP and eye size. (**G**) Retinal length correlated with measures of IOP, but to a lesser degree. For complete *n* please see **Methods**. Scale bars = 1 mm in **E** (*upper panel*), 20 μm in **E** (*lower panel*), * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

(Following page) **Figure 5: OHT results in microglia remodelling and activation.** (**A**) Morphologies of individual microglia from the NFL/GCL were assessed by high resolution confocal imaging (*top row* and *x-y* projections *below*) and Imaris reconstructions (*middle row* and insets *bottom row*). (**B**) Multiple metrics of microglia morphology demonstrated significant changes in microglia between all groups suggesting progressive activation from NT/NT > NT/OHT > OHT/NT > OHT/OHT. (**C-D**) These data were mirrored in microglia from the IPL. (**E**) Hierarchical clustering of microglia metrics by Euclidean distance was used to cluster microglia, generating 4 independent clusters (Cluster 1-4). The breakdown of each cluster is shown *below*. Clusters broadly represented groups of activation and demonstrates that microglial morphological measurements can distinguish biologically meaningful groups of microglia independently of disease grouping. For complete *n* please see **Methods**. Scale bars = 30 μm in **A** and **C**, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 5

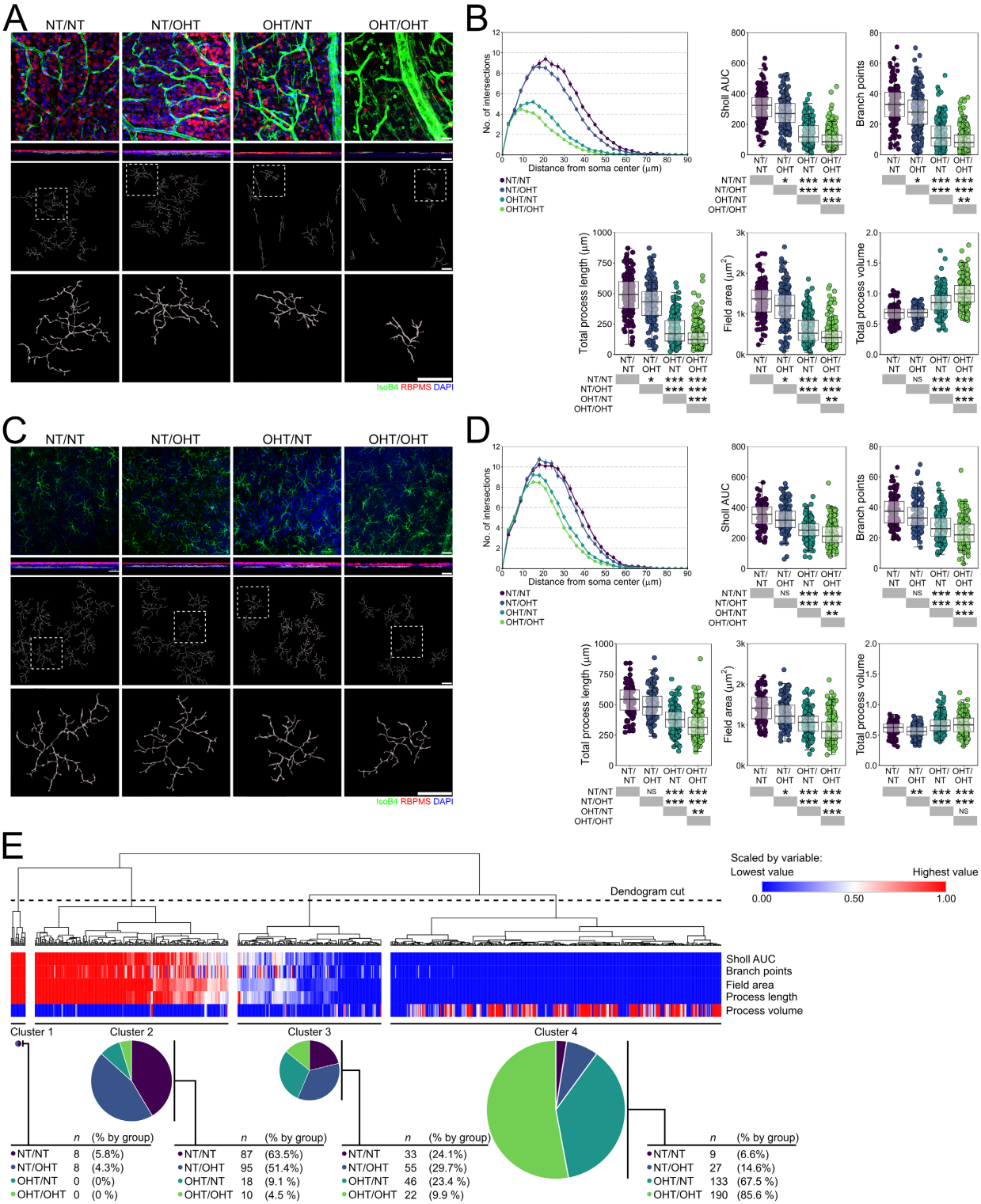
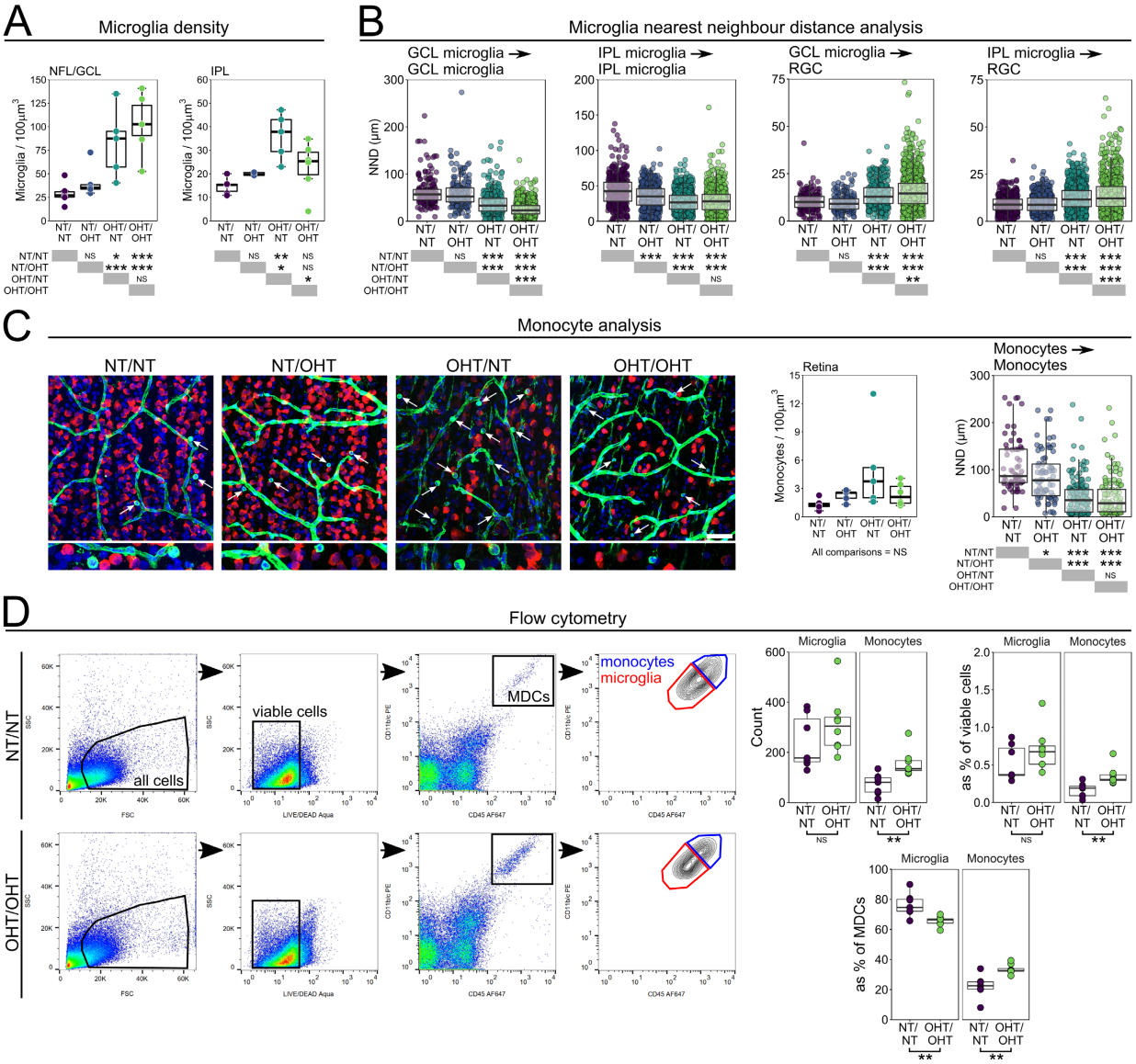

A
NT/NT
NT/OHT
OHT/NT
OHT/OHT
IsoB4 RBPMS DAPI
B
No. of intersections
Distance from soma center (μm)
NT/NT
NT/OHT
OHT/NT
OHT/OHT
Sholl AUC
Branch points
Total process length (μm)
Field area (μm²)
Total process volume
C
D
E
Scaled by variable:
Lowest value
Highest value
0.00
0.50
1.00
Dendogram cut
Sholl AUC
Branch points
Field area
Process length
Process volume
Cluster 1
Cluster 2
Cluster 3
Cluster 4
n (% by group)
NT/NT 8 (5.8%)
NT/OHT 8 (4.3%)
OHT/NT 0 (0%)
OHT/OHT 0 (0 %)
NT/NT 87 (63.5%)
NT/OHT 95 (51.4%)
OHT/NT 18 (9.1 %)
OHT/OHT 10 (4.5 %)
NT/NT 33 (24.1%)
NT/OHT 55 (29.7%)
OHT/NT 46 (23.4 %)
OHT/OHT 22 (9.9 %)
NT/NT 9 (6.6%)
NT/OHT 27 (14.6%)
OHT/NT 133 (67.5 %)
OHT/OHT 190 (85.6 %)

Figure 6


A
Microglia density
NFL/GCL
IPL
Microglia / 100µm3
NT/NT
NT/OHT
OHT/NT
OHT/OHT
B
Microglia nearest neighbour distance analysis
GCL microglia → GCL microglia
IPL microglia → IPL microglia
GCL microglia → RGC
IPL microglia → RGC
NND (µm)
C
Monocyte analysis
NT/NT
NT/OHT
OHT/NT
OHT/OHT
Retina
Monocytes / 100µm3
All comparisons = NS
Monocytes → Monocytes
NND (µm)
D
Flow cytometry
all cells
viable cells
MDCs
monocytes
microglia
FSC
SSC
LIVE/DEAD Aqua
CD45 AF647
CD11b/c PE
Microglia
Monocytes
Count
as % of viable cells
as % of MDCs

(Previous page) **Figure 6: Monocytes enter OHT retina early following IOP elevation.** (**A**) Following 14 days of OHT microglia numbers were increased in both the NFL/GCL and IPL of the retina. (**B**) NND analysis demonstrates that microglia get closer to each other but are diffuse around RGCs suggesting that microglia do not cluster around surviving RGCs. (**C**) Ameboid monocytes were present in all conditions after 14 days of OHT with small NNDs suggestion monocyte clumping in local regions of damage. (**D**) To begin to assess whether this increase in microglia number was due to infiltrating monocytes entering the tissue and become microglia-like monocyte derived macrophages, whole retina homogenate was assessed by flow cytometry. Retinas were sampled at 3 days post-injection (the point of peak IOP in this model). At this time point microglia numbers were not increased but there was a significant increase in monocyte numbers (both by raw count and as a percentage of other cell types). MDCs = myeloid derived cells. For complete *n* please see **Methods**. Scale bars = 100 µm in **C**, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 7

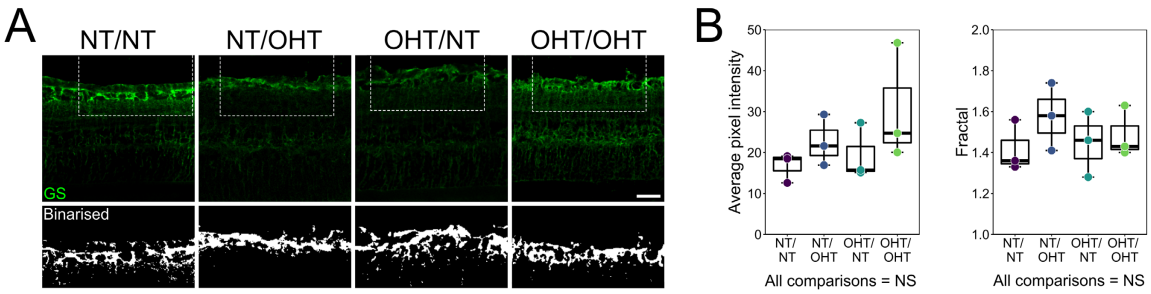


Figure 7: No activation of Müller glia identified by glutamine synthetase labelling. (**A**) Müller glia were labelled in cryo-sections by glutamine synthetase (GS). GS content was assessed in the NFL/GCL/IPL by measuring average pixel intensity and by fractal analysis of binarized crops. (**B**) No significant difference in average pixel intensity or fractal was observed across groups, suggesting no gross Müller glia activation at this time point in this model. Scale bars = 50 µm in **A**, NS = non-significant ($P > 0.05$).

Figure 8

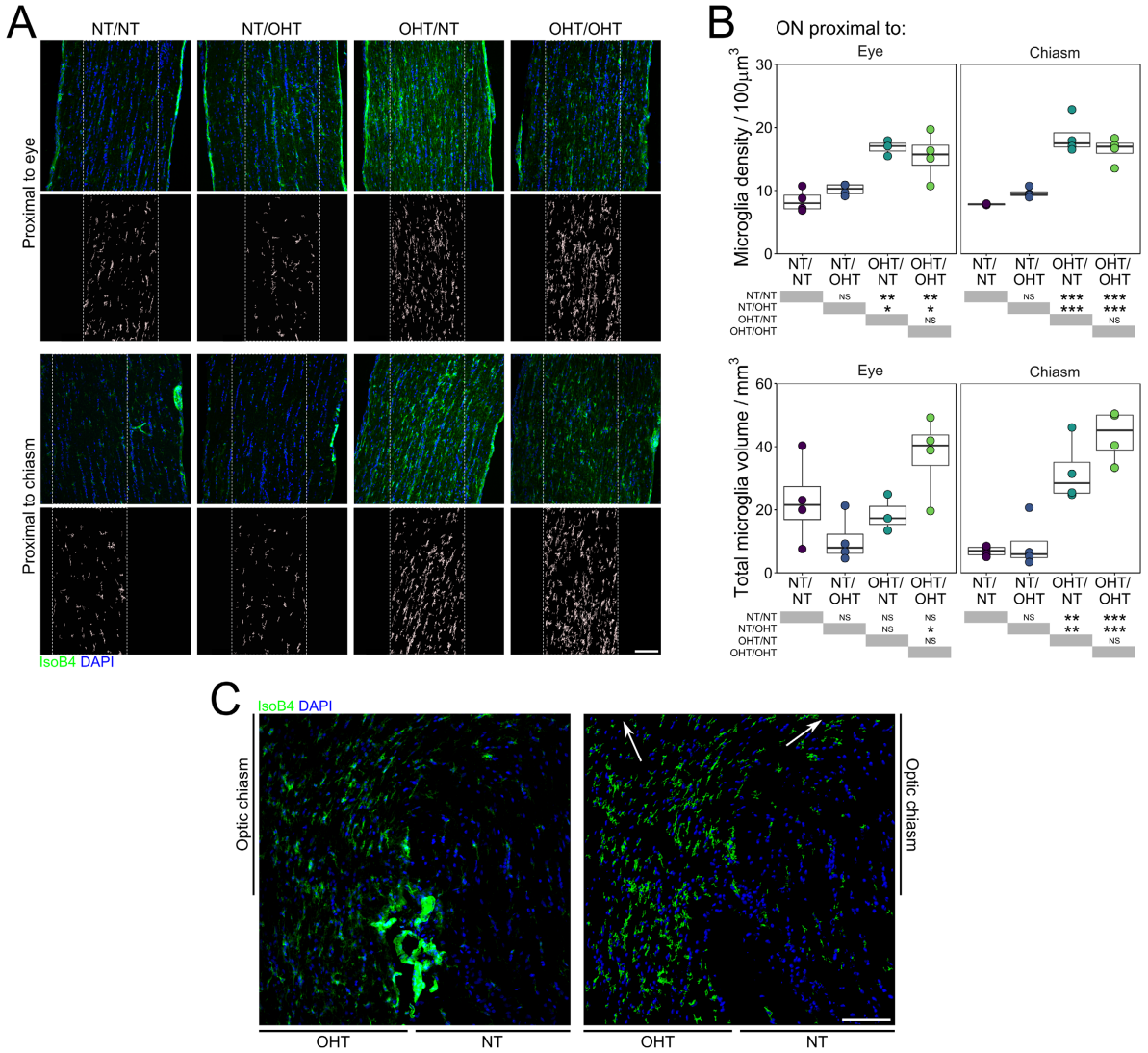
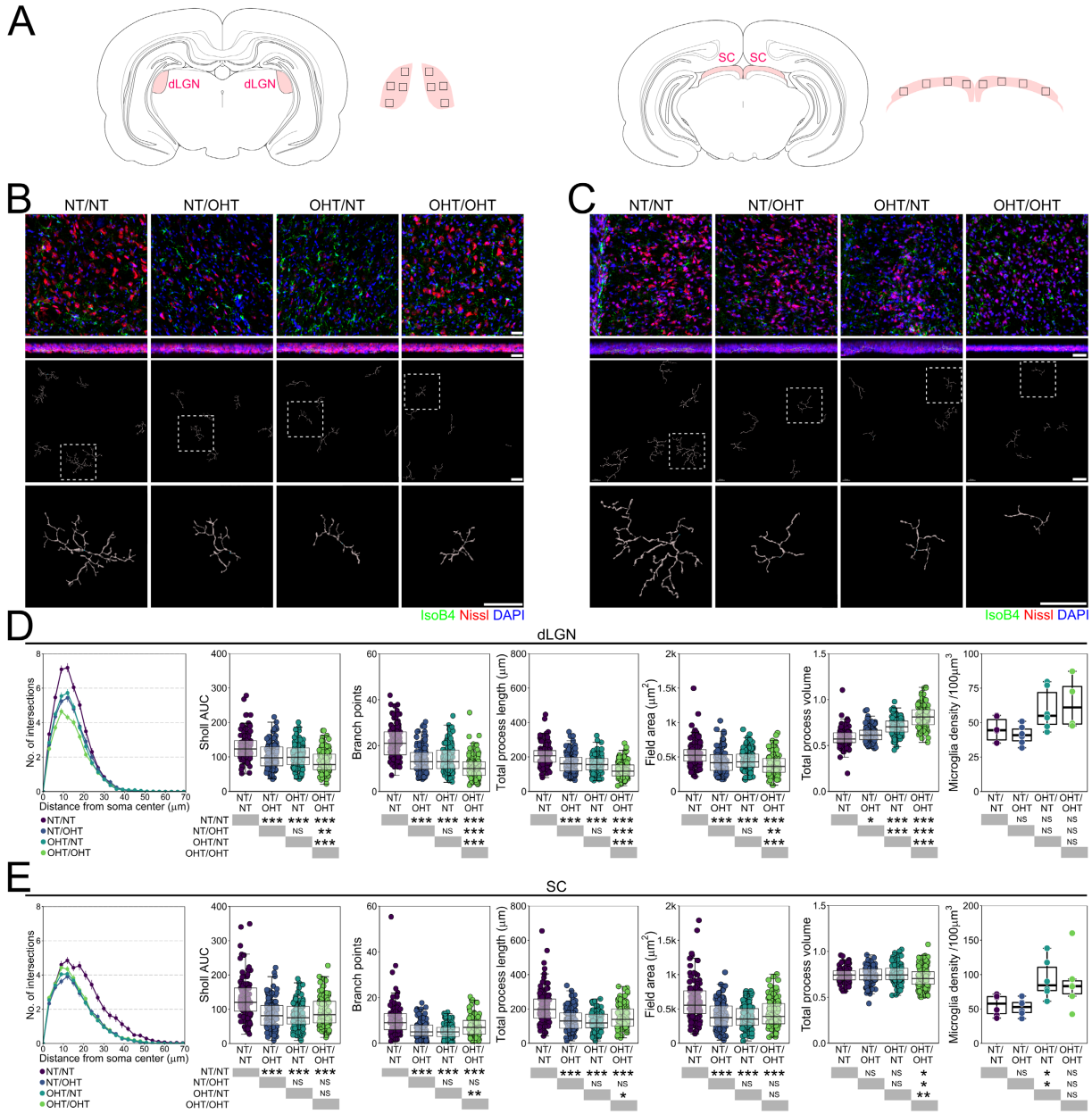


Figure 8: Microglia activation extends into the optic nerve and optic chiasm. (**A**) Optic nerves were transversally sectioned and overall microglial labelling was reconstructed (*insets*) and analysed. (**B**) There exists significant microglia activation in the optic nerve proximal to the eye and proximal to the chiasm. (**C**) Microglial activation is most evidently demonstrated at the chiasm where RGC axons from unilateral OHT animals decussate, with the appearance of activated microglia following both optic tracts post-chiasm (*white arrows* in reconstruction; *right*). Scale bars = 100 μm in **A** and **C**, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 9


A
dLGN
dLGN
SC
SC
B
NT/NT
NT/OHT
OHT/NT
OHT/OHT
IsoB4 Nissl DAPI
C
NT/NT
NT/OHT
OHT/NT
OHT/OHT
IsoB4 Nissl DAPI
D
dLGN
No. of intersections
Distance from soma center (μm)
NT/NT
NT/OHT
OHT/NT
OHT/OHT
Sholl AUC
Branch points
Total process length (μm)
Field area (μm²)
Total process volume
Microglia density /100μm³
E
SC
No. of intersections
Distance from soma center (μm)
NT/NT
NT/OHT
OHT/NT
OHT/OHT
Sholl AUC
Branch points
Total process length (μm)
Field area (μm²)
Total process volume
Microglia density /100μm³

(Previous page) **Figure 9: Microglia activation persists into terminal visual thalami following OHT.** (**A**) Microglia were assessed from dLGN and SC sections. Regions of images are shown by black inset boxes in the schematics. Morphologies of individual microglia from the dLGN (**B**) and SC (**C**) were assessed by high resolution confocal imaging (*top row* and *x-y* projections *below*) and Imaris reconstructions (*middle row* and insets *bottom row*). (**D-E**) Multiple metrics of microglia morphology demonstrated significant changes in microglia morphology between NT/NT and all other groups. These results were more binary (*i.e.* damage to a single retina drives neuroinflammatory insults to both terminating hemispheres) suggesting that neuroinflammatory signatures propagate even when only a small percentage of axons are damaged or dystrophic. For complete *n* please see **Methods**. Scale bars = 30 µm in **B** and **C**, * = $P < 0.05$, ** = $P < 0.01$, *** $P < 0.001$, NS = non-significant ($P > 0.05$).

Figure 10

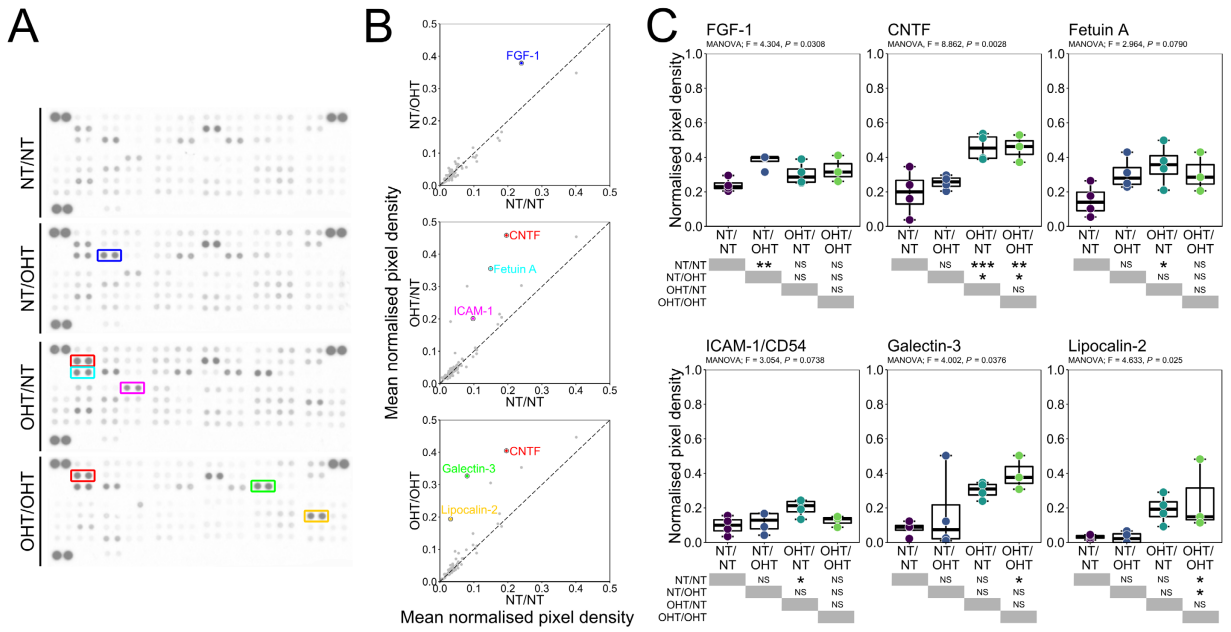


Figure 10: OHT drives neuroinflammatory cytokine expression in the retina. (**A**) To assess cytokine and chemokine expression in the retina following OHT, retinas were homogenized and assessed by protein array. (**B-C**) Densitometry analysis identified 1 changed cytokine (FGF-1) between NT/NT and NT/OHT groups, 3 changed cytokines (CNFT, Fetuin A, ICAM-1) between NT/NT and OHT/NT groups, and 3 changed cytokines (CNTF, Galectin-3, Lipocalin-2) between NT/NT and OHT/OHT groups. All cytokines were increased compared to NT/NT groups and may represent ideal candidates for further experimentation or as biomarker candidates. * = $P < 0.01$, ** = $P < 0.05$, *** $P < 0.01$, NS = non-significant ($P > 0.05$).

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