INTRODUCTION

Neurodegeneration is a key component of multiple sclerosis (MS) pathology which is understood to be the structural correlate of progressive neurological disability (Trapp & Nave, 2008), however, despite this, neuroprotective therapies for MS remain an unmet clinical need (Compston & Coles, 2008). Among various compartments of the central nervous system (CNS), the visual pathway is frequently affected in MS.

Abstract

Autoimmune optic neuritis (AON), a model of multiple sclerosis-associated optic neuritis, is accompanied by degeneration of retinal ganglion cells (RGCs) and optic nerve demyelination and axonal loss. In order to investigate the role of N-methyl-D-aspartate (NMDA) receptors in mediating RGC degeneration, upstream changes in the optic nerve actin cytoskeleton and associated deterioration in visual function, we induced AON in Brown Norway rats by immunization with myelin oligodendrocyte glycoprotein. Subsequently, visual acuity was assessed by recording visual evoked potentials and electroretinograms prior to extraction of optic nerves for western blot analysis and retinas for quantification of RGCs. As previously reported, in Brown Norway rats RGC degeneration is observed prior to onset of immune cell infiltration and demyelination of the optic nerves. However, within the optic nerve, destabilization of the actin cytoskeleton could be seen as indicated by an increase in the globular to filamentous actin ratio. Interestingly, these changes could be mimicked by intravitreal injection of glutamate, and similarly blocked by application of the NMDA receptor blocker MK-801, leading us to propose that prior to optic nerve lesion formation, NMDA receptor activation within the retina leads to retinal calcium accumulation, actin destabilization within the optic nerve as well as a deterioration of visual acuity during AON.

KEYWORDS

actin cytoskeleton, glutamate excitotoxicity, NMDA receptors, optic nerve, optic neuritis, retinal ganglion cells
Inflammatory demyelination of optic nerves, termed optic neuritis, is a common syndrome associated with MS. This is accompanied by degenerative changes in the retina, consisting of a loss of retinal ganglion cells (RGCs) together with thinning of the retinal nerve fibre and inner nuclear layers in patients (Galetta et al., 2015; Green, McQuaid, Hauser, Allen, & Lyness, 2010; Kuper-Smith, Garvin, Wang, Durbin, & Kardon, 2016; Sye et al., 2012). Interestingly, retinal nerve fibre layer thinning has also been reported in MS patients in the absence of clinically defined optic neuritis (Bock et al., 2010; Petzold et al., 2010; Talman et al., 2010).

One neurodegenerative mechanism suggested to be involved in MS pathology is glutamate excitotoxicity (Kwach & Lassmann, 2017; Macrez, Sty, Denis, Lipton, & Fabian, 2016). Indeed, increased levels of glutamate have been detected in all lesion stages of MS pathology (Srinivasan, Sailasuta, Hurd, Nelson, & Pelletier, 2005; Tisell et al., 2013). In the retina, RGCs are especially vulnerable to increased glutamate levels, since these cells have a high density of dendritic glutamate receptors (Aiazenman, Frosch, & Lipton, 1988; Massey & Miller, 1990). Ionotropic glutamate receptors are key players in the process of excitotoxicity (Choi, 1988; Tymianski, 1996), and their over-stimulation is thought to lead to prolonged intracellular calcium increases capable of activating downstream pathways leading to cell death (Obrenovitch, Urenjak, Zikha, & Jay, 2000; Olney, 1969).

In order to investigate the mechanisms underlying early retinal neurodegeneration in optic neuritis, we have used the model of myelin oligodendrocyte glycoprotein (MOG) immunization of Brown Norway rats. This model of experimental autoimmune encephalomyelitis (EAE) has a high incidence of autoimmune optic neuritis (AON) as well as other clinical and pathological features similar to those in MS patients (Meyer et al., 2001; Storch et al., 1998). In addition, RGC loss has been shown in this model to precede inflammatory demyelination of optic nerves (Fairless et al., 2012), to correlate with the timing of increases in retinal calcium levels and calpain activity (Hoffmann et al., 2013), and to involve NMDA receptor activity (Sühs et al., 2014). We also previously showed that ultrastructural changes in RGC axons of the optic nerve were present during the onset of disease (Fairless et al., 2012) as was elongation of Nodes of Ranvier (Stojic, Bojcevski, Williams, Diem, & Fairless, 2018).

In this study, in order to investigate further the impact of NMDA receptor activity on RGC degeneration in AON, we have correlated the timing of RGC degeneration with changes in the actin cytoskeleton of the optic nerve and visual disturbances. Through intravitreal injection of glutamate and also intravitreal application of an NMDA receptor blocker into rats with AON, we demonstrate that retinal NMDA receptors contribute to upstream changes in the optic nerve, probably by regulating retinal calcium levels.

2 | MATERIALS AND METHODS

2.1 | Animals

Female Brown Norway rats (8–10 weeks old, 140–160 g; Charles River, Sulzfeld, Germany; RRID:RGD_737972) were used in all experiments. All animals were kept under environmentally controlled pathogen-free conditions with free access to food and water, and housed in groups of four in UNO polycarbonate type IV cages under a 12 hr light/dark cycle. Animals were acclimatized for 1 week prior to experiments, which were then performed during the hours of 09:00 and 18:00. Animal experiments were performed in an approved animal facility according to the relevant laws and institutional guidelines of the local ethics committees of Baden-Württemberg and Saarland, Germany (approved protocols C-1.2.4.2.1, 35-9185.81/G-36/12, 35-9185.81/G-172/14 and 35-9185.81/G-36/17). The study was not preregistered. Assignment of animals to study groups was randomly performed by arbitrarily marking tails with a skin marker (Fine Science Tools) before using the Randbetween function in Windows Excel. A total of 160 rats were used in this study, of which 4 were excluded (but not replaced) because of the exclusion criteria of unsatisfactory signal-to-noise ratios during magnetic resonance imaging (MRI). Experiments were exploratory in nature with animal group sizes kept to a minimum to avoid animal suffering, and were based on previous studies conducted within the laboratory (no sample calculation was performed). The different animal groups and the experimental timelines are shown in Figure 1.

2.2 | MOG-EAE induction and animal scoring

Figure 1 gives an overview of the study design. Brown Norway rats were immunized with whole recombinant rat MOG (a kind gift of Prof. Stadelmann, Dept. of Neuropathology, University of Göttingen). Immunization emulsion contained 1:1 ratio of 100 μg MOG in phosphate-buffered saline (Sigma-Aldrich) and complete Freund’s adjuvant (Sigma-Aldrich) containing 100 μg of heat-inactivated mycobacterium tuberculosis H37RA (Difco Microbiology). Rats were anaesthetized with 5% isoflurane inhalation (chosen for its rapid on- and offset) and injected intradermally at the tail with 200 μl of immunization emulsion. No analgesics were applied following injection because of the potential to interfere with local inflammation and subsequent EAE. Rats were observed daily for clinical signs of EAE that reflect spinal cord pathology. The grading system used to score neurological symptoms were: grade 0, no signs; grade 0.5, distal paresis of the tail; grade 1, complete tail paralysis; grade 1.5, paresis of the tail and mild hind leg paresis; grade 2.0, unilateral severe hind leg paresis; grade 2.5, bilateral severe hind limb paresis; grade 3.0, complete bilateral hind limb paralysis; grade 3.5, complete bilateral hind limb paralysis and paresis of one front limb; grade 4, complete paralysis (tetraplegia), moribund state, or death. Approved protocols required animals exceeding a clinical score of 2.5 for over 48 hr to be euthanized, a criterion that was not met. The clinical onset of EAE coincides with the pathological hallmark of AON—the appearance of inflammatory demyelination in the optic nerve; the period referred to as clinical AON (cAON). However, in this MOG-EAE model RGC loss precedes cAON (Fairless et al., 2012); the period referred to as induction AON (iAON).

At desired time-points during AON (day 10 post-immunization (p.i.) for iAON, or day 1–3 after clinical onset of EAE (approximately day 14 p.i.) for cAON) animals were sacrificed by inhalation of 5% isoflurane followed
by decapitation. Retinas were further processed for retinal whole-mount immunofluorescence and optic nerves for Western blotting.

### 2.3 Retina excitotoxicity model — intravitreal injection of glutamate

In order to induce a primary retinal insult, healthy rats received intravitreal injection of glutamate or saline as control. During injections, animals were kept under anaesthesia by inhalation of 5% isoflurane. In total 100 nmoles of glutamate (4 μl of 25 mM glutamate (Sigma-Aldrich) in sterile saline) was injected intravitreally by puncturing the eye at the cornea-sclera junction with a 33G needle attached to a 10 μl NanoFil syringe and injecting over the course of 5 min. Subsequently, eyes were treated with a 0.75% povidone-iodine solution (Braunol; B Braun Melsungen AG). Twenty-four hours (24 hr) or 7 days following intravitreal injection of glutamate, animals were sacrificed and tissue was processed as described in the previous section.

### 2.4 Measurement of visual evoked potentials and electroretinograms

Five to seven days prior to the first recording, holes were drilled in the skull for the placement of electrodes. Rats were anaesthetized by intraperitoneal injection (i.p.) of ketamine (60 mg/kg; Atarost GmbH and Co.) and xylazine (12 mg/kg Albrecht) (anaesthetics chosen for longevity during procedure, as well as analgesic properties), skin was incised medially, and holes were drilled above both visual cortices 1 mm frontal and 3.5 mm lateral to lambda (for recording electrodes) and above the motor cortex (1 mm frontal and lateral from bregma for reference electrode). Following surgery, rats were provided drinking water containing tramadol (100 mg/L) for the next 24 hr to minimize potential pain, and the wound was treated with 7.5% povidone-iodine to minimize the risk of infection.

Measurements of visual evoked potentials (VEPs) were performed using a UTAS Visual Diagnostic System (LKC Technologies). Prior to recording, animals were anaesthetized with ketamine and
Autoimmune optic neuritis is accompanied by RGC loss and disturbances in the optic nerve actin cytoskeleton. (a) Representative images showing myelin (LFB) and axons (Bielschowsky) in the optic nerve (longitudinal sections), and RGC density (Rbpms+ cells) in the retina (flat-mounted). The histopathological hallmarks of AON (demyelination and axonal loss) correlate with the onset of cAON (identified by EAE clinical impairment of the spinal cord). (b) Quantification of RGC densities in retinal whole-mounts during AON reveals a decrease starting during iAON prior to inflammatory demyelination in optic nerves (healthy n = 7; iAON n = 7; cAON n = 6 retinas). (c) Western blot with an antibody against actin (42 kDa) following fractionation of optic nerve lysates into globular and filamentous forms (G/F-actin). (d) Quantification of the G/F-actin ratio reveals its increase during disease progression, beginning at iAON (healthy n = 10; iAON, n = 14; cAON n = 11 animals). (e) Western blotting of optic nerve lysates with antibodies against gelosin (98 kDa) and the actin-cleavage product, fractin (32 kDa), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa) as a loading control. (f) Gelsolin levels decrease during AON (healthy n = 9; iAON n = 13; cAON n = 8 animals). (g) Fractin levels increase during AON (healthy n = 8; iAON n = 11; cAON n = 9 animals). Scale bars 100 µm. *p < .05; **p < .01, ***p < .001 (B, D, F, ANOVA; G, Kruskal–Wallis). iAON, induction phase of autoimmune optic neuritis; cAON, clinical phase of autoimmune optic neuritis.
Intravitreal injection of glutamate induces RGC loss, perturbs the optic nerve actin cytoskeleton and induces visual deterioration comparable to IACN by 24 hr post-injection. (a) Representative anti-Rbpm immunostaining (green) of whole-mounted retinas to identify RGCs following saline or glutamate injection. (b) Quantification of Rbpm’s cells following intravitreal injection of glutamate reveals the induction of significant RGC degeneration in comparison to their respective saline controls (healthy n = 7, saline 24 hr n = 6, glutamate 24 hr n = 5 retinas, saline 7 days n = 13, glutamate 7 days n = 15). (c) Western blotting with an antibody against actin (42 kDa) following fractionation of optic nerve lysates (24 hr after intravitreal injections) into globular and filamentous forms (G/F-actin). (d) The G/F-actin ratio is significantly increased in optic nerves of glutamate-injected rats compared to either saline-injected or healthy (both glutamate- and saline-injected, n = 4; healthy n = 10 animals). (e) Western blot of optic nerve lysates (24 hr after intravitreal injections) with antibodies against gelsolin (98 kDa) and the actin-cleavage product, fractin (32 kDa), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa) as a loading control. (f) Gelsolin was not significantly altered in optic nerves of rats receiving intravitreal glutamate injection (both glutamate- and saline-injected, n = 8; healthy n = 9 animals), but (g) fractin was significantly elevated, compared to optic nerves from rats either receiving only saline or healthy (both glutamate- and saline-injected, n = 7; healthy n = 8 animals). (h) Representative fVEP waveforms from saline-/glutamate-injected eyes 24 hr post-injection. There is no significant change in fVEP amplitudes (i) or latencies (j) between these two groups (saline n = 3; glutamate n = 8, single eye recorded per rat). (k) Representative pVEP waveforms following pattern stimulation at different spatial frequencies recorded in saline-/glutamate-injected eyes after 24 hr. (l) Quantification revealed a significant decrease in pVEP amplitudes in glutamate-injected compared to saline-injected eyes (saline n = 5; glutamate n = 6, single eye recorded per rat). (m) Representative pERG waveforms following pattern stimulation at different spatial frequencies recorded 24 hr following saline or glutamate intravitreal injection. (n) Quantification revealed a significant decrease in pERG amplitudes in glutamate-injected compared to saline-injected eyes (both glutamate- and saline-injected rats, n = 8, single eye recorded per rat). *p < .05, **p < .01, ***p < .001 (Student’s t-test or ANOVA). fVEP, flash visual evoked potential; pERG, pattern electroretinogram; pVEP, pattern visual evoked potential.

2.8 | Manganese-enhanced magnetic resonance imaging

Magnetic resonance imaging (MRI) measurements were performed with a system designed for small animal research ( Biospec Avance III 9.4/20; Bruker Biospin GmbH), with a static magnetic field strength of 9.4 Tesla. A linear single channel volume coil (inner diameter 72 mm) was employed for radio frequency transmission and a saddle-shaped surface coil developed for imaging of the rat brain, with a 2 × 2 array setup was used as receiver. Anaesthesia was induced with 4% isoflurane in oxygen (at a rate of 2 litres per minute) and maintained during the procedure with 2% isoflurane. An aqueous cream was applied to the cornea to prevent desiccation. Animals were placed in prone position on a custom-designed animal cradle, including a water-driven warming system connected to a thermostat. Respiration was monitored via a pressure transducer (Graseb infant respiration sensor; Smith Medical Germany) placed under the abdomen. Physiological status was monitored throughout the procedure using PC-SAM32 software (Small Animal Instruments Inc.).

Thirty minutes prior to MRI, animals received either saline (250 μl) or MK-801 (0.15 mg/kg body weight in 250 μl; dosage previously used in Sühs et al., 2014) by intraperitoneal injection. Intravitreal injections were avoided so as not to disrupt retinal tissue integrity and induce imaging artefacts. Animals were then placed in the magnet, and correct positioning was verified using a tri-directional Fast Low Angle Shot scan. To identify the retina, MRI was performed with a T2-weighted sagittal Rapid Acquisition with Relaxation Enhancement (RARE) sequence (TR/TE, 5,000 ms/57 ms; RARE factor 17) and positioning checked with a T2-weighted coronal RARE sequence (TR/TE, 2,300 ms/39.6 ms; RARE factor 8). Manganese enhancement was measured using a T1-weighted multislice multi-echo scan (TR/TE, 320 ms/9.1 ms; FOV, 4.8 × 3.55 cm; matrix, 380 × 380; averages, 5; inter-slice distance 0.2 mm). After
acquisition of pre-contrast T1-weighted images, an aqueous solution of MnCl₂ (20 mg/kg; Sigma-Aldrich) was injected via vein catheter. Mn²⁺-enhanced, T1-weighted scans were then made 1 hr after MnCl₂ administration.

For quantitative image analyses, regions of interest were selected, and signal intensities normalized to the intensity of background brain tissue using Paravision 5.1 software (Bruker). To calculate manganese enhancehance, the increase in normalized signal intensities after manganese injection was calculated as a percentage of the signal before manganese injection. The signal increase following manganese injection in iAON rats was then compared to that occurring in healthy controls. False colour images were generated using ImageJ software (National Institutes of Health), by application of rainbow red- Syc-blue colour scaling.

2.9 | Statistics

All data are presented as their mean values ± standard error of the mean (SEM). Statistical analyses were made using SigmaPlot 13.0 software (Systat Software Inc.). Where two experimental groups were compared, if data were normally distributed (as assessed by the Shapiro-Wilk test), statistical significance was assessed by two-tailed Student’s t test. If data failed the Shapiro-Wilk test, statistical significance was assessed by Mann-Whitney rank sum test. Where more than two groups were compared in normally distributed data (as assessed by the Shapiro-Wilk test), ANOVA combined with post hoc Tukey’s method was used. If data failed the Shapiro-Wilk test, Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn’s method was used. No test for outliers was performed. Exact p values are given in the results section with three levels of significance defined: *p ≤ .05 was considered significant, **p ≤ .01 was considered strongly significant; ***p ≤ .001 was considered highly significant. All data were used for analysis unless a clear reason for exclusion was apparent — criteria for exclusion included damage to the retina during dissection (e.g. if the area around the optic disc was damaged or missing), or bleeding from the eye during intravitreal injection. Experiments were performed at least twice, with all data pooled for analysis.

3 | RESULTS

3.1 | Progressive RGC loss during AON is detectable with anti-Rbpms labelling

Following MOG-immunization, there are two distinct stages of AON. The first is the induction or pre-clinical stage (which we term iAON; in this study d10 p.i. has been used) during which there are no signs of spinal cord injury (i.e. no clinical deficit reflected in the classical EAE scoring system). During this period, no changes in the optic nerve in terms of demyelination or axonal loss are detectable (Fairless et al., 2012), as confirmed in this study (Figure 2a). However, it has previously been reported that progressive retinal deterioration, indicated by a loss of RGCs, already begins during this stage (Fairless et al., 2012; Hobom et al., 2004). This was confirmed in the current study through immunolabelling of RGCs within retinal whole-mounts with an antibody against the relatively novel RGC marker RNA-binding protein with multiple splicing (Rbpms, Rodriguez et al., 2014). A significant decrease in RGCs was observed during iAON of about 7% compared to healthy, unimmunized controls (iAON, 2,875 ± 46 Rbpms⁻ cells/mm², n = 7; healthy, 3,076 ± 53 Rbpms⁻ cells/mm², n = 7; p = .047; Figure 2b).

The second stage of AON is the clinical phase (which we term cAON; in this study days 1 to 3 after EAE onset has been used, approximately day 14 p.i.), which is characterized by optic nerve demyelination, immune cell infiltration and axonal loss (Figure 2a), and typically occurs in parallel to the onset of clinical EAE symptoms reflecting spinal cord lesions (Fairless et al., 2012; Meyer et al., 2001). During this stage, RGC loss had progressed to about 11% compared to healthy controls (cAON, 2,720 ± 72 Rbpms⁻ cells/mm², n = 6; p = .001; Figure 2b).

3.2 | Optic nerve actin cytoskeleton is remodelled during AON

Cytoskeletal and axonal transport deficits have been proposed as a possible early mechanism that contributes to MS neurodegeneration (Lingor, Koch, Tönges, & Bähr, 2012). Therefore, we wanted to determine the impact of AON on the actin cytoskeleton network dynamics in the optic nerve, where RGC axons are located. The globular to filamentous actin ratio (G/F-actin ratio) was assessed by ultracentrifugation of optic nerve lysates to separate the actin pool into its globular and filamentous fractions prior to Western blotting. This ratio increased with disease progression (iAON 0.958 ± 0.109, n = 14, p = .003; cAON 1.032 ± 0.123, n = 11, p = .002) compared to healthy controls (0.429 ± 0.058, n = 10; Figure 2d). Gelsolin, an F-actin-binding protein, is known to be a calcium-activated anti-apoptotic factor (Harms et al., 2004) that is at the same time cleaved by caspase/calpain proteases (Kothakota et al., 1997). In AON, gelsolin protein levels were decreased with disease progression (iAON 0.548 ± 0.045 compared to GAPDH, n = 13, p = .019; cAON, 0.342 ± 0.062, n = 8, p < .001) compared to healthy controls (0.770 ± 0.066, n = 9; Figure 2f). Fractin is a 32 kDa cleaved G-actin monomer product that accumulates following caspase/calpain activity (Sokolowski et al., 2014; Utsumi, Sakurai, Nakano, & Ishisaka, 2003). In AON, fractin protein levels increased with disease progression (iAON 0.477 ± 0.071 compared to GAPDH, n = 11, p = .019; cAON 0.637 ± 0.097, n = 9, p < .001) compared to healthy (0.175 ± 0.036, n = 8; Figure 2g). Since the actin cytoskeleton, along with the microtubule network, are involved in mediating intracellular transport (Franker & Hoogenraad, 2013), we also analysed transportation deficits in the optic nerve as indicated by accumulation of transported proteins such as β-amyloid precursor protein and synaptophysin, as well as the microtubule motor protein kinesin. No accumulation was seen in these proteins during iAON, but only in cAON samples (Figure S1).
3.3 | Intravitreal injection of glutamate mimics retinal and optic nerves changes during iAON

In the retina, glutamate excitotoxicity can be induced following intravitreal injection of glutamate (Schori et al., 2001; Sisk & Kuwabara, 1985; Zhou et al., 2007). In order to explore whether the changes that we observe during AON (Figure 2) reflect over-activation of glutamate receptors, glutamate was injected into the eye of healthy rats and retina and optic nerves were subsequently analysed.

Quantification of RGC densities was performed revealing a significant decrease in the number of surviving cells already at 24 hr following glutamate injections (2,879 ± 40 Rbpms+ cells/mm², n = 5) compared to both, healthy and saline-injected controls (healthy 3,076 ± 53 Rbpms+ cells/mm², n = 7, p = .019; Saline 3,094 ± 48 Rbpms+ cells/mm², n = 6, p = .011). Saline-injected controls at this time-point were comparable to healthy RGC counts (p = .998; Figure 3b). Similarly, at day 7 post-glutamate injection, surviving RGCs decreased further (2,695 ± 19 Rbpms+ cells/mm², n = 13, p < .0001 compared to healthy) which were significantly reduced compared to their respective saline control (2,877 ± 31 Rbpms+ cells/mm², n = 15, p = .0004). For further analysis, we have focused on 24 hr post-injection since this best reflected the early, degenerative changes observed in iAON. Other studies have also shown that NMDA receptor activation (through intravitreal application of NMDA) at this time-point can result in significant RGC degeneration (Maekawa et al., 2017; Zheng et al., 2015).

We next investigated components of the actin cytoskeleton in optic nerves from rats receiving either saline or glutamate intravitreal injection. Similar to that seen during AON, the G/F-actin ratio was significantly higher in 24 hr glutamate-injected animals (1.652 ± 0.214, n = 4) compared to 24 hr saline-injected controls (0.654 ± 0.132, n = 4, p < .001) and healthy (0.429 ± 0.058, n = 10, p < .001; Figure 3d). Although gelsolin protein levels were not significantly changed between the saline- (0.633 ± 0.080 compared to GAPDH, n = 8), healthy (0.770 ± 0.065, n = 9) and glutamate-injected groups (0.630 ± 0.043, n = 8, p = .229; Figure 3f), the fractin protein level was significantly increased in glutamate-injected animals (0.505 ± 0.059 compared to GAPDH, n = 7) compared to both, saline-injected controls (0.315 ± 0.053, n = 7, p = .039) and healthy (0.175 ± 0.036, p < .001; Figure 3g). Interestingly, saline- and glutamate-injected G/F-actin ratios as well as fractin levels were quantitatively comparable to healthy and iAON values (Figure 2) respectively. This suggests that the saline injection did not adversely affect these parameters, and that glutamate injection could replicate the changes in the G/F-actin ratio and fractin levels observed during iAON.

3.4 | Intravitreal injection of glutamate leads to a deterioration in visual acuity

Next, we investigated the effect of intravitreal injection of glutamate on visual acuity. To address this we first performed recordings of fVEPs. In this study, we observed no change in fVEP amplitudes following intravitreal injection of glutamate compared to saline-injected controls 24 hr after the injection (Saline, 160 ± 30 µV, n = 3, Glutamate, 147 ± 17 µV, n = 8, p = .694; Figure 3i). The same was true for the fVEP latencies (Saline, 38 ± 1 ms, n = 3; Glutamate, 35 ± 1 ms, n = 8, p = .233; Figure 3j), and was also observed at 7 days post-injection (Figure S2c).

However, since fVEP responses reflect the activity of all cells in the pathway from photoreceptors to the visual cortex, we next performed recordings of pVEPs which specifically give information about the pathway from RGCs to the visual cortex, and are considered a more sensitive parameter, for example for assessment of MS pathology (Halliday & Mushin, 1980). Following intravitreal injection of glutamate we observed a significant decrease in pVEP amplitudes compared to saline-control 24 hr post-injection (Table 1; Figure 3i), and a similar decrease was observed at 7 days post-injection (Figure S2e).

pVEGs were then recorded to give specific information regarding RGC activity within the retina without involvement of the optic nerve (Pocciatti, 2015). Similar to the pVEP measurements, pERG amplitudes following intravitreal injection of glutamate were significantly decreased compared to saline-control group 24 hr post-injection period (Table 1; Figure 3n), and a similar decrease was observed at 7 days post-injection (Figure S2g).

**TABLE 1** pVEP and pERG amplitudes following pattern stimulation at the indicated spatial frequencies, measured 24 hr following intravitreal injection of either glutamate or saline

| Spatial frequency (c/deg) | Noise  | 0.056  | 0.11  | 0.22  | 0.44  | 0.89  |
|-------------------------|--------|--------|-------|-------|-------|-------|
| pVEP                    |        |        |       |       |       |       |
| Saline (n = 5)          | 29 ± 6 µV | 188 ± 18 µV | 153 ± 23 µV | 134 ± 13 µV | 120 ± 11 µV | 50 ± 10 µV |
| Glutamate (n = 6)       | 27 ± 3 µV | 125 ± 25 µV | 101 ± 17 µV | 86 ± 14 µV  | 75 ± 13 µV  | 54 ± 6 µV  |

| pERG                    |        |        |       |       |       |       |
|-------------------------|--------|--------|-------|-------|-------|-------|
| Saline (n = 8)          | 4 ± 1 µV  | 17 ± 4 µV  | 15 ± 2 µV  | 13 ± 1 µV  | 10 ± 1 µV  | 9 ± 2 µV  |
| Glutamate (n = 8)       | 6 ± 1 µV  | 9 ± 1 µV   | 11 ± 2 µV  | 7 ± 2 µV   | 5 ± 1 µV   | 5 ± 1 µV   |

*p* values are given comparing glutamate with saline using Student’s t test. For graphical representation of values, see Figure 3l,n.

Abbreviations: pERG, pattern electroretinogram; pVEP, pattern visual evoked potential.
3.5 | NMDA receptor is involved in RGC neurodegeneration during iAON

In order to determine the potential role of NMDA receptors in mediating RGC degeneration and upstream optic nerve actin cytoskeletal changes in iAON, the potent use-dependent blocker of NMDA receptors MK-801 was chosen (Huettner & Bean, 1988; McKay, Bengtson, Bading, Wyllie, & Hardingham, 2013). Treatment with MK-801 (or saline for controls) was performed by intravitreal injection on days 4 and 7 p.i., with iAON samples taken at day 10 p.i. The density of RGCs at iAON was significantly increased following MK-801 (2,805 ± 22 Rbps/mm², n = 16) compared to saline treatment (2,703 ± 22 Rbps/mm², n = 16, p = .023). However, in both groups RGC counts were significantly lower compared to healthy controls (iAON – saline p < .001; iAON – MK-801 p < .001; Figure 4b). Next, the optic nerve G/F-actin ratio was investigated and found to be significantly decreased in iAON following MK-801 (0.246 ± 0.039, n = 6) compared to saline treatment (0.822 ± 0.195, n = 6, p = .006). Most importantly, the G/F-actin ratio in the MK-801-treated group was indistinguishable from the ratio in healthy controls (0.429 ± 0.058, n = 10, p = .431), contrary to the saline-treated group where the G/F-actin ratio was significantly elevated compared to healthy controls (p = .035; Figure 4d). In addition, whereas saline-treated gelsolin levels during iAON were significantly reduced compared to the healthy values (saline, 0.426 ± 0.027 compared to GAPDH, n = 5; healthy, 0.770 ± 0.066, n = 9, p = .003; Figure 4f), delivery of MK-801 prevented this decrease (MK-801 0.697 ± 0.035, n = 4; p = .704 compared to healthy), being significantly higher than that seen following saline treatment (p = .042). The optic nerve fractin level was decreased in iAON - MK-801 (0.123 ± 0.025 compared to GAPDH, n = 5) versus iAON - saline treatment (0.324 ± 0.031, n = 4, p = .008). As for gelsolin and the G/F-actin ratio, fractin levels following MK-801 treatment were indistinguishable from healthy controls (0.175 ± 0.036, n = 8, p = .542), contrary to the iAON - saline-treated group where fractin was significantly higher (p = .030; Figure 4g). Therefore, the changes observed during AON (increases in the G/F-actin ratio and fractin levels, and decreases in gelsolin) could be reversed by MK-801 demonstrating these to be NMDA receptor-dependent events.

3.6 | MK-801 reduces retinal manganese-enhanced MRI signal intensities during iAON

In order to further explore the role of NMDA receptor signalling during AON, we used manganese-enhanced MRI to visualize retinal calcium entry pathways, as has been widely used to study the optic tract (Gadjanski et al., 2009; Yang et al., 2016). We have previously reported that retinal manganese-enhanced MRI signals are increased in iAON animals compared to healthy controls, indicating increased tissue calcium levels during the preclinical stage of AON (Hoffmann et al., 2013). In this study, whereas saline-treated iAON animals had increased manganese-enhanced MRI signal intensities compared to healthy (4.25 ± 1.00%, n = 10), this was reduced in iAON animals receiving MK-801 (~0.61 ± 0.208%, n = 8; p = .044, Figure 4j). Thus, it appears that increased NMDA receptor activation is responsible for the increased retinal calcium levels in iAON.

3.7 | MK-801 treatment improves visual acuity during iAON

Following MK-801 or saline treatment during iAON, no differences were seen in fVEP amplitudes between these two groups, nor upon comparison to healthy controls (healthy, 217 ± 19 µV, n = 8; iAON - saline, 175 ± 15 µV, n = 6; iAON – MK-801, 161 ± 11 µV, n = 6; p = .062; Figure 5b). Similarly fVEP latencies were also not significantly altered (Healthy, 36 ± 1 ms, n = 8; iAON – saline, 37 ± 2 ms, n = 6; iAON – MK-801, 38 ± 1 ms, n = 6; p = .458; Figure 5c).

As for the pVEPs, there was a significant difference in pVEP amplitudes between the three groups; with healthy values being the highest, followed by iAON – MK-801 and lastly by iAON – saline group (Table 2; Figure 5e). The same was true for the pVEP.
amplitudes (Table 2; Figure 5g), suggesting that MK-801 treatment reduced the decrease in visual acuity observed during AON.

4 | DISCUSSION

In this study we demonstrate that, during the induction phase of AON, a decrease in visual acuity and changes in the optic nerve cytoskeleton (as indicated by alterations in actin treadmilling and expression of its regulatory proteins) occur along with RGC degeneration which can be mimicked by intravitreal injection of glutamate. To test the hypothesis that NMDA receptor activation during the induction phase of AON, prior to inflammatory demyelination of the optic nerve (Sühs et al., 2014), leads to these changes, the potent NMDA receptor blocker MK-801 was applied by intravitreal injection during AON. Indeed, this resulted in a stabilization of optic nerve actin dynamics and a restoration of visual functions, along with neuroprotection of RGCs. This is further supported by the observations that the retinal calcium increase...
TABLE 2 pVEP and pERG amplitudes following pattern stimulation at the indicated spatial frequencies, measured in healthy rats, or rats with iAON treated either with saline or MK-801

| Spatial frequency (c/deg) | Noise  | 0.056 | 0.11 | 0.22 | 0.44 | 0.89 |
|--------------------------|--------|-------|------|------|------|------|
| pVEP                     |        |       |      |      |      |      |
| Healthy (n = 5)           | 30 ± 6 µV | 243 ± 13 µV | 209 ± 16 µV | 224 ± 21 µV | 177 ± 18 µV | 71 ± 16 µV |
| iAON – saline (n = 5)     | 28 ± 4 µV | 103 ± 16 µV | 106 ± 15 µV | 110 ± 12 µV | 96 ± 16 µV | 48 ± 9 µV |
| p compared to healthy     | <.001  |       |      |      |      |      |
| iAON – MK-801 (n = 5)     | 18 ± 3 µV | 183 ± 15 µV | 179 ± 28 µV | 119 ± 20 µV | 101 ± 10 µV | 60 ± 13 µV |
| p compared to healthy     | .04    | .071  | .003 |      |      |      |
| iAON – saline (n = 5)     | 5 ± 1 µV | 18 ± 3 µV | 15 ± 3 µV | 11 ± 1 µV | 11 ± 2 µV | 5 ± 1 µV |
| p compared to healthy     | .009   | .048  | .043 |      |      |      |
| pVEP                     | 3 ± 1 µV | 16 ± 3 µV | 18 ± 4 µV | 13 ± 1 µV | 11 ± 1 µV | 7 ± 1 µV |
| p compared to healthy     | .038   | .874  |      |      |      |      |
| iAON – saline (n = 5)     | 3 ± 1 µV | 16 ± 3 µV | 18 ± 4 µV | 13 ± 1 µV | 11 ± 1 µV | 7 ± 1 µV |
| p compared to healthy     | .01    | .003  |      |      |      |      |
| pVEP                     | 5 ± 1 µV | 18 ± 3 µV | 15 ± 3 µV | 11 ± 1 µV | 11 ± 2 µV | 5 ± 1 µV |
| p compared to healthy     | .009   | .048  | .043 |      |      |      |
| iAON – saline (n = 5)     | 3 ± 1 µV | 16 ± 3 µV | 18 ± 4 µV | 13 ± 1 µV | 11 ± 1 µV | 7 ± 1 µV |
| p compared to healthy     | .01    | .003  |      |      |      |      |
| pVEP                     | 5 ± 1 µV | 18 ± 3 µV | 15 ± 3 µV | 11 ± 1 µV | 11 ± 2 µV | 5 ± 1 µV |
| p compared to healthy     | .009   | .048  | .043 |      |      |      |
| iAON – MK-801 (n = 5)     | 3 ± 1 µV | 16 ± 3 µV | 18 ± 4 µV | 13 ± 1 µV | 11 ± 1 µV | 7 ± 1 µV |
| p compared to healthy     | .01    | .003  |      |      |      |      |

*p values are given comparing groups as indicated using an ANOVA with post hoc Tukey's test. For graphical representation of values, see Figure 5e,g. Abbreviations: iAON, induction autoimmune optic neuritis; pVEP, pattern visual evoked potential.

which occurs during this same period during AON (as indicated by manganese-enhanced MRI; Hoffmann et al., 2013), could be significantly reduced by MK-801 treatment (Figure 4j). Although MK-801 has also been suggested to interfere with monoamine uptake (Callado, Hopwood, Hancock, & Stamford, 2000) and to block other receptors such as the nicotinic acetylcholine receptor (Amador & Dani, 1991), since the AON changes could also be mimicked by glutamate injection, this would suggest that the NMDA receptor is the likely candidate.

The role of glutamate receptors in mediating neurodegeneration has been well described in many different pathophysiological conditions such as brain trauma, cerebral ischaemia and Alzheimer’s disease (Lipton, 2006). This is also true of different pathological conditions of the retina, such as diabetic retinopathy (Gu et al., 2014; Santiago et al., 2009; Santiago, Hughes, Kamphuis, Schlingemann, & Ambrósio, 2008), retinal vein thrombosis (Mosingler et al., 1991; Nivison-Smith, Kho, Acosta, & Kaloniatis, 2018) and glaucoma (Fu & Sretavan, 2012; Ju et al., 2015). In AON, we have previously demonstrated that systemic NMDA receptor blockade leads to RGC neuroprotection (Sühs et al., 2014), but this study goes further by delivering an NMDA receptor blocker by intravitreal injection, thus limiting the potential targets of the drug to the retina and therefore revealing more about the mechanisms of action. However, it should be noted that therapeutically, systemic administration of NMDA receptor blockers is more attractive than intravitreal delivery both in terms of avoiding potential discomfort and the ability to target multiple disease processes in parallel.

In addition, we demonstrate that the alterations we describe in early AON can be modelled through intravitreal delivery of glutamate. However, it remains unclear to what extent over-stimulation of NMDA receptors specifically located on RGCs contribute directly to their damage since many other retinal cells express NMDA receptor subunits throughout all layers of the retina (Fan, Xing, Zhong, Chen, & Shen, 2013). The involvement of other retinal cell types might also be inferred from the modest decrease in RGCs observed by Rbpms immunolabelling, despite the robust changes in both visual attributes and optic nerve axon cytoskeletal parameters. Thus, other cell types which are also sensitive to NMDA receptor stimulation might be involved. For example, brain endothelial cells are known to express NMDA receptors whose activation leads to an increase in blood–brain barrier permeability (Sharp et al., 2003; Vazana et al., 2016), and thus it is possible that retinal endothelial cells similarly express NMDA receptors that contribute to pathological mechanisms in AON. Intravitreal MK-801 treatment might therefore reverse blood-retinal barrier-induced damage by decreasing the influx of proinflammatory compounds such as fibrin (Davalos et al., 2012) or immunomodulatory cytokines. However, independent of the cellular retinal target of MK-801, its application resulted in a significant protection of RGCs and visual parameters associated with RGC function, as well as a stabilization of the actin cytoskeleton upstream in the optic nerve. In a similar vein, it is currently unclear whether the cytoskeletal changes observed are occurring in axons or glia of the optic nerve. Previously, we have seen evidence of cellular stress within the optic nerve at iAON occurring within both axons and oligodendrocytes (Stojic et al., 2019). Again, the influence of retinal NMDA receptors suggests that it might be axonal, but the contribution of other cell types cannot be ruled out.

Regarding our observations of disturbances in the actin cytoskeletal dynamics of the optic nerve during progression of AON, they may have implications for RGC degeneration since mounting evidence indicate that actin is at the same time both a sensor and a mediator of apoptosis (Desouza, Gunning, & Stehn, 2012; Franklin-Tong & Gourly, 2008). Both NMDA receptor stimulation (Halpain, Hipolito, & Saffer, 1998) and increased intracellular calcium...
(Furukawa, Smith-Swintosky, & Mattson, 1995; Neely & Gesemann, 1994) cause F-actin disassembly, leading to a destabilization of the actin network. Significant increases in intracellular calcium also activate calcium-dependent proteases such as calpains and caspasas, which further destabilize the actin cytoskeleton. One such protease activated by calcium is the actin-severing protein gelsolin (Yin & Stossel, 1979). At the same time, gelsolin is cleaved by calpain/caspase (Kothakota et al., 1997) which, as an anti-apoptotic factor (Harms et al., 2004), may leave the cell more vulnerable to NMDA receptor activity. In addition, fractin, a calpain/caspase-cleaved actin monomer product which accumulates following induction of apoptosis (Brown, Bailey, & Savill, 1997), also plays a functional role in apoptotic signalling (Schulz, Vogel, Mashima, Tsuruo, & Kriegstein, 2009). Both our findings of gelsolin and fractin changes are in agreement with our previous report of increased calpain activity during iAON (Hoffmann et al., 2013). Moreover, changes in actin network dynamics might be involved in the restructuring of Nodes of Ranvier following both intravitreal injections of glutamate and during early AON, as has been recently reported (Stojic et al., 2018). However, no changes in gelsolin were observed following injection of glutamate despite the protective effects of MK-801 in iAON, suggesting that a decrease in gelsolin levels might be dependent upon NMDA receptor activation in addition to other, currently unidentified, disease-related factors. Conversely, since gelsolin is protective against apoptosis (Harms et al., 2004), it is conceivable that following glutamate-mediated stress its expression might be up-regulated, but for reasons that are unclear (but might reflect the complexity of the disease scenario) this appears to have failed in AON.

Different visual tests can be used to provide insight into the function of different visual pathway components. In general, fVEPs are considered to reflect the activity of all cells in the pathway from photoreceptors to the visual cortex, with alterations closely correlating with optic nerve damage (Halliday, McDonald, & Mushin, 1972 and 1973). As such, it is a key parameter for optic neuritis with changes in latency and amplitude reflecting the extent of myelination and axonal injury respectively (You, Klistorner, Thie, & Graham, 2011). In our study, no changes in fVEPs were detected, which fits with the absence of inflammatory-driven demyelination or axonal loss during the induction phase of AON (Fairless et al., 2012). In contrast, the more sensitive measure of pVEPs is believed to reflect the signalling from RGCs to the visual cortex. The most specific technique, however, for assessing RGC function is through measurement of pERGs (Poricatti, 2015), and has been shown to correlate with the degree of RGC degeneration in AON (Hobom et al., 2004; Mayer et al., 2018; Meyer et al., 2001). Furthermore, this technique is highly sensitive being able to detect RGC dysfunction prior to the onset of death, as demonstrated in models of glaucoma (Poricatti, 2015).

The early degeneration that is seen in this model prior to the demyelination and inflammatory infiltration that characterize optic neuritis is in contrast to the classical concept that secondary degeneration of RGCs occurs as a result of axonal damage in the demyelinated optic nerve (Shindler, Ventura, Dutt, & Rostami, 2008). In this manner, the Brown Norway rat model also differs from the mouse model where significant RGC loss is not seen until after onset of optic nerve demyelination. An explanation for this is probably the early and robust antibody response observed in Brown Norway rats (Stefferl et al., 1999), which has been demonstrated to accumulate in the optic nerve head during the induction phase prior to immune cell infiltration and demyelination (Stojic et al., 2019), a time when subtle changes in the axo-glial junctions are also observed (Stojic et al., 2018). It is also interesting that in MS patients, RGC degeneration in the absence of optic nerve demyelination may also be occurring as evidenced by the observations of retinal nerve fibre layer thinning even in the absence of optic neuritis (Bock et al., 2010; Petzold et al., 2010; Talman et al., 2010). Thus, it is conceivable that subtle, subclinical optic nerve changes may initiate retinal degenerative processes that in turn affect visual performance in an NMDA receptor-mediated manner.

In conclusion, data presented in this study support the hypothesis that in early AON retinal events can lead to anterograde optic nerve changes in actin cytoskeletal dynamics, probably mediated by calcium accumulation and activation of actin-regulatory proteases. In addition, we provide further evidence that NMDA receptor modulation may prove a therapeutically relevant strategy for achieving retinal neuroprotection under autoimmune neuro-inflammatory conditions. Most importantly, MK-801, chosen in this study because of its high specificity and persistent inhibitory kinetics (Halliwell, Peters, & Lambert, 1989; Huettner & Bean, 1988; McKay et al., 2013), was able to protect against visual disturbances in iAON affected eyes. Even though MK-801 is known to have side effects precluding its therapeutic use such as interfering with long-term potentiation (Frankiewicz, Potier, Bashir, Collingridge, & Parsons, 1996) and induction of psychosis (Andine et al., 1999), if applied locally, as in this study was done by intravitreal injection, this blocker might be reconsidered in future therapeutic studies.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICTS OF INTEREST

The authors have no competing financial interests.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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