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NLRP3 Inflammasome in NMDA-induced Retinal Excitotoxicity

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Abstract

N-methyl-D-aspartate (NMDA)-induced excitotoxicity is an acute form of experimental retinal injury as a result of overactivation of glutamate receptors. NLRP3 (nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3) inflammasome, one of the most studied sensors of innate immunity, has been reported to play a critical role in retinal neurodegeneration with controversial implications regarding neuroprotection and cell death. Thus far, it has not been elucidated whether NMDA-mediated excitotoxicity can trigger NLRP3 inflammasome *in vivo*. Moreover, it is unknown if NLRP3 is beneficial or detrimental to NMDA-mediated retinal cell death. Here, we employed a murine model of NMDA-induced retinal excitotoxicity by administering 100 nmoles of NMDA intravitreally, which resulted in massive TUNEL⁺ (TdT-dUTP terminal nick-end labelling) cell death in all retinal layers and especially in retinal ganglion cells (RGCs) 24 hours post injection. NMDA insult in the retina can potentiate macrophage/microglia cell infiltration, prime the NLRP3 inflammasome in a transcription-dependent manner and induce the expression of interleukin-1 β (IL-1 β). However, despite NLRP3 inflammasome upregulation, systemic deletion of *Nlrp3* or *Casp1* (caspase-1) did not significantly alter the NMDA-induced, excitotoxicity-mediated TUNEL⁺ retinal cell death at 24 hours (acute phase). Similarly, the deletion of the two aforementioned genes did not alter the survival of the Brn3a⁺ (brain-specific homeobox/POU domain protein 3A) RGCs at 3- or 7-days post injection (long-term phase). Our results indicate that NMDA-mediated retinal excitotoxicity induces immune cell recruitment and NLRP3 inflammasome activity even though inflammasome-mediated neuroinflammation is not a leading contributing factor to cell death in this type of retinal injury.

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Author Contributions

DGV conceived the idea and supervised the study. PT and DGV were responsible for the experimental design. PT conducted experiments, analyzed the data and wrote the manuscript. PRB, XC and EIP conducted experiments. KK and JWM provided intellectual input. XC, BT and PB helped with the quantification and the statistical analysis. All authors have critically reviewed and edited the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

Keywords

excitotoxicity; Neuroinflammation; neurodegeneration; neuroprotection; microglia; immune cells

1. Introduction

Cell death of the inner retina and the retinal ganglion cells (RGCs) is a hallmark of a broad spectrum of retinal diseases, including optic neuropathies, diabetic retinopathy (DR) and glaucoma (Almasieh et al., 2012; Kern and Barber, 2008; Yu-Wai-Man et al., 2014). N-Methyl- D-aspartate receptors (NMDARs) are the primary glutamate receptors in the central nervous system (CNS) (Iacobucci and Popescu, 2017; Ozawa et al., 1998), including both the outer and inner primate retina with most robust expression in the latter (Shen et al., 2006). Excessive stimulation of NMDARs initiates a massive intracellular influx of Ca^{2+} that consequently leads to mitochondrial damage, oxidative stress and cell death, a process termed excitotoxicity (LUCAS and NEWHOUSE, 1957; Michaelis, 1998; Olney, 1969). N-Methyl-D-aspartate (NMDA), the agonist molecule of glutamate that binds selectively to NMDARs, has been extensively used in the experimental model of NMDA-induced retinal excitotoxicity, an acute form of retinal injury, that results in severe and rapid cell death of RGCs and the inner retina. However, although inhibition of NMDARs is neuroprotective in many *in vivo* models of glaucoma and optic neuropathies (Dong et al., 2008; Hare et al., 2001; Lambuk et al., 2017; Qiu et al., 2010; Russo et al., 2008; Seki and Lipton, 2008) and elevated levels of glutamate have been reported in the vitreous of patients with DR (Lu et al., 2007), clinical trials using memantine (the most common inhibitor of NMDARs) have failed to achieve neuroprotection (Lipton, 2006; Osborne, 2009). Therefore, understanding the mechanisms of NMDA-mediated cell death could be particularly beneficial for many neurological and retinal disorders in which excitotoxicity is considered to be contributory to their pathophysiology.

Neuroinflammation is a fundamental and tightly regulated mechanism of innate immunity in the CNS. It has been reported that NMDA-mediated injury activates resident microglia (Al-Gayyar et al., 2011) and Müller glial cells (El-Azab et al., 2014) and mediates CD45⁺ cell infiltration (Aoki et al., 2015) in the rodent retina, yet, the role of macrophage infiltration and consequent inflammasome activation has not been systematically studied. NLRP3 (nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3) inflammasome is a cytoplasmic molecular platform that upon activation leads to the cleavage of caspase-1 and subsequent secretion of the mature forms of the pro-inflammatory cytokines, interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) (Guo et al., 2015; Latz et al., 2013; Martinon et al., 2002; Srinivasula et al., 2002). NLRP3-mediated IL-1 β and IL-18 driven inflammation has been recently linked with retinal diseases, with controversial implications regarding cell death and survival (Doyle et al., 2015, 2014, 2012; Kataoka et al., 2015; Puyang et al., 2016; Viringipurampeer et al., 2016). It has been shown that NMDA-treated primary Müller cells express elevated levels of NLRP3, cleaved caspase-1 and IL-1 β (El-Azab et al., 2014), however, very little is known about the activation of NLRP3 inflammasome as a result of NMDA-induced cell death *in vivo*.

In the current study, we employed a mouse model of NMDA-induced retinal excitotoxicity and we examined if NMDA-mediated retinal injury can induce macrophage/microglia infiltration and upregulate the NLRP3 inflammasome activity. Additionally, we investigated whether *Nlrp3* or *Casp1* (caspase-1) systemic deletion can alter NMDA-induced TUNEL⁺ (TdT-dUTP terminal nick-end labeling) retinal cell death and Brn3a⁺ (brain-specific homeobox/POU domain protein 3A; cell-specific marker, expressed from the vast majority of RGCs' subsets) cell survival in the acute or in the long-term phase of the injury.

2. Materials and Methods

2.1. Animals

All animal experiments were conducted in accordance with the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in ophthalmic and vision research and were approved by the Animal Care Committee of Massachusetts Eye and Ear Infirmary. *Nlrp3*^{-/-} (B6.129S6-Nlrp3^{tm1Bhk/J}; Stock No: 021302) (Kovarova et al., 2012), *Casp1*^{-/-} (B6N.129S2-Casp1^{tm1Flv/J}; Stock No: 016621) (Kuida et al., 1995) and age-matched WT (C57BL/6J; Stock No: 000664) male mice (7–10 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and had free access to food and water in an air-conditioned room with a 12-h light/12-h dark cycle.

2.2. Experimental Model of N-Methyl-D-aspartic acid (NMDA) Excitotoxicity

Mice were anesthetized with an intraperitoneal injection of Avertin, 125 mg per kg of body weight of 2,2,2-tribromoethanol diluted in 2-methyl-2-butanol (#T48402; and #240486; respectively, MilliporeSigma, Burlington, MA, USA), followed by proparacaine eye drops (0.5% Proparacaine Hydrochloride Ophthalmic Solution; Sandoz Inc., Princeton, NY, USA) for topical anesthesia. Pupils were dilated by instillation of 5% phenylephrine and 0.5% tropicamide eye drops (Massachusetts Eye and Ear Infirmary Pharmacy, Boston, MA, USA). Consequently, a conjunctival incision was made temporally and a sclerotomy was created approximately 3–4 mm from the limbus. A 33-gauge needle (Hamilton Custom Needles: Length: 10.00mm/Point Style: 4/Angle: 20, #7803–05; Hamilton Company, Reno, NV, USA) connected to a 10- μ l syringe (Hamilton, 701RN SYR, #7635–01; Hamilton Company, Reno, NV, USA) was then inserted into the intravitreal cavity and 2 μ l of NMDA (100 nmoles, #M3262; MilliporeSigma, Burlington, MA, USA) or vehicle (phosphate buffer saline-PBS) were injected slowly into the intravitreal space. Special care was given to avoid hitting the lens. At the end of the procedure antibiotic ophthalmic ointment (Bacitracin Zinc Ointment; Fougere Pharmaceuticals Inc., Melville, NY, USA) was applied to prevent potential infection. Any eyes with cataract and/or hemorrhage were excluded from the analysis.

2.3. TUNEL (TdT-dUTP terminal nick-end labeling) assay

Mice were euthanized 24 hours post NMDA injury and eyes were enucleated, embedded in O.C.T. compound (Tissue Tek; Sakura Finetek, Torrance, CA, USA) and fresh-frozen at –80°C. Cryosections were cut at 10 μ m-thickness in the optic nerve in the sagittal plane on a cryostat (Leica CM1850; Leica Biosystems, Buffalo Grove, IL, USA). For each eye, 3–4 serial sections were taken with a step of 50 μ m. TUNEL was performed according to the

manufacturer's instructions (ApopTag Fluorescein In Situ Apoptosis Detection Kit #S7110; MilliporeSigma, Burlington, MA, USA) and sections were counterstained with TO-PRO-3 Iodide (642/661) (Life Technologies #T3605; Thermo Fisher Scientific, Waltham, MA, USA), mounted (Fluoromount-G; SouthernBiotech, Birmingham, AL, USA) and coverslipped. Images were taken using an upright AXIO Imager.M2 Zeiss fluorescence microscope under a 20x/0.8 lens (Zeiss PLAN-APOCHROMAT) and were analyzed using the ZEN software (Carl Zeiss Inc., Thornwood, NY, USA). TUNEL⁺ cells were quantified in all three nuclear layers of the retina (ganglion cell layer, inner nuclear layer and outer nuclear layer) using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) and TUNEL⁺ cell density was calculated as the ratio of TUNEL⁺ cells/mm of the length of the layer of interest. Random images were also counted by masked observers as described above to verify the results, while TUNEL⁺ cell density in outer and inner nuclear layer was also calculated using an automated ImageJ macro as previously described (Maidana et al., 2015).

2.4. Immunofluorescence

For the CD11b staining, animals were euthanized 24 hours post NMDA or PBS intravitreal injection, eyes were enucleated and cryosections were prepared as described above. Subsequently, sections were fixed with acetone, blocked with 5% milk and incubated overnight at 4°C with rat anti-CD11b polyclonal antibody (1:50, BD Pharmingen #550282; San Jose, CA, USA). Following the primary incubation, sections were stained with the secondary antibody (1:500, Alexa Fluor 488 goat anti-rat #A-11006; Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA), counterstained with DAPI and mounted as described above. Imaging was performed as described above under a 10x/0.3 lens (Zeiss EC-PLAN NEOFLUAR; Carl Zeiss Inc., Thornwood, NY, USA) and CD11b⁺ cells were quantified peripapillary and at the optic nerve head (ONH) per section using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

For the Brn3a staining, animals were euthanized 3- or 7-days post NMDA intravitreal injection, eyes were enucleated, anterior segment was removed and eyecups were fixed in 4% paraformaldehyde (PFA) at 4°C for 2 hours. Eyecups from untreated animals were also collected following the same procedure. Subsequently, eyecups were washed and the choroid and sclera were removed in order to isolate the retina tissue. Each retina was divided into 4 quadrants and was incubated with blocking buffer containing 0.5% bovine serum albumin (BSA) and 0.003% Triton-X-100 in PBS for 30 min at 4°C. Following, retinas were incubated overnight at 4°C with goat anti-Brn3a polyclonal antibody (1:350, Santa Cruz #sc-31984; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in blocking buffer containing also 2% normal donkey serum (NDS), washed and then incubated again with the secondary antibody (1:500, Alexa Fluor 647 donkey anti-goat #A-21447; Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) for 2 hours, rotating, at room temperature. Finally, retinas were washed again and mounted (retinal whole mount) with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Imaging was performed as described above under a 20x/0.8 lens (Zeiss PLAN-APOCHROMAT) and images were analyzed using the ZEN software (Carl Zeiss Inc., Thornwood, NY, USA). Brn3a⁺ cells were quantified in 3–4

areas (posterior, mid peripheral and far peripheral) in each of the 4 quadrants of the retina using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Quantification was cross-checked by a second masked observer.

2.5. Quantitative Real-Time RT-PCR

Mice were euthanized 12, 24, 72 or 168 hours post NMDA injury or 24 hours post PBS injection, eyes were enucleated and retinas were isolated and immersed in RNA stabilization reagent (RNA_{later} #76104; Qiagen, Valencia, CA, USA). RNA was isolated with RNeasy mini kit (#74104; Qiagen, Valencia, CA, USA) and cDNA was synthesized with SuperScript III First-Strand Synthesis System for RT-PCR using Oligo(dT) primers (#18080051; Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) according to manufacturers' protocols. PCR reaction was performed with TaqMan fast advanced master mix (#4444557; Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) in a QuantStudio5 Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA). TaqMan primers (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) were used to assess the mRNA expression levels of the following genes: *Nlrp3* (Mm00840904_m1), *Il18* (Mm00434225_m1), *Il1β* (Mm00434228_m1) and *Casp1* (Mm00438023_m1). The average cycle threshold (C_T) value was used in the analysis and the relative quantity of mRNA expression was calculated by C_T method normalized to *18s rRNA* as endogenous control (TaqMan Mm03928990_g1; Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

To assess protein expression in the whole eye, mice were euthanized 24 hours post NMDA or PBS intravitreal injection, eyes were enucleated and immersed in RIPA lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitors (cOmplete #11836170001; MilliporeSigma, Burlington, MA, USA). To assess the protein expression in the retina, mice were euthanized 24 hours post NMDA injury, cornea and lens were removed without enucleation and retina was eviscerated en bloc with as much vitreous as possible, and subsequently immersed in RIPA lysis buffer as described above. Tissues were homogenized, centrifuged and supernatants were collected to proceed to analysis for detection of total protein of Interleukin-1 β or MCP-1 by ELISA (#MLB00C and #MJE00 respectively; R&D Systems, Minneapolis, MN, USA) as described by the manufacturer.

2.7. Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by post analysis with Tukey HSD test (Fig. 2, 4 and 5) or Student's *t*-test (Fig.1 and Fig.3). Data were presented as mean $\pm$ SD. Statistical significance was determined at $P < 0.05$.

3. Results

3.1. NMDA-mediated excitotoxicity induces macrophage/microglia infiltration and MCP-1 production.

We first investigated whether NMDA-induced excitotoxicity potentiates inflammatory markers and immune cell infiltration in the retina. To answer our question, we checked if the monocyte chemoattractant protein-1 (MCP-1) was elevated in retinal lysates and if CD11b⁺ positive cells could be detected in retinal sections 24 hours after NMDA injury. As seen in Fig. 1A and B, the number of CD11b⁺ cells was robustly increased both peripapillary and at the optic nerve head (4 ± 1 cells/section vs. 25.9 ± 7.2 cells/section; *** $P < 0.001$ and 0 cells/section vs. 48.2 ± 15.7 cells/section; *** $P < 0.001$ respectively; $n = 10-14$). Similarly, MCP-1 levels were significantly elevated (6.23 ± 13.19 pg/ml vs. 113.9 ± 36.42 pg/ml; ** $P < 0.01$, $n = 4-5$, Fig. 1C) at 24 hours. Our data strongly suggest that NMDA-mediated excitotoxicity can induce immune cell infiltration in the retina.

3.2. NMDA-mediated excitotoxicity triggers NLRP3 inflammasome priming.

Given the immune cell infiltration observed, we further examined whether NMDA injury led to NLRP3 inflammasome upregulation. As seen in Fig. 2A-D, NMDA injury resulted in modest elevation of *Nlrp3* mRNA and a more notable elevation of *Casp1* mRNA. For both genes, induction of mRNA expression started after the first 12 hours post injury and peaked at day 7 (0.9-fold at 12 hours, 1.6-fold at 24 hours, 2-fold at 72 hours and 2.4-fold at 168 hours for *NLRP3*, $n = 4$, Fig. 2A and 1.3-fold at 12 hours, 6.5-fold at 24 hours, 8.5-fold at 72 hours and 18.8-fold at 168 hours; * $P < 0.05$ for *Casp1*, $n = 4$, Fig. 2B). Furthermore, as seen in Fig. 2C, NMDA insult led to a robust induction of *Il1 β* mRNA that started early at 12 hours (19-fold, $n = 3-4$) and peaked at 24 hours (38-fold; ** $P < 0.01$, $n = 3-4$). However, it was later (72 hours) significantly reduced almost to baseline (4.7-fold; * $P < 0.05$, $n = 3-4$) to have a secondary remarkable elevation at 168 hours (26.9-fold, * $P < 0.05$, $n = 3-4$). On the contrary, transcriptional expression of *Il18* remained stable before and after injury throughout the whole duration of 7 days that we examined ($n = 4$, Fig. 2D). Our data indicate that NMDA-mediated retinal excitotoxicity can trigger the NLRP3 inflammasome transcription-dependent priming, and particularly, in the NLRP3/IL-1 β axis.

3.3. NMDA-mediated excitotoxicity induces IL-1 β expression.

To examine whether NMDA-mediated injury not only primes the NLRP3 inflammasome but also induces IL-1 β production, we measured total protein levels of IL-1 β by ELISA in retinal and whole-eye lysates. Following NMDA injury, IL-1 β levels were significantly elevated in whole eye lysates (38.20 ± 16.72 pg/ml vs. 107.65 ± 40.67 pg/ml; * $P < 0.05$, $n = 3-4$, Fig. 3B) and showed a trend in retinal lysates ($n = 3-4$, Fig. 3A). These results suggest that NMDA-mediated injury induces upregulation of IL-1 β in the mouse eye.

3.4. Inhibition of NLRP3 or caspase-1 does not alter TUNEL⁺ cell death 24 hours after NMDA injury.

Depending on the retinal injury model, NLRP3 inflammasome activation can be either detrimental or neuroprotective (Doyle et al., 2015, 2014, 2012; Kataoka et al., 2015; Puyang

et al., 2016; Viringipurampeer et al., 2016). To address whether NLRP3 inflammasome upregulation is detrimental, protective or indifferent in NMDA-mediated, retinal cell death we administered NMDA intravitreally in wild type or *Nlrp3*^{-/-} or *Casp1*^{-/-} mice and assessed TUNEL⁺ cell death. As seen in Fig. 4A, NMDA induces robust cell death primarily in the ganglion cell layer, but also in a portion of the inner nuclear layer and in a small number of photoreceptors. Deletion of *Nlrp3* or *Casp1* did not lead to a significant change in cell death in any layer of the retina ($n = 11-15$, Fig. 4A and B), suggesting that NLRP3 inflammasome does not play a significant role in NMDA excitotoxicity-induced cell death in the acute phase of the injury.

3.5. Inhibition of NLRP3 or caspase-1 does not alter the survival of Brn3a⁺ retinal ganglion cells (RGCs) at 3- or 7-days post NMDA injury.

To further investigate if NLRP3 inflammasome upregulation has an effect on NMDA-mediated, excitotoxicity-induced retinal cell death overall, we administered NMDA in wild type or *Nlrp3*^{-/-} or *Casp1*^{-/-} mice and we examined the total number of Brn3a⁺ cells (Brn3a is a widely-accepted, cell-specific marker expressed from the vast majority of retinal ganglion cells' subsets) in untreated and NMDA-treated retinas 3- and 7-days post injury. A single injection of 100 nmoles NMDA resulted in almost 90% loss of Brn3a⁺ RGCs 3- and 7- days post injection ($n = 4-5$, Fig. 5A and B). However, deletion of *Nlrp3* or *Casp1* did not significantly alter the survival of the Brn3a⁺ cells at 3- or 7- days post injury ($n = 4-6$, Fig. 5A and B). Our results confirm our previous findings regarding the acute phase of the injury (24 hours) and implicate that inflammasome-mediated neuroinflammation is not a leading contributing factor to NMDA-mediated retinal cell death. Nonetheless, it is important to note that *Casp1* deletion led to a significant reduction of Brn3a⁺ RGCs in the untreated retina (WT: $654,1 \pm 29$; *Nlrp3*^{-/-}: $608,7 \pm 22,7$; and *Casp1*^{-/-}: $531,8 \pm 24,4$ Brn3a⁺ cells/retinal whole mount; ** $P < 0.01$ for WT vs. *Casp1*^{-/-} and * $P < 0.05$ for *Nlrp3*^{-/-} vs. *Casp1*^{-/-}, $n = 4-5$, Fig. 5B).

4. Discussion

In this study we demonstrated that N-methyl-D-aspartate (NMDA)-induced retinal excitotoxicity can trigger macrophage/microglia recruitment and can induce NLRP3 (nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3) inflammasome transcription-dependent priming and interleukin-1 β (IL-1 β) production.

Macrophage/microglia infiltration has been reported to play a pivotal role in many retinal degenerative diseases as a component of a strong interplay between cell death/survival, inflammation and clearance (Kataoka et al., 2015; London et al., 2011; H Matsumoto et al., 2014; H. Matsumoto et al., 2014; Murakami et al., 2014; Okunuki et al., 2018; Trichonas et al., 2010; Tsoka et al., 2018). We and others, have previously shown that monocyte chemoattractant protein 1 (MCP-1) initiates the inflammatory reaction following retinal injury and that elevated MCP-1 levels result in increased macrophage/microglia recruitment (H Matsumoto et al., 2014; Matsumoto et al., 2015; Nakazawa et al., 2007). Indeed, in our study, MCP-1 levels were significantly elevated 24 hours after NMDA injury similarly to

CD11b⁺ cells, both peripapillary and at the optic nerve head (ONH), suggesting that NMDA insult results in upregulation of MCP-1, which in turn leads to increased infiltration of macrophages/microglia into the retina. In the NMDA-injured eyes, accumulation of CD11b⁺ cells was primarily in the vitreous and the ONH, contrary to the vehicle-treated eyes in which there were only a few CD11b⁺ cells throughout the different retinal layers and none in the vitreous or ONH, suggesting that the latter is the primary route of immune cell infiltration in that type of injury. In line with our results, a previous study has reported increased CD45⁺ cell infiltration in the retina following administration of 200 nmoles of NMDA (Aoki et al., 2015), while several other studies have shown that NMDA-mediated retinal cell death induces the activation of resident microglia (Al-Gayyar et al., 2011; Aoki et al., 2015) and Müller cells (Al-Gayyar et al., 2011; El-Azab et al., 2014).

Canonical activation of the NLRP3 inflammasome requires two different signals, known as 'signal 1' and 'signal 2' with the first being responsible for priming and the second being responsible for activation. During priming, transcriptional and post-translational mechanisms must be activated to achieve a suitable level of NLRP3 inflammasome genes' expression in the cytosol (Bauernfeind et al., 2009; Juliana et al., 2012; Py et al., 2013). In our study, *Nlrp3* and *Casp1* (caspase-1) mRNA levels were modestly raised following NMDA injury to an extent somewhat similar to that reported in previous works that investigated the induction of transcriptional-dependent priming as a result of experimental retinal degeneration in rodents (Liu et al., 2013; Mohamed et al., 2014; Rivera et al., 2013). In particular, elevation of transcriptional expression of *Nlrp3* and *Casp1* started after the first 12 hours post injury and peaked on day 7. *Il1β* (interleukin- 1β) mRNA followed a robust increase post NMDA injury that started early at 12 hours, peaked at 24 hours, was significantly reduced later on at 72 hours and was again notably elevated on day 7. Interestingly, the elevation that we observed, was greater than that observed in other models of retinal injury (Devi et al., 2012; Liu et al., 2013; Rivera et al., 2013) and followed a biphasic expression pattern. The results of the three afore- mentioned genes indicate that inflammation remains active in the retina even 7 days after NMDA injury. Nonetheless, we were not able to detect any differences in the levels of *Il18* (interleukin-18) mRNA following NMDA injury on any time point that we examined, perhaps due to higher baseline levels of expression of IL-18 compared to IL- 1β (Puren et al., 1999), or, to differential expression of these two inflammatory cytokines. The biphasic robust elevation of IL-1β in combination with the lack of transcriptional upregulation of IL-18 strongly suggest that NMDA-mediated insult in the retina triggers only the IL-1β axis of the inflammasome-mediated neuroinflammation.

To further investigate if NMDA-induced excitotoxicity can lead to increased production of the end-product of the NLRP3 inflammasome, we measured the total protein levels of IL-1β. Given the technical limitations of sampling/isolating the mouse vitreous, which does not exceed 7–10 μl in total volume, we decided to check total levels of IL-1β in both retina- and whole eye- samples after NMDA injury. The mean concentration of total IL- 1β in the retina was lower than that in the whole eye, suggesting that the vast majority of secreted IL-1β is located in the vitreous, and thus, its main source is the infiltrating immune cells and not the injured retinal neurons. However, more extensive studies are necessary in order to elucidate the mechanism of NLRP3 priming and activation by NDMA in the retina. Furthermore,

IL-1 β production is the final downstream molecule of other inflammasomes (canonical and noncanonical) (Guo et al., 2015) so further research is needed in order to clarify if IL-1 β production in NMDA-induced retinal excitotoxicity is mediated by NLRP3. Finally, we were not able to detect cleaved IL-1 β by western blot (WB), most likely due to the very small number of immune cells proportionally to the total number of cells in the eye (we performed the WB analysis in whole eye samples in order to include the vitreous).

Prolonged stimulation of NMDARs allows massive intracellular influx of Ca²⁺, which in turn activates calpain-1 and neuronal nitric oxide synthase (nNOS) that lead to necrosis and/or apoptosis depending on the severeness of the insult and the mitochondrial function (Ankarcrona et al., 1995; Fujikawa, 2015). Furthermore, NMDA-induced retinal excitotoxicity upregulates tumor necrosis factor (TNF)-alpha expression and downstream signalling of its death receptor TNFR (Ishimaru et al., 2017; Lebrun-Julien et al., 2009; Maekawa et al., 2017) and has been extensively correlated with endoplasmic reticulum (ER) stress (Awai et al., 2006; Maekawa et al., 2017; Shimazawa et al., 2007). However, deficiency in proteins downstream of the above-mentioned pathways did not offer neuroprotection in RGCs according to a study published last year (Fahrenthold et al., 2018). Recently, high-mobility group box-1 (HMGB1), a damage-associated molecular pattern (DAMP) molecule secreted only from necrotic cells, has also been found to be involved in NMDA-mediated retinal cell death (Sakamoto et al., 2015) and to induce nuclear factor kappa B (NF- κ B) activation and NF- κ B-mediated excitotoxicity (Sakamoto et al., 2017).

Taken into consideration that intrinsic and extrinsic cell death signalling as well as programmed necrosis have been strongly correlated with inflammasome upregulation (Guo et al., 2015; Kataoka et al., 2015) and that the role of inflammasomes in excitotoxicity has not been fully investigated, we wanted to examine the role of NLRP3 inflammasome in NMDA-induced, excitotoxicity-mediated retinal cell death. The exact role of NLRP3 inflammasome is not yet entirely understood and remains a controversial subject (Guo et al., 2015; Kosmidou et al., 2018; Ozaki et al., 2015). It has been reported to play a dual role in retinal injury; inhibition of NLRP3 has led to reduced TUNEL⁺ cell death in a mouse model of retinal detachment (Kataoka et al., 2015) and to increased cone survival in a mouse model of inherited retinal degeneration (Viringipurampeer et al., 2016). Moreover, deficiency of *Nlrp3* leads to significant delay of retinal ganglion cells' (RGCs) loss in an experimental model of optic neuropathy (Puyang et al., 2016). On the contrary, NLRP3-mediated IL-18 induction was neuroprotective in experimental models of age-related macular degeneration in rodents and non-human primates (Doyle et al., 2015, 2014, 2012).

In our study, genetic deletions of *Nlrp3* or *Casp1* did not significantly alter TUNEL⁺ cell death or the survival of Brn3a⁺ RGCs (Brn3a is a widely-accepted cell-specific marker for RGCs) (Nadal-Nicolás et al., 2009) at 24 hours or 7 days post injury respectively, indicating that pyroptosis and/or inflammasome-mediated neuroinflammation is not a dominant contributing factor to cell death in this type of injury, or that NMDA insult at doses commonly used by many investigators (100 nmoles) is so irreversibly aggressive to the retinal neurons that cell death can be induced even in the absence of *Nlrp3* or *Casp1*. Consistent with our results, inhibition of NLRP3 inflammasome failed to prevent neuronal loss in models of autoimmune/neurodegenerative diseases under intense stimuli (Inoue et al.,

2016, 2012). However, it is important to note that 100 nmoles of intravitreal administration of NMDA results to massive cell death in the retina, contrary to lower doses (e.g. 40 nmoles) and that perhaps *Nlrp3* or *Casp1* deficiency could lead to protection in a mild NMDA-mediated insult. Nonetheless, systemic deletion of *Casp1* resulted in significantly fewer Brn3a⁺ cells in the untreated retinas compare to wild type and/or *Nlrp3*^{-/-} mice. This finding, in combination with the recently confirmed incidental deficiency of *Casp4* (caspase 4) in *Casp1*^{-/-} mice (according to Jackson Laboratories, Bar Harbor, ME, USA), might indicate that deletion of these two genes play a detrimental role towards maturation and survival of RGCs. On the other hand, somewhat interestingly, deletion of *Nlrp3* or *Casp1* led to a significant reduction of immune cell infiltration ($n = 7-10$).

Previous studies have shown that in other models of retinal degenerations (e.g. retinal detachment or double-stranded RNA-induced retinal degeneration) when one cell death pathway is inhibited, retinal neurons switch towards another pathway of cell death (Murakami et al., 2014; Trichonas et al., 2010). NLRP3 inflammasome complex has been highly associated with receptor interacting proteins (RIP) that regulate necroptosis, which are also considered molecular switches for necrosis and inflammation (Moriwaki and Chan, 2013; Newton, 2015). Furthermore, in a study published in 2015, inflammasome pathway (NLRP3-mediated) and necroptosis pathway (RIP3-mediated) were both active in an experimental model of retinal detachment (Kataoka et al., 2015). It has been well established that NMDA-induced excitotoxicity results in programmed necrosis (Ankarcrona et al., 1995; Fujikawa, 2015), while recently, NMDA insult has also been associated with autophagy (Roux et al., 2015; Wang et al., 2014), which has a strong interplay with NLRP3 inflammasome (Zhong et al., 2016). Taken all into account, it is very possible that when inflammasome complex and consequently pyroptosis are blocked, regulated necrosis and/or different types of cell death could be augmented in NMDA-mediated retinal excitotoxicity and should be part of subsequent studies.

Finally, we also highlight that NMDA injury is not restricted to the RGCs and the inner retina and also affects the outer retina. It has been well established that N-Methyl-D-aspartate receptors (NMDARs) are robustly expressed in the RGCs as well as in some subsets of amacrine cells (Shen et al., 2006). However, a few studies show evidence that photoreceptors, and specifically cones, might express NMDARs as well (Fletcher et al., 2000; Kalloniatis et al., 2004). Nonetheless, NMDARs' expression in photoreceptors has been so far only poorly understood, and thus we do not know if photoreceptor cell death is due to overactivation of NMDARs that are expressed in those cells or is secondary to the released harmful endogenous products of the NMDARs⁺ dying cells.

In summary, our study shows for the first time that NMDA-mediated excitotoxic insult in the mouse retina can trigger NLRP3 inflammasome activity and result in upregulation of transcription-dependent priming and in increased production of IL-1 β . We further show that inhibition of NLRP3 or caspase-1 does not alter the NMDA-mediated, excitotoxicity-induced cell death, assessed by TUNEL or Brn3a⁺ cell survival, indicating that inflammasomes are not a key player in the acute or in the long-term phase of NMDA-induced cell death in retinal neurons. However, given that inflammasome-mediated

neuroinflammation is still active 7 days following injury, NLRP3 inflammasome might still have a different role in this type of injury.

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- NMDA excitotoxicity induces immune cell infiltration in the retina.
- NMDA retinal excitotoxicity triggers NLRP3 inflammasome priming.
- *Nlrp3*^{-/-} or *Casp1*^{-/-} do not have major impact on overall neuronal loss after NDMA.

Figure 1. NMDA-mediated excitotoxicity induces macrophage/microglia infiltration and MCP-1 production.

(A) Representative pictures of CD11b (red) and DAPI (blue) staining at 24 hours post NMDA injury. Areas marked with solid line depict the two parts of the retina in which CD11b⁺ cells were counted peripapillary. Area marked with scattered line depicts the area of the optic nerve head (ONH) in which CD11b⁺ cells were also counted. (B) Quantification of CD11b⁺ cells/section ($n=10-14$). (C) ELISA to detect MCP-1 levels 24 hours post NMDA injury ($n=4-5$). The graphs show mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$. NMDA, N-methyl-D-aspartate; PBS, phosphate buffer saline; MCP-1, monocyte chemoattractant protein-1; ONH, optic nerve head.

Figure 2. NMDA-mediated excitotoxicity triggers NLRP3 inflammasome priming.

(A-D) Relative expression of mRNA levels of (A) *Nlrp3*, (B) *Casp1*, (C) *Il1β* and (D) *Il18* of untreated retinas (intravitreal injection of vehicle, $n = 4-5$) and NMDA-treated retinas (intravitreal injection of 100 nmoles of NMDA, $n = 3-5$) 12, 24, 72 and 168 hours post injury. The graphs show mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. NMDA, N-methyl-D- aspartate; PBS, phosphate buffer saline; *Nlrp3*, nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3; *Casp1*, caspase-1; *Il1β*, interleukin-1 β ; *Il18*, interleukin-18.

Figure 3. NMDA-mediated excitotoxicity induces IL-1 β production.

The graphs show IL-1 β levels detected by ELISA 24 hours post intravitreal injection of control (PBS- treated) and injured (NMDA 100 nmoles-treated) eyes in (A) retinal ($n = 3-4$) and in (B) whole eye ($n = 3-4$) lysates. The graphs show mean $\pm$ SD. * $P < 0.05$. NMDA, N-methyl- D-aspartate; PBS, phosphate buffer saline; IL-1 β , interleukin-1 β .

Figure 4. Inhibition of NLRP3 or caspase-1 does not alter TUNEL⁺ cell death 24 hours post NMDA injury.

(A) TUNEL (green) and TO-PRO-3 (blue) staining of mouse retinal sections from NMDA (100 nmoles)-treated eyes 24 hours post injury in WT, *Nlrp3*^{-/-} and *Casp1*^{-/-} animals. (B) Quantification of TUNEL⁺ cells/mm per retinal layer 24 hours post injury in WT, *Nlrp3*^{-/-} and *Casp1*^{-/-} animals ($n = 11-15$). Red color dots represent the number of TUNEL⁺ cells/layer of the pictures depicted above. NMDA, N- methyl-D-aspartate; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; WT, wild type; *Nlrp3*^{-/-}, nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3 (NLRP3) knockout; *Casp1*^{-/-}, caspase-1 knockout.

Figure 5. Inhibition of NLRP3 or caspase-1 does not alter the survival of Brn3a⁺ retinal ganglion cells (RGCs) at 3- or 7-days post NMDA injury.

(A) Representative pictures of Brn3a (red) staining of retinal whole-mounts of untreated and NMDA-treated eyes 7 days post injury in WT, *Nlrp3*^{-/-} and *Casp1*^{-/-} animals ($n = 4-6$). (B) Quantification of Brn3a⁺ RGCs/retinal whole-mount in untreated retinas and in NMDA-treated retinas 3- and 7-days post injury in WT, *Nlrp3*^{-/-} and *Casp1*^{-/-} animals ($n = 4-6$). The graphs show mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. NMDA, N-methyl-D-aspartate; RGCs, retinal ganglion cells; WT, wild type; *Nlrp3*^{-/-}, nucleotide-binding domain, leucine-rich-repeat containing family, pyrin domain containing-3 (NLRP3) knockout; *Casp1*^{-/-}, caspase-1 knockout; Brn3a, brain-specific homeobox/POU domain protein 3A.