

1 **Comparison of ion channel inhibitor combinations for limiting secondary degeneration**
2 **following partial optic nerve transection**

3 Lillian M. Toomey¹, Carole A. Bartlett¹, Maimuna Majimbi², Gopana Gopalasingam¹,
4 Jennifer Rodger¹ and Melinda Fitzgerald^{1,2,3}

5
6 *¹Experimental and Regenerative Neurosciences, School of Biological Sciences, The*
7 *University of Western Australia, 35 Stirling Hwy, Perth 6009 Western Australia, Australia.*

8 *²Curtin Health Innovation Research Institute, Curtin University and ³Perron Institute for*
9 *Neurological and Translational Science, Sarich Neuroscience Research Institute, 8 Verdun*
10 *St, Nedlands 6009 Western Australia, Australia*

11
12
13
14 Correspondence:

15 name: Melinda Fitzgerald

16 phone: +61 467 729 300

17 mail: lindy.fitzgerald@curtin.edu.au

18
19 Word count: 43,114 + 4 Figures = 50,314

20
21 Funding sources: MF is supported by an NHMRC Career Development Fellowship (APP1087114).

23 **Abstract**

24 Following neurotrauma, secondary degeneration of neurons and glia adjacent to the injury
25 leads to further functional loss. A combination of ion channel inhibitors (lomerizine + oxATP
26 + YM872) has been shown to be effective at limiting structural and functional loss due to
27 secondary degeneration. Here we assess efficacy of the combination where oxATP is
28 replaced with Brilliant Blue G (BBG), a more clinically applicable P2X₇ receptor inhibitor.
29 Partial optic nerve transection was used to model secondary degeneration in adult female rats.
30 Animals were treated with combinations of lomerizine + YM872 + oxATP or lomerizine +
31 YM872 + BBG, delivered *via* osmotic mini pump directly to the injury site. Outcomes
32 assessed were Iba1+ and ED1+ microglia and macrophages, oligodendroglial cell numbers,
33 node/paranode structure and visual function using the optokinetic nystagmus test. The
34 lomerizine + BBG + YM872 combination was at least as effective at the tested
35 concentrations as the lomerizine + oxATP + YM872 combination at preserving
36 node/paranode structure and visual function when delivered locally. However, neither ion
37 channel inhibitor combination significantly improved microglial/macrophage nor
38 oligodendroglial numbers compared to vehicle treated controls. In conclusion, a locally
39 delivered combination of ion channel inhibitors incorporating lomerizine + BBG + YM872 is
40 at least as effective at limiting secondary degeneration following partial injury to the optic
41 nerve as the combination incorporating oxATP.

42

43

44 **Keywords:** secondary degeneration; neurotrauma; ion channel inhibitor; myelin; visual
45 function

46

47 **Introduction**

48 Following neurotrauma, a series of metabolic and structural changes are propagated in
49 initially undamaged tissue, associated with increased intracellular Ca^{2+} , oxidative stress and
50 apoptotic cell death of neurons and glia (Dong et al. 2009). Since the initial insult is often
51 unavoidable, treatments for functional recovery after neurotrauma focus heavily on limiting
52 this secondary damage (Doan et al. 2016). However, despite extensive research, effective
53 pharmacotherapeutic treatments for secondary degeneration following neurotrauma are
54 limited (Kwon et al. 2011). In order to successfully limit secondary degeneration following
55 neurotrauma, it is important to test efficacy of treatments in appropriate animal models of
56 injury. Partial optic nerve transection is an established and useful model for investigating
57 secondary degeneration, where the dorsal optic nerve of adult rats is partially transected,
58 allowing for spatial separation between the primary and subsequent secondary degeneration
59 (Levkovitch-Verbin et al. 2003; Blair et al. 2005). The model has been further characterised
60 and employed to assess efficacy of pharmacotherapeutics for secondary degeneration,
61 delivered directly to the injury site using osmotic mini-pumps (Fitzgerald et al. 2009a;
62 Fitzgerald et al. 2009b; Savigni et al. 2013; O'Hare Doig et al. 2017).

63 Secondary degeneration is characterised by a myriad of reactive metabolic pathways,
64 including inflammation, excitotoxicity, mitochondrial dysfunction and oxidative stress,
65 associated with structural deficits, dysmyelination and apoptotic cell death (Tymianski and
66 Charles 1996; Dong et al. 2009; Maxwell 2013). Ca^{2+} overload is considered to be a major
67 trigger for the toxic mechanisms of secondary degeneration (Farooqui et al. 2008). Using the
68 partial optic nerve transection model, we have previously demonstrated that a locally
69 delivered combinatorial treatment strategy to limit excess Ca^{2+} influx through voltage gated
70 calcium channels, P2X_7 receptors and Ca^{2+} permeable AMPA receptors with lomerizine,
71 oxATP and YM872 respectively, reduced myelin decompaction, preserved node/paranode

72 structure and visual function (Savigni et al. 2013). Acute outcomes indicated that early
73 preservation of node/paranode structure and OPC numbers was associated with longer term
74 preservation of visual function (O'Hare Doig et al. 2017).

75 Following mild traumatic brain injury, it is currently unclear as to whether there is a blood
76 brain barrier breach, with studies reporting varying degrees of compromise of blood brain
77 barrier integrity following injury (Deford et al. 2002; Tomkins et al. 2011; Zetterberg et al.
78 2013). Therefore, pharmacotherapies designed to treat all but the most severe cases of
79 neurotrauma, need to be able to travel across the closed blood brain barrier following
80 systemic delivery. oxATP does not appear to be able to cross the blood brain barrier (Peng et
81 al. 2009). Thus, for clinical applicability of the lomerizine, oxATP and YM872 combination,
82 oxATP needs to be substituted with a blood brain barrier permeable P2X₇ receptor
83 antagonist. Here we introduce an alternative P2X₇ receptor inhibitor Brilliant Blue G (BBG)
84 to the combination. BBG has previously shown therapeutic effects following neurotrauma
85 (Peng et al. 2009; Kimbler et al. 2012; Wang et al. 2015), and importantly, can cross the
86 closed blood brain barrier (Wong et al. 2011). This study compared the efficacy of the ion
87 channel inhibitor combination of lomerizine + BBG + YM872 to a combination with
88 lomerizine + oxATP + YM872 for limiting secondary degeneration and restoring function
89 following partial optic nerve transection.

90

91 **Methods**

92 *Animals and study design*

93 Thirty-eight adult, female PVG rats were obtained from the Animal Resource Centre in
94 Murdoch, Western Australia. The animals were housed under 12-hour light/dark cycles with
95 ad libitum access to food and water. All procedures were approved by the University of

96 Western Australia Animal Ethics Committee (approval number RA3/100/1485) and were in
97 accordance with the National Health and Medical Research Council (NHMRC) of Australia
98 Code of Practice for use of Animals for Scientific Purposes. The animals were divided into
99 four experimental groups, a sham injured, vehicle treated group (n = 8); an injured, vehicle
100 treated group (n = 10); an injured, lomerizine + oxATP + YM872 treated group (n = 10); and
101 an injured, lomerizine + BBG + YM872 treated group (n = 10); with the sham group serving
102 as an uninjured, vehicle treated control.

103 *Surgical procedures*

104 Partial optic nerve transection (day 1) and left eyelid suturing (day 3) were performed as
105 previously described (Fitzgerald et al. 2009a), under Ketamine (Ketamil, 50mg/kg, Troy
106 Laboratories) and Xylazine (Ilium Xylazil, 10mg/kg, Troy Laboratories) anaesthesia
107 administered intraperitoneally. In brief, for the partial transection surgery: the skin overlying
108 the skull behind the right eye was incised. The optic nerve was accessed and the nerve
109 parenchyma exposed by making a longitudinal cut in the sheath using fine iridectomy
110 scissors. About 1mm behind the right eye, the dorsal aspect of the optic nerve was partially
111 lesioned to a depth of approximately 200µm with a diamond radial keratotomy knife
112 (Geuder); the depth determined by the protrusion of the blade beyond the surrounding guard.
113 Sham injury included all procedures except the cut in the sheath and the partial optic nerve
114 lesion. Surgical implantation of Alzet osmotic mini-pumps was performed as described
115 (Savigni et al. 2013). Immediately following surgery, subcutaneous injections of analgesia
116 (2.8mg/kg carprofen, Norbrook) and 1mL sterile phosphate buffered saline (PBS) were
117 administered.

118 *Treatments*

119 Lomerizine (30mg/kg, LKT Labs©) was orally administered in butter vehicle twice daily 8
120 hours apart, until end of experiment, commencing once animals were ambulatory following
121 surgery as previously described (Fitzgerald et al. 2009a). oxATP (1mM), BBG (540µM) and
122 YM872 (240µM) were delivered *via* osmotic mini-pump at 0.5µL/h in PBS vehicle.
123 Concentrations employed for oxATP and YM872 were consistent with our previous studies
124 where efficacy was demonstrated (Savigni et al. 2013), and the BBG dose was chosen with
125 reference to the literature describing efficacy of BBG and YM872 in related models
126 (Takahashi et al. 2002; Diaz-Hernandez et al. 2012; Cervetto et al. 2013). The sham injured
127 and partial optic nerve transection injured, vehicle treated experimental groups both received
128 PBS *via* osmotic mini-pump and butter orally, administered as described for the inhibitor
129 treated groups.

130 ***Behavioural assessment***

131 On day 3, the animals were anaesthetised as described above and their uninjured left eyelids
132 sutured shut. The optokinetic nystagmus assessment of visual function was performed on day
133 4 for all animals, in accordance with established procedures (Fitzgerald et al. 2010b).
134 Animals were videoed and number of responses per unit time engaged in the task was
135 determined by a single investigator blinded to animal identity. Note that due to a procedural
136 error, n = 5 for the injured, vehicle treated group. Responses were categorised as either
137 smooth pursuits or fast resets. Smooth pursuits are characterised as an elongated head
138 rotation tracking the stripes, and fast resets as a rapid, realigning head movement; both
139 elements are an indication of visual ability of the animal (Abdeljalil et al. 2005).

140 ***Tissue processing and immunohistochemistry***

141 Immediately following behavioural assessment, rats were euthanised with pentobarbitone
142 sodium (160mg/kg, Delvet), transcardially perfused with 0.9% saline, followed by 4%

143 paraformaldehyde (Sigma-Aldrich) in 0.1M PBS. Optic nerves were dissected and fixation
144 continued overnight by immersion in 4% paraformaldehyde. Tissue was transferred into 15%
145 sucrose (Chem Supply) in PBS, then cryosectioned in longitudinal orientation at a thickness
146 of 14 μ m and collected onto Superfrost Plus glass microscope slides. Immunohistochemistry
147 was conducted in accordance with established procedures(Fitzgerald et al. 2010a) using
148 primary antibodies recognising: microglial activation markers Iba1 (1:500; Abcam, goat
149 Ab5076) and ED1 (1:500; Merck Millipore, mouse MAB1435); oligodendroglial indicators
150 oligodendrocyte transcription factor 2 (Olig2; 1:500; R&D Systems, goat AF2418) and
151 platelet-derived growth factor alpha receptor (PDGF α R; 1:500; Abcam Ab96806); and for
152 paranode and node of Ranvier structures Caspr (1:500; Abcam, rabbit Ab34151), and β -III
153 tubulin (1:500; Merck Millipore, mouse MAB1637). Antibodies were diluted in PBS
154 containing 0.2% TritonTM X-100 and 5% normal donkey serum. Secondary antibodies were
155 Alexa Flour 488 or 555 (1:400; Thermo Fisher ScientificTM), together with Hoechst 3342
156 (1:1000; Thermo Fisher ScientificTM) diluted in PBS containing 0.2% TritonTM X-100.
157 Finally, the sections were mounted and cover slipped using Fluoromount-G (Thermo Fisher
158 Scientific).

159 *Imaging and Analysis*

160 The ventral optic nerve directly below the site of injury was visualised, with one field of view
161 from one section per animal imaged for each outcome measure. The slides were viewed using
162 either a Nikon Ni-E confocal fluorescence microscope (Nikon Corporation) or a Nikon
163 Eclipse Ti inverted microscope. A series of 13 optical images were taken at 0.5 μ m
164 increments along the z-axis, and deconvoluted using Nikon Elements AT software. Imaging
165 for each outcome measure was performed in a single sitting with consistent capture settings.
166 All image analysis was performed on Fiji image processing software (NIH) by a single
167 investigator blinded to section identity. Due to poor fixation and tissue processing in a few

168 animals, numbers of animals per group analysed for immunohistochemistry outcomes were:
169 sham injured, vehicle treated group (n = 8); injured, vehicle treated group (n = 10); injured,
170 lomerizine + oxATP + YM872 treated group (n = 8); and injured, lomerizine + BBG +
171 YM872 treated group (n =9).

172 Total numbers of Iba1+ resident reactive microglia, ED1+ activated microglia/macrophages,
173 Olig2+ oligodendroglia and Olig2+/PDGF α R+ oligodendrocyte precursor cells (OPCs) were
174 counted within a region of interest in a 20x image of the ventral nerve directly beneath the
175 primary injury site and expressed as the mean number of cells/mm². For node/paranode
176 analyses, a single 60x z-stack image per animal was divided into a 3 x 3 grid and all
177 complexes with clearly defined Caspr immunostaining in a single randomly selected grid
178 square assessed; at least 30 node/paranode complexes were analysed per animal. Outcome
179 measures were the length of the paranodal gap, defined as the distance between two Caspr+
180 areas; paranode length, as defined by the length of Caspr+ areas; and the percentages of
181 atypical nodal complexes, as previously described (Szymanski et al. 2013).

182 ***Statistics***

183 Results were analysed using IBM SPSS software. Outliers were detected using the Tukey
184 Outlier Detection Model, whereby data that were greater than 1.5 interquartile ranges outside
185 of the first and third quartiles were considered outliers and removed from the data set. No
186 more than two outliers were removed per treatment group for each outcome measure, details
187 provided in figure legends. A single one-way ANOVA encompassing all four treatment
188 groups were performed for each outcome measure. Normality was assumed, Levene's test
189 was used to assess homogeneity of variances for each data set ($\alpha = 0.05$). If Levene's test
190 showed equal variance, the Tukey post-hoc was used; for unequal variance, the Games-
191 Howell post-hoc was applied; both used $p \leq 0.05$ to indicate statistical significance.

192

193 **Results**

194 *Effects of ion channel inhibitor combinations on microglia and macrophages*

195 Numbers of inflammatory cells in ventral optic nerve vulnerable to secondary degeneration
196 were quantified using Iba1 for resident microglia, ED1 for infiltrating
197 microglia/macrophages, and colocalised Iba1+/ED1+ for infiltrating microglial cells (Wu et
198 al. 2005). The numbers of Iba1+ activated resident microglia were different in the various
199 treatment groups (Figure 1a; $F = 3.483$, $DF = 3$, $p = 0.028$), with the only statistically
200 significant difference an increase in the number of Iba1+ cells when animals were treated
201 with lomerizine + BBG + YM872 compared to the sham injured, vehicle treated group ($p =$
202 0.029). There was a trend towards increased Iba1+ cells with injury when comparing the
203 injured, vehicle treated group with the sham injured, vehicle treated group ($p = 0.071$). There
204 was no significant difference between the two ion channel inhibitor combinations in the
205 number of Iba+ cells ($p = 0.938$).

206 In contrast, there was a significant difference in numbers of ED1+ infiltrating microglia/
207 macrophages (Figure 1b; $F = 2.5$, $DF = 3$, $p = 0.079$), with the numbers of the injured, vehicle
208 treated group significantly increased compared to sham injured, vehicle treated animals ($p =$
209 0.049). The number of ED1+ cells in the groups treated with either of the ion channel
210 inhibitor combinations were not significantly different to either the injured, vehicle treated
211 group or the sham injured, vehicle treated group ($p > 0.05$). There was no significant
212 difference between the two ion channel inhibitor combinations in the number of ED1+ cells
213 ($p = 0.994$).

214 Similarly, the number of Iba1+/ED1+ infiltrating microglial cells differed with experimental
215 treatment (Figure 1c; $F = 2.912$, $DF = 3$, $p = 0.041$). A significant increase in the numbers of

216 Iba1+/ED1+ cells was observed in the injured, vehicle treated group compared to the sham
217 injured, vehicle treated group ($p = 0.024$). Neither of the ion channel inhibitor treatment
218 groups had significantly reduced numbers of Iba1+/ED1+ cells compared to the injured,
219 vehicle treated group ($p > 0.05$). There was no significant difference between the two ion
220 channel inhibitor combinations in the number of Iba1+/ED1+ cells ($p = 0.988$).
221 Representative images of Iba1+ cells, ED1+ cells and Iba1+/ED1+ cells are shown (Figure
222 1d).

223 *Effects of ion channel inhibitor combinations on oligodendroglia*

224 The densities of Olig2+ oligodendroglia were not different in any of the experimental groups
225 (Figure 2a; $F = 2.042$, $DF = 3$, $p = 0.128$), example cell shown (Figure 2b). However, when
226 the analysis was refined to include immunoreactivity to PDGF α R, thereby detecting OPCs
227 more specifically, there were significant differences between experimental groups (Figure 2c,
228 d; $F = 4.681$, $DF = 3$, $p = 0.008$). As expected (O'Hare Doig et al. 2017), there was a
229 significant decrease in the number of OPCs in the injured, vehicle treated group compared to
230 the sham injured, vehicle treated group ($p = 0.007$). However, neither ion channel inhibitor
231 combination groups had significantly increased numbers of OPCs compared to the injured,
232 vehicle treated group ($p > 0.05$), and there was no significant difference between the two ion
233 channel inhibitor combinations in the number of OPCs ($p = 0.599$).

234 *Effects of ion channel inhibitor combinations on node/paranode complexes*

235 Significant differences in the length of the paranode between experimental groups were
236 observed (Figure 3a; $F = 52.445$, $DF = 3$, $p = 0.0001$). Partial optic nerve transection resulted
237 in a significant increase in paranode length in the injured, vehicle treated group compared to
238 the sham injured, vehicle treated group ($p = 0.0001$), as expected from previous studies
239 (Szymanski et al. 2013). While treatment with lomerizine + oxATP + YM872 reduced

240 paranode length ($p = 0.0001$), paranodes remained longer than in the sham injured, vehicle
241 treated group ($p = 0.0001$). Treatment with the lomerizine + BBG + YM872 combination also
242 resulted in significantly reduced paranode lengths compared to the injured, vehicle treated
243 group ($p = 0.0001$), to levels significantly different to the sham injured, vehicle treated group
244 ($p = 0.001$). Largely similar outcomes were observed when measuring the length of the
245 paranodal gap, indicative of the length of the node of Ranvier (Figure 3b; $F = 20.367$, $DF = 3$,
246 $p = 0.0001$). There was no significant difference between the two ion channel inhibitor
247 combinations in the length of the paranode ($p = 0.084$) or the paranodal gap ($p = 0.122$).

248 There were significant differences between experimental groups in the percentage of atypical
249 nodal complexes (Figure 3c-e; $F = 374.951$, $DF = 3$, $p = 0.0001$). Atypical nodal complexes
250 were defined as either a heminode, characterised as a β -III tubulin+ area flanked by only one
251 Caspr+ area, or a single paranode, defined as a Caspr+ area not associated with a β -III
252 tubulin+ area (Figure e) (Szymanski et al. 2013). Injury resulted in an increase in the
253 percentage of atypical nodal complexes in the injured, vehicle treated group compared to the
254 sham injured, vehicle treated group ($p = 0.0001$), which was significantly reduced by both
255 lomerizine + oxATP + YM872 ($p = 0.0001$) and lomerizine + BBG + YM872 ($p = 0.0001$).

256 However, both ion channel inhibitor combination groups still had significantly increased
257 levels of atypical nodal complexes compared to the sham injured, vehicle treated group ($p =$
258 0.0001). There was no significant difference between the two ion channel inhibitor
259 combinations in the number of atypical nodal complexes ($p = 0.668$).

260 *Effects of ion channel inhibitor combinations on the optokinetic nystagmus reflex*

261 The optokinetic nystagmus test of visual function, revealed significant differences in the
262 number of smooth pursuits and fast resets following injury and treatment (Figure 4a; smooth
263 pursuits $F = 7.05$, $DF = 3$, $p = 0.001$; fast resets $F = 7.656$, $DF = 3$, $p = 0.001$). Injury resulted

264 in a significant decrease in the numbers of both smooth pursuits and fast resets by the injured,
265 vehicle treated group compared to the sham injured, vehicle treated group ($p = 0.002$, $p =$
266 0.001 respectively). Animals treated with lomerizine + oxATP + YM872 made significantly
267 more smooth pursuits than the injured, vehicle treated group ($p = 0.035$), but fewer fast resets
268 than the sham injured, vehicle treated group ($p = 0.006$). Animals treated with lomerizine +
269 BBG + YM872 made significantly more smooth pursuits ($p = 0.002$) and fast rests ($p =$
270 0.039) than the injured, vehicle treated group; outcomes were not different from the sham
271 injured, vehicle treated group ($p = 0.992$, $p = 0.284$ respectively). There was no significant
272 difference between the two ion channel inhibitor combinations in the number of smooth
273 pursuits ($p=0.422$) or fast resets ($p = 0.222$).

274 To control for the length of the tracking motions, the time engaging in tracking behaviour
275 was also assessed, giving similar outcomes (Figure 4b; $F = 11.458$, $DF = 3$, $p = 0.0001$).
276 Injury resulted in a significantly lower proportion of time spent engaging in smooth pursuits
277 by the injured, vehicle treated group compared to the sham injured, vehicle treated group ($p =$
278 0.0001). Animals treated with lomerizine + oxATP + YM872 spent more time tracking than
279 the injured, vehicle group ($p = 0.01$), but still less time than the sham injured, vehicle treated
280 group ($p = 0.045$). Animals treated with lomerizine + BBG + YM872 also spent significantly
281 longer performing smooth pursuits than the injured, vehicle treated group ($p = 0.0001$), and
282 the time spent tracking was not significantly different to the sham injured, vehicle treated
283 group ($p = 0.161$). There was no significant difference between the two ion channel inhibitor
284 combinations in the time spent tracking ($p = 0.601$).

285

286 **Discussion**

287 The aim of this study was to determine if the combination of lomerizine + oxATP + YM872,
288 shown to effectively limit functional loss associated with secondary degeneration of the optic
289 nerve (Savigni et al. 2013), would be as effective if oxATP was replaced with BBG, a more
290 clinically applicable P2X₇ receptor inhibitor that can cross the closed blood brain barrier. It
291 was found that the lomerizine + BBG + YM872 combination was as effective, or marginally
292 more effective at the tested concentrations, than the lomerizine + oxATP + YM872
293 combination, at preserving node/paranode structure and visual function when delivered
294 locally. However, neither therapeutic combination affected numbers of microglia and
295 macrophages, or the number of OPCs or oligodendrocytes. The data suggest an associative
296 relationship between preservation of myelin structure and maintenance of visual function
297 following injury.

298 The observed increase in nodal and paranodal lengths following partial optic nerve injury is
299 in line with previous findings (Szymanski et al. 2013; O'Hare Doig et al. 2017) and is
300 suggestive of myelin retraction and a breakdown of the paranodal junction
301 (Arancibia- Carcamo and Attwell 2014). Increased P2X₇ receptor activation on the myelin
302 sheath has also been associated with myelin degradation following injury, however the
303 underlying cellular mechanisms remain unclear (Matute 2008). The lomerizine + BBG +
304 YM872 combination restored nodal and paranodal structure to dimensions closer to the sham
305 control group than the lomerizine + oxATP + YM872 combination. The lomerizine + BBG +
306 YM872 combination was the only treatment to not be different to the sham control group for
307 visual function. BBG is a more potent and selective antagonist of P2X₇ receptors than oxATP
308 (Donnelly-Roberts and Jarvis 2007), which may explain this marginally greater efficacy of
309 the BBG containing combination in preserving myelin structure and visual function
310 compared to the control groups in the current study. However, titration of doses of these
311 agents relative to their specific inhibitory concentrations for P2X₇ receptors is required to

312 definitively compare efficacy of the two agents within the context of this treatment
313 combination and injury model. We have previously established that locally delivered oxATP
314 alone does not preserve node/paranode structure nor visual function in the partial optic nerve
315 transection model (Savigni et al. 2013). The addition of lomerizine and YM872 to the
316 combination is required for full beneficial effects (Savigni et al. 2013), emphasising that
317 limiting excess Ca^{2+} flux through voltage gated calcium channels and Ca^{2+} permeable AMPA
318 receptors is also important.

319 Following injury to the CNS, there is a high influx of Ca^{2+} into myelin *via* AMPA receptors
320 (Fowler et al. 2003), which results in increased Ca^{2+} binding to the catalytic core of calpain,
321 enhancing calpain activation (Croall and Demartino 1991; Khorchid and Ikura 2002). An
322 increase in calpain activation can induce myelin degradation, *via* cleavage of myelin basic
323 protein and myelin-associated glycoprotein (Banik et al. 1985; Shields et al. 1997; Fu et al.
324 2007). Myelin degradation has been associated with paranodal loop eversion and sheath
325 retraction and thus increased nodal and paranodal lengths (Ouyang et al. 2010). Therefore,
326 observed preservation of nodal and paranodal length by both ion channel combinations may
327 be due to inhibition of this Ca^{2+} -dependent calpain mechanism through the antagonistic
328 activity of YM872 on AMPA receptors.

329 Furthermore, when the axolemma becomes exposed following myelin sheath retraction, and
330 paranodal splitting, there is an increase in Ca^{2+} entry into axons *via* sub-myelin L-type
331 VGCCs, which are normally hidden underneath the myelin sheath (Zhang and David 2015).
332 This contributes to neuronal Ca^{2+} overload, associated with oxidative stress, caspase-
333 mediated apoptosis and decreased function (Annunziato et al. 2003). Lomerizine-mediated
334 inhibition of these sub-myelin VGCCs from beneath the sheath, together with exposure of
335 fewer L-type VGCCs by prevention of the myelin retraction, may be a further therapeutic
336 mechanism of the combinations of inhibitors.

337 Myelin structure is integral to the capacity of nerves to propagate action potentials, with the
338 lengthening of the node associated with slower conduction velocities in a variety of
339 pathologies (Howell et al. 2006; Reimer et al. 2011; Sun et al. 2012). Abnormal myelination
340 at a single internode can be sufficient to block neural signal transduction for an entire axon
341 (Baumann and Pham-Dinh 2001). Previous studies have also hypothesised that abnormalities
342 in the node of Ranvier proteins, associated with increased nodal length, may result in
343 decreased synchronicity of neuronal firing (Arancibia-Carcamo and Attwell 2014).
344 Preservation of myelin integrity by the ion channel inhibitor combinations may be facilitating
345 action potential propagation along axons, associated with preservation of function following
346 injury.

347 However, in the current study myelin structure and visual function were only partly preserved
348 by the ion channel inhibitor treatments, which suggests some aspects of myelin breakdown
349 following injury are mediated via alternative mechanisms of damage. One potential
350 mechanism is immune-cell mediated depletion of OPCs. The current study found that
351 following injury, there is a significant increase in infiltrating microglia and macrophages, but
352 not resident microglial cells, indicative of an infiltrating immune response. However,
353 treatment did not show a significant effect at ameliorating this infiltrating immune response.
354 Following neurotrauma, inflammatory cells produce cytokines and chemokines, as well as
355 reactive oxygen species, resulting in oxidative damage of surrounding tissue (Anderson
356 2002). OPCs are especially vulnerable to oxidative stress and apoptosis following injury
357 (Thorburne and Juurlink 1996; Giacci et al. 2018), which may be why the combinations of
358 ion channel inhibitors were unable to ameliorate the loss of OPCs in this study. OPCs are
359 required for oligodendrogenesis and remyelination following injury (Mirron et al. 2011).
360 OPCs also contribute to the formation of myelin nodal structures (Butt et al. 2004). It may be
361 that significantly preserving OPCs following injury, perhaps through preventing this

362 infiltrating immune response, would be associated with a complete preservation of myelin
363 structure and thus visual function. Furthermore, given previous studies have found an
364 increased therapeutic effect of the lomerizine + oxATP + YM872 combination after three
365 months of administration compared to three days (Savigni et al. 2013; O'Hare Doig et al.
366 2017), it may be that a longer duration of treatment would provide further improvements to
367 these outcomes following injury.

368 This study showed that the combination of lomerizine + BBG + YM872, which has the
369 potential to be delivered systemically following injury, shows promise for limiting secondary
370 degeneration following neurotrauma, however further work remains to be done. While BBG
371 is a highly selective P2X₇ receptor antagonist, it is 30 to 50 times more potent in rats than
372 humans (Jiang et al. 2000), which will necessitate careful titration of dosages before clinical
373 translation will be feasible. Lomerizine is currently used in clinical practice (Hara et al.
374 1999), YM872 is more soluble than other Ca²⁺ permeable AMPA receptor inhibitors
375 (Takahashi et al. 2002) and has been trialled in stroke with an acceptable safety profile
376 (Labiche and Grotta 2004) and BBG has no-observed-adverse-effect level of 8966mg/kg per
377 day in a mouse model of lifetime toxicity (Borzelleca et al. 1990). Nevertheless, while no
378 adverse effects have been observed in our rodent model, the combination of three ion channel
379 inhibitors will need to be carefully assessed for toxicity in humans before a trial of efficacy
380 following neurotrauma can be contemplated. Furthermore, given the clinical need for
381 systemically administered drug delivery following neurotrauma, the efficacy of this blood
382 brain barrier permeable combination of lomerizine + BBG + YM872 needs to be tested when
383 systemically delivered following injury.

384

385 **Acknowledgements**

386 MF is supported by an NHMRC Career Development Fellowship (APP1087114). The authors
387 declare that they have no conflict of interest.

388 **References**

389 Abdeljalil J, Hamid M, Abdel-Mouttalib O, et al. (2005) The optomotor response: A robust first-line
390 visual screening method for mice. *Vision Research* 45:1439-1446 doi:
391 10.1016/j.visres.2004.12.015

392 Anderson A (2002) Mechanisms and pathways of inflammatory responses in CNS trauma: Spinal cord
393 injury. *Journal of Spinal Cord Medicine* 25:70-79

394 Annunziato L, Amoroso S, Pannaccione A, et al. (2003) Apoptosis induced in neuronal cells by
395 oxidative stress: Role played by caspases and intracellular calcium ions. *Toxicology Letters*
396 139:125-133 doi: 10.1016/S0378-4274(02)00427-7

397 Arancibia- Carcamo I, Attwell D (2014) The node of Ranvier in CNS pathology. *Acta Neuropathology*
398 128:161-175 doi: 10.1007/s00401-014-1305-z

399 Banik NL, McAlhaney WW, Hogan EL (1985) Calcium-stimulated proteolysis in myelin: Evidence for a
400 Ca²⁺-activated neutral proteinase associated with purified myelin of rat CNS. *Journal of*
401 *Neurochemistry* 45:581-588 doi: 10.1111/j.1471-4159.1985.tb04026.x

402 Baumann N, Pham-Dinh D (2001) Biology of oligodendrocyte and myelin in the mammalian central
403 nervous system. *Physiological Reviews* 81:871-927

404 Blair M, Pease ME, Hammond J, Valenta D, Kielczewski J, Levkovitch-Verbin H, Quigley H (2005)
405 Effect of glatiramer acetate on primary and secondary degeneration of retinal ganglion cells
406 in the rat. *Investigative Ophthalmology & Visual Science* 46:884-884 doi: 10.1167/iovs.04-
407 0731

408 Borzelleca JF, Depukat K, Hallagan JB (1990) Lifetime toxicity/carcinogenicity studies of FD & C
409 Blue No. 1 (brilliant blue FCF) in rats and mice. *Food and Chemical Toxicology* 28:221-234

410 Butt A, Pugh M, Hubbard P, James G (2004) Functions of optic nerve glia: axoglia signaling in
411 physiology and pathology. *Eye* 18:1110-1121

412 Cervetto C, Frattaroli D, Maura G, Marcoli M (2013) Motor neuron dysfunction in a mouse model of
413 ALS: Gender-dependent effect of P2X7 antagonism. *Toxicology* 311:69-77 doi:
414 10.1016/j.tox.2013.04.004

415 Croall DE, Demartino GN (1991) Calcium-activated neutral protease (calpain) system: Structure,
416 function, and regulation. *Physiological Reviews* 71:813-847

417 Deford SM, Wilson MS, Rice AC, et al. (2002) Repeated mild brain injuries result in cognitive
418 impairment in B6C3F1 mice. *Journal of Neurotrauma* 19:427-438

419 Diaz-Hernandez JI, Gomez-Villafuertes R, León-Otegui M, et al. (2012) In vivo P2X7 inhibition reduces
420 amyloid plaques in Alzheimer's disease through GSK3 β and secretases. *Neurobiology of*
421 *Aging* 33:1816-1828 doi: 10.1016/j.neurobiolaging.2011.09.040

422 Doan N, Patel M, Doan H, Janich K, Nguyen HS, Shabani S (2016) Traumatic brain injury. *International*
423 *Journal of Physical Medicine and Rehabilitation* 4:120-121 doi: 10.4172/2329-
424 9096.1000e120

425 Dong X-X, Wang Y, Qin Z-H (2009) Molecular mechanisms of excitotoxicity and their relevance to
426 pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin Acta Pharmacologica Sinica*
427 30:379-387 doi: 10.1038/aps.2009.24

428 Donnelly-Roberts DL, Jarvis MF (2007) Discovery of P2X7 receptor-selective antagonists offers new
429 insights into P2X7 receptor function and indicates a role in chronic pain states. *British*
430 *Journal of Pharmacology* 151:571-579 doi: 10.1038/sj.bjp.0707265

431 Farooqui AA, Ong W-Y, Horrocks LA (2008) *Neurochemical Aspects of Excitotoxicity*. Springer, New
432 York

433 Fitzgerald M, Bartlett CA, Evill L, Rodger J, Harvey AR, Dunlop SA (2009a) Secondary degeneration of
434 the optic nerve following partial transection: The benefits of lomerizine. *Experimental*
435 *Neurology* 216:219-230 doi: 10.1016/j.expneurol.2008.11.026

436 Fitzgerald M, Bartlett CA, Harvey AR, Dunlop SA (2010a) Early events of secondary degeneration
437 after partial optic nerve transection: An immunohistochemical study. *Journal of*
438 *Neurotrauma* 27:439-452

439 Fitzgerald M, Bartlett CA, Payne SC, Hart NS, Rodger J, Harvey AR, Dunlop SA (2010b) Near Infrared
440 Light Reduces Oxidative Stress and Preserves function in CNS Tissue Vulnerable to Secondary
441 Degeneration following Partial Transection of the Optic Nerve. *Journal of Neurotrauma*
442 27:2107-2119 doi: 10.1089/neu.2010.1426

443 Fitzgerald M, Payne SC, Bartlett CA, Evill L, Harvey AR, Dunlop SA (2009b) Secondary retinal ganglion
444 cell death and the neuroprotective effects of the calcium channel blocker lomerizine.
445 *Investigative Ophthalmology and Visual Science* 50:5456-5462 doi: 10.1167/iovs.09-3717

446 Fowler J, McCracken E, Dewar D, McCulloch J (2003) Intracerebral injection of AMPA causes axonal
447 damage in vivo. *Brain Research* 991:104-112 doi: 10.1016/J.BRAINRES.2003.08.004

448 Fu Y, Wang H, Huff TB, Shi R, Cheng J-X (2007) Coherent anti-Stokes Raman scattering imaging of
449 myelin degradation reveals a calcium-dependent pathway in lyso-PtdCho-induced
450 demyelination. *Journal of Neuroscience Research* 85:2870-2881 doi: 10.1002/jnr.21403

451 Giacci MK, Bartlett CA, Smith NM, et al. (2018) Oligodendroglia are particularly vulnerable to
452 oxidative damage after neurotrauma in vivo. *Journal of Neuroscience*:1898-1917

453 Hara H, Shimazawa M, Sasaoka M, et al. (1999) Selective effects of lomerizine, a novel
454 diphenylmethylpiperazine Ca²⁺ channel blocker, on cerebral blood flow in rats and dogs.
455 *Clinical and Experimental Pharmacology and Physiology* 26:870-876 doi: 10.1046/j.1440-
456 1681.1999.03154.x

457 Howell OW, Palser A, Polito A, et al. (2006) Disruption of neurofascin localization reveals early
458 changes preceding demyelination and remyelination in multiple sclerosis. *Brain* 129:3173-
459 3185 doi: 10.1093/brain/awl290

460 Jiang L-H, Mackenzie AB, North RA, Surprenant A (2000) Brilliant Blue G selectively blocks ATP-gated
461 rat P2X7 receptors. *Molecular Pharmacology* 58:82-88

462 Khorchid A, Ikura M (2002) How calpain is activated by calcium. *Nature Structural Biology* 9:239-241
463 doi: 10.1038/nsb0402-239

464 Kimbler DE, Shields J, Yanasak N, Vender JR, Dhandapani KM, Kleinschnitz C (2012) Activation of
465 P2X7 promotes cerebral edema and neurological injury after traumatic brain injury in mice.
466 *PLoS ONE* 7 doi: 10.1371/journal.pone.0041229

467 Kwon BK, Okon E, Hillyer J, et al. (2011) A systematic review of non-invasive pharmacologic
468 neuroprotective treatments for acute spinal cord injury. *Journal of Neurotrauma* 28:1545-
469 1588

470 Labiche LA, Grotta JC (2004) Clinical trials for cytoprotection in stroke. *NeuroRx* 1:46-70 doi:
471 10.1602/neurorx.1.1.46

472 Levkovitch-Verbin H, Quigley HA, Martin KRG, Zack DJ, Pease ME, Valenta DF (2003) A model to
473 study differences between primary and secondary degeneration of retinal ganglion cells in
474 rats by partial optic nerve transection. *Investigative Ophthalmology & Visual Science* 44:3388-
475 3388 doi: 10.1167/iovs.02-0646

476 Matute C (2008) P2X7 receptors in oligodendrocytes: A novel target for neuroprotection. *Molecular*
477 *Neurobiology* 38:123-128 doi: 10.1007/s12035-008-8028-x

478 Maxwell WL (2013) Damage to myelin and oligodendrocytes: A role in chronic outcomes following
479 traumatic brain injury? *Brain Sciences* 3:1374-1394 doi: 10.3390/brainsci3031374

480 Mirron V, Kuhlmann T, Antel J (2011) Cells of the oligodendroglial lineage, myelination and
481 remyelination. *Biochimica et Biophysica Acta* 1812:184-193

482 O'Hare Doig R, Chiha W, Giacci M, et al. (2017) Specific ion channels contribute to key elements of
483 pathology during secondary degeneration following neurotrauma. *BMC Neuroscience* 18

484 Ouyang H, Sun W, Fu Y, Li J, Cheng J-X, Nauman E, Shi R (2010) Compression induces acute
485 demyelination and potassium channel exposure in spinal cord. *Journal of Neurotrauma*
486 27:1109-1120 doi: 10.1089/neu.2010.1271

487 Peng W, Cotrina ML, Han X, et al. (2009) Systemic administration of an antagonist of the ATP-
488 sensitive receptor P2X7 improves recovery after spinal cord injury. *Proceedings of the*
489 *National Academy of Sciences of the United States of America* 106:12489-12493
490 Reimer MM, McQueen J, Searcy L, et al. (2011) Rapid disruption of axon–glial integrity in response to
491 mild cerebral hypoperfusion. *Journal of Neuroscience* 31:18185-18194 doi:
492 10.1523/JNEUROSCI.4936-11.2011
493 Savigni DL, O'Hare Doig RL, Szymanski CR, Bartlett CA, Lozić I, Smith NM, Fitzgerald M (2013) Three
494 Ca²⁺ channel inhibitors in combination limit chronic secondary degeneration following
495 neurotrauma. *Neuropharmacology* 75:380-390 doi: 10.1016/j.neuropharm.2013.07.034
496 Shields DC, Deiber GE, Banik NL (1997) Calpain: A mediator of myelin breakdown in demyelinating
497 diseases. *Annals of the New York Academy of Sciences* 825:128-130 doi: 10.1111/j.1749-
498 6632.1997.tb48422.x
499 Sun W, Fu Y, Shi Y, Cheng J-X, Cao P, Shi R (2012) Paranodal myelin damage after acute stretch in
500 guinea pig spinal cord. *Journal of Neurotrauma* 29:611-619 doi: 10.1089/neu.2011.2086
501 Szymanski CR, Chiha W, Morellini N, et al. (2013) Paranode abnormalities and oxidative stress in
502 optic nerve vulnerable to secondary degeneration: Modulation by 670 nm light treatment.
503 *PLoS One* 8 doi: 10.1371/journal.pone.0066448
504 Takahashi M, Kohara A, Shishikura J-I, et al. (2002) YM872: A selective, potent and highly water-
505 soluble alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor antagonist. *CNS*
506 *Drug Reviews* 8:337-352
507 Thorburne S, Juurlink B (1996) Low glutathione and high iron govern the susceptibility of
508 oligodendroglial precursors to oxidative stress. *Journal of Neurochemistry* 67:1014-1022
509 Tomkins O, Feintuch A, Benifla M, Cohen A, Friedman A, Shelef I (2011) Blood-brain barrier
510 breakdown following traumatic brain injury: a possible role in posttraumatic epilepsy.
511 *Cardiovasc Psychiatry Neurol* 2011:765923 doi: 10.1155/2011/765923
512 Tymianski M, Charles T (1996) Normal and abnormal calcium homeostasis in neurons: A basis for the
513 pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery*
514 38:1176-1195
515 Wang Y-C, Cui Y, Cui J-Z, et al. (2015) Neuroprotective effects of brilliant blue G on the brain
516 following traumatic brain injury in rats. *Molecular Medicine Reports* 12:2149-2154 doi:
517 10.3892/mmr.2015.3607
518 Wong HE, Qi W, Choi H-M, Fernandez EJ, Kwon I (2011) A safe, blood-brain barrier permeable
519 triphenylmethane dye inhibits amyloid- β neurotoxicity by generating nontoxic aggregates.
520 *ACS Chemical Neuroscience* 2:645-657 doi: 10.1021/cn200056g
521 Wu D, Miyamoto O, Shibuya S, et al. (2005) Different expression of macrophages and microglia in rat
522 spinal cord contusion injury model at morphological and regional levels. *Acta Medica*
523 *Okayama* 59:121-127 doi: 10.18926/AMO/31950
524 Zetterberg H, Smith DH, Blennow K (2013) Biomarkers of mild traumatic brain injury in cerebrospinal
525 fluid and blood. *Nat Rev Neurol* 9:201-210 doi: 10.1038/nrneurol.2013.9
526 Zhang Z, David G (2015) Stimulation-induced Ca²⁺ influx at nodes of Ranvier in mouse peripheral
527 motor axons. *The Journal of Physiology* 594:39-57 doi: 10.1113/JP271207

528

529 **Figure Legends**

530 **Fig. 1** Effects of ion channel inhibitor combinations on densities of Iba1+ and ED1+ cells.

531 Densities of Iba1+ (a), ED1+ (b) and Iba1+/ED1+ (c) cells in the ventral optic nerve from

532 sham injured, vehicle treated animals, injured, vehicle treated animals, and ion channel

533 inhibitor treated animals 3 days after partial optic nerve transection. N = 7 - 10 rats per group;

534 graphs display min to max values, with the central line representing the median data point.

535 Significant differences are indicated by * ($p \leq 0.05$). (d) Representative images of Iba1+,

536 ED1+ and Iba1+/ED1+ cells, indicated with arrow heads; scale bar = 10 μ m

537

538 **Fig. 2** Effects of ion channel inhibitor combinations on oligodendroglial cells. Densities of

539 Olig2+ oligodendroglial cells (a) and PDGF α R+/Olig2+ OPCs (c) in the ventral optic nerve

540 from sham injured, vehicle treated animals, injured, vehicle treated animals, and ion channel

541 inhibitor treated animals, 3 days after partial optic nerve transection. N = 8 - 10 rats per

542 group. Graphs display min to max values, with the central line representing the median data

543 point. Significant differences are indicated by ** ($p \leq 0.01$). Representative image of Olig2+

544 cells (b) and OPCs (d), indicated with arrow heads; scale bar = 15 μ m and 10 μ m respectively

545

546 **Fig. 3** Effect of ion channel inhibitor combinations on node/paranode complexes. Paranode

547 length (a), paranodal gap length (b) and percentage of atypical node/paranode complexes (c)

548 from thirty nodal complexes per animal in the ventral optic nerve from sham injured, vehicle

549 treated animals, injured, vehicle treated animals, and ion channel inhibitor treated animals 3

550 days after partial optic nerve transection. Graphs display min to max values, with the central

551 line representing the median data point; N = 6 - 9 rats per group. Significant differences

552 indicated by * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. Differences compared to the sham

553 injured, vehicle treated group are indicated by *, differences compared to the injured, vehicle
554 treated group are indicated by #. (d) Representative image of two Caspr+ areas flanking a β -
555 III tubulin+ area i.e. a typical nodal complex; scale bar = $2\mu\text{m}$. (e) Representative image of
556 one Caspr+ area flanking a β -III tubulin+ area denoting an atypical nodal complex /
557 heminode; scale bar = $2\mu\text{m}$

558

559 **Fig. 4** Effects of injury and ion channel inhibitor combinations on the number of responses in
560 the optokinetic nystagmus test of visual function. Total number of smooth pursuits and fast
561 resets per minute engaged in the task (a) and proportion of time paying attention to task
562 engaged in smooth pursuits (b) by sham injured, vehicle treated animals; injured, vehicle
563 treated animals; or injured ion channel inhibitor treated animals. Graphs display min to max
564 values, with the central line representing the median data point; N = 5 - 10 rats per group.
565 Significant differences are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.
566 Differences compared to the sham injured, vehicle treated group are indicated by *,
567 differences compared to the injured, vehicle treated group are indicated by #