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Endothelin signalling mediates experience-dependent myelination in the CNS

1   **Title**

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4   **Authors**

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15

16   **Abstract**

17

18   Experience and changes in neuronal activity can alter CNS myelination, but the signalling pathways

19   responsible remain poorly understood. Here we define a pathway in which endothelin, signalling

20   through the G protein-coupled receptor endothelin receptor B and PKC epsilon, regulates the

21   number of myelin sheaths formed by individual oligodendrocytes in mouse and zebrafish. We show

22   that this phenotype is also observed in the prefrontal cortex of mice following social isolation, and is

23   associated with reduced expression of vascular endothelin. Additionally, we show that increasing

24   endothelin signalling rescues this myelination defect caused by social isolation. Together, these

25   results indicate that the vasculature responds to changes in neuronal activity associated with

26   experience by regulating endothelin levels, which in turn affect the myelinating capacity of

27   oligodendrocytes. This pathway may be employed to couple the metabolic support function of

28   myelin to activity-dependent demand and also represents a novel mechanism for adaptive

29   myelination.

30

31   **Introduction**

32

33   There is increasing evidence that experience regulates CNS myelination. For example, social

34   interactions, sensory stimulation and several forms of learning have been shown to alter white

35   matter and myelin structure in both humans and animal models (Scholz et al., 2009, Makinodan et

36   al., 2012, Liu et al., 2012, Sampaio-Baptista et al., 2013, McKenzie et al., 2014, Etxeberria et al.,

37   2016, Xiao et al., 2016, Hughes et al., 2018). At the level of individual neurons and axons, increasing

38   the level of activity by using optogenetics or chemogenetics enhances the generation of myelin-

39   forming oligodendrocytes and increases the amount of myelin they form (Gibson et al., 2014, Mitew

40   et al., 2018), whilst preventing synaptic vesicular release from axons reduces myelin formation

41   (Hines et al., 2015, Mensch et al., 2015, Koudelka et al., 2016). Together, these findings have led to a

42   new concept of CNS plasticity - adaptive myelination. This concept posits that changes in neuronal

43   activity in response to experience of the extrinsic environment lead to local changes in myelination.

44   Such changes could in turn contribute to the alterations in conduction that underpin CNS neural

45   circuit plasticity (Sampaio-Baptista and Johansen-Berg, 2017, Foster et al., 2019, Suminaite et al.,

46   2019).

47   Exploring this important concept requires that we understand the mechanisms that can link changes

48   in neuronal activity to the regulation of myelination. In addition to the communication between

49   axons of active neurons and the oligodendrocytes that myelinate them, neuronal activity might also

50   affect myelination indirectly in the local area through signals from other glial cells or from vascular

51   cells that can respond to dynamic changes in neuronal activity. Such indirect signalling has been

52   implied by in vitro studies, wherein increased levels of LIF secreted by cultured astrocytes in the

53   presence of neuronal activity enhances myelination (Ishibashi et al., 2006). However, whether the

54   vasculature might relay information about neuronal activity and in turn influence myelination by

55   oligodendrocytes in vivo is not known.

56   Here we set out to test the hypothesis that the vasoactive peptide endothelin (EDN) enables blood

57   vessels to play such a role in indirectly linking activity and myelination. This hypothesis is based on

58   two sets of prior data. First, EDN expression by endothelial cells increases with enhanced blood flow

59   (Yanagisawa et al., 1988, Dancu et al., 2004, Walshe et al., 2005, Pandit et al., 2015), which occurs in

60   response to increased CNS activity. Second, the EDN G-protein coupled receptor (GPCR) endothelin

61   receptor B (EDNRB) enhances myelination in slice cultures (Yuen et al., 2013). Here we show that

62   EDN is expressed by CNS vascular cells, and that this expression lessens in the medial prefrontal

63   cortex following social isolation, which we confirm also leads to impaired cortical myelination.

64   Correspondingly, we show, by manipulating EDNRB signalling in rodent and zebrafish models, that

65   reduced EDN signalling decreases the number of myelin sheaths formed by individual

66   oligodendrocytes in vivo. Finally, we rescue the reduction in myelination associated with

67   environmental social deprivation by intranasal administration of an EDNRB agonist to activate EDN

68   signalling within the CNS. Together our data indicate that the vasculature responds to environmental

69   signals associated with changes in neuronal activity and can, in turn, affect the myelinating capacity

70   of oligodendrocytes in vivo. In this way, we propose a novel mode by which active neurons may

71   regulate oligodendrocyte behaviour and provide a mechanism for adaptive myelination.

72

73 **Results**

74

75 *The levels of EDN expression in blood vessels are reduced following social isolation*

76 If EDN signalling from CNS blood vessels provides a link between circuit function and myelination,

77 then an initial prediction would be that EDN expression by these vessels will be responsive to

78 extrinsic environmental changes that alter neuronal activity and myelination. To test this prediction,

79 we used a model in which the extrinsic environment has been shown to regulate CNS myelination:

80 social isolation (Liu et al., 2012, Makinodan et al., 2012).

81 Prior work has established that social isolation in mice during a critical period comprising 2 weeks

82 after weaning reduces both the excitability of specific subtypes of pyramidal neurons of the medial

83 prefrontal cortex (mPFC) and oligodendrocyte formation and myelination in the same area (Liu et al.,

84 2012, Makinodan et al., 2012, Yamamuro et al., 2018) We repeated this protocol, confirming the

85 previously-described effect on circuit function by showing that isolated mice spent significantly less

86 time than socially-experienced controls interacting with a novel mouse (figure 1A-C). To quantify the

87 myelination defects in these mice immediately following the isolation period we used a labelling

88 strategy in which oligodendrocyte cell bodies, processes and myelin sheaths are revealed by CNPase

89 immunostaining. The sparsely-myelinated layers II/III of the mPFC, a region myelinated during this

90 isolation period (figure 1D), enabled individual oligodendrocyte morphologies to be analysed for

91 myelin sheath number and length (figure 1D-E, figure 1 - figure supplement 1). Using this approach

92 we showed that individual oligodendrocytes made fewer myelin sheaths (figure 1F-H), although the

93 length of the myelin sheaths formed by oligodendrocytes in these mice was unaffected (figure 1 –

94 figure supplement 2E-F). Further, we found that the isolated mice also generated fewer

95 oligodendrocytes in the mPFC (figure 1 - figure supplement 2C-D). In contrast, social isolation had no

4

96   effect on myelin sheath number in the visual cortex (figure 1 - figure supplement 3A-B),

97   demonstrating the region-dependent effects of this protocol on myelination.

98   Having validated our social isolation protocol by demonstrating experience-dependent changes in

99   myelination, we next analysed EDN expression in the mPFC. We performed in situ hybridization

100   studies examining the expression of all three EDN ligands (*Edn1-3*) in controls and following

101   isolation. We confirmed robust *Edn1* and *Edn3* mRNA expression in controls, localised to laminin-

102   positive blood vessels, which co-expressed the endothelial/pericyte marker *Pecam1* mRNA (figure

103   2A) (Yanagisawa et al., 1988, Hammond et al., 2014). While previous studies have observed *Edn1*

104   expression also localised to astrocytes following demyelination (Hammond et al., 2014) and

105   microglia (Zhang et al., 2014), here we observed no *Edn1*, *Edn2* or *Edn3* mRNA in S100β positive

106   astrocytes or IBA1 positive microglia (figure 2 - figure supplement 1A-B), indicating that endothelial

107   cells and/or pericytes are the main source of *Edn* in the healthy mouse brain. Following social

108   isolation, both the number of endothelial cells expressing *Edn1* and *Edn3* mRNA and the expression

109   level of *Edn1* mRNA within each cell was significantly reduced in the mPFC (figure 2B-D, figure 2 -

110   figure supplement 2F-G). However, the vascular area and the number of cells expressing *Pecam1*

111   mRNA were unaffected (figure 2 - figure supplement 2A-E). In contrast to the mPFC, there was no

112   effect of social isolation on *Edn* expression by *Pecam1* cells in the visual cortex (figure 1 - figure

113   supplement 3C). We therefore conclude that the environmental deprivation associated with social

114   isolation reduces vascular *Edn* production in the mouse mPFC.

115

116   ***EDNRB loss reduces the number of myelin sheaths formed by individual oligodendrocytes in vivo***

117   Next, we asked whether a reduction in EDN signalling could potentially contribute to the reduced

118   levels of oligodendrocyte generation and myelination in the isolated mice. To do this, we defined the

119   role of EDN signalling in myelination by performing genetic loss of function experiments to remove

120   the relevant receptor in conditional knockout mice. We targeted the EDN receptor EDNRB due to the

121   high levels of expression in forebrain oligodendrocytes and our own previous work identifying

122   EDNRB as a regulator of myelination in vitro (Yuen et al., 2013, Horiuchi et al., 2017, Marques et al.,

123   2018). We generated a conditional knock out (cKO) using a floxed *Ednrb* mouse line crossed with a

124   line expressing cre recombinase driven by the *Pdgfra* promoter to delete *Ednrb* from

125   oligodendrocytes and their precursor cells through development (figure 3 - figure supplement 1A).

126   Comparison of the number of oligodendrocyte lineage cells in the mPFC in the control (*Pdgfra*-

127   cre;*Ednrb*^wt/wt^) mice shown in figure1 with EDNRB cKO (*Pdgfra*-cre;*Ednrb*^flox/flox^) mice revealed no

128   significant difference in the oligodendrocyte progenitor cell (OPC) or oligodendrocyte generation

129   (figure 3A, figure 3 - figure supplement 1B-D). However, loss of EDNRB in the oligodendroglial

130   lineage significantly reduced the number of myelin sheaths generated by individual oligodendrocytes

131   (figure 3B-D). EDNRB cKO oligodendrocytes demonstrated a 22% reduction in myelin sheath number

132   compared to wild type littermates, but the lengths of the remaining sheaths were unchanged (figure

133   3 - figure supplement 1E-F). A similar effect of EDNRB loss on myelin sheath number was also seen in

134   the visual cortex (figure 1 - figure supplement 3A-B). These experiments show that oligodendroglial

135   EDNRB regulates the number of myelin sheaths formed by individual oligodendrocytes and,

136   strikingly, that this effect of deleting EDNRB was identical to the myelination deficits of individual

137   oligodendrocytes within the mPFC following social isolation in wild-type animals.

138   By contrast, the numbers of oligodendrocytes within the mPFC differs between the two

139   experimental manipulations – reduced by social isolation (figure 1 - figure supplement 2C-D) but not

140   affected by deleting oligodendroglial EDNRB. Given these similarities and difference in the cellular

141   phenotypes of social isolation and EDNRB deletion, we therefore next asked to what extent the

142   changes in behaviour following isolation were phenocopied in the EDNRB cKO mice. Surprisingly,

143   despite the additional effects of social isolation on oligodendrocyte number, the cell type specific

144   loss of EDNRB from the oligodendrocyte lineage was sufficient to cause a significant reduction in the

145   amount of time that cKO mice spent with the novel mouse in the social interaction task, although, as

146   might have been expected, the effect size was smaller than in socially isolated mice, with a 33%

147 reduction following social isolation and 20% following EDNRB CKO as compared to wild type mice

148 (figure 3E, figure 3 - figure supplement 1G-H). We conclude that loss of EDNRB signalling in

149 oligodendrocyte lineage cells phenocopies a specific component of the response of oligodendrocytes

150 to social isolation and that this, in turn, contributes to the behavioural deficits that result from social

151 isolation.

152

153 ***EDRNB signalling enhances myelin sheath number in 3D microfiber cultures***

154 Given that cell-type specific loss of EDNRB results in oligodendrocytes making fewer myelin sheaths

155 per cell, we wanted to ask whether activation of EDRNB signalling in oligodendrocytes might have

156 the complementary effect and promote myelin sheath generation. To do this, we turned to a

157 reductionist in vitro system in which myelination takes place in the absence of confounding

158 influences from other cell types - a 3-dimensional microfiber culture system in which

159 oligodendrocytes form myelin sheaths (Lee et al., 2012a, Bechler et al., 2015). In these cultures we

160 tested both a peptide agonist (BQ2030) and a small molecule antagonist (BQ788) of the receptor.

161 Rat oligodendrocyte precursors were seeded onto poly-l-lactic acid-coated microfibers and allowed

162 to differentiate for 3 days before being treated with BQ2030 or BQ788. We saw a significant increase

163 in the number of myelin sheaths produced by individual oligodendrocytes 11 days later in response

164 to the EDNRB agonist (figure 4A-C). Similar results were obtained with wild type mouse

165 oligodendrocytes (*Pdgfra*-cre;*Ednrb*[wt/wt]) while BQ3020 had no effect on EDNRB cKO (*Pdgfra*-

166 cre;*Ednrb*[flox/flox]) oligodendrocytes (figure 4F-G), confirming the specificity of the agonist for EDNRB.

167 In contrast to the agonist, treatment with the EDNRB antagonist had no effect on myelin sheath

168 number in these cultures (figure 4A-C), as might be expected given the lack of EDN-expressing blood

169 vessel cells, and neither agonist nor antagonist altered sheath length (figure 4D). We conclude from

170 these experiments that the BQ2030 agonist selectively activates EDNRB-mediated signalling

171 pathways in oligodendrocytes that increase myelin sheath number in vitro.

172

173 ***EDRNB promotes myelin sheath formation through Protein Kinase C ε.***

174 The observation that we can stimulate myelination by oligodendrocytes in an EDNRB-dependent

175 manner in vitro enabled a biochemical approach to identify the downstream signalling mechanisms

176 by which this was mediated. We therefore next used a forward-phase phosphorylated-protein

177 antibody array of mouse oligodendroglial cultures treated with the EDNRB agonist as an initial

178 screen to identify these downstream pathways. As shown in supplementary file 1, one of the largest

179 changes in protein phosphorylation following EDNRB activation was an increase in the

180 phosphorylation of serine-729 of PKC epsilon, an isozyme of the Protein Kinase C (PKC) family. We

181 confirmed an instructive role of PKCε in two ways. First, we examined myelination in

182 oligodendrocyte microfiber cultures treated with FR 236924, a specific activator of PKCε, which

183 promoted phosphorylation of PKCε at serine-729 (figure 5A) and significantly increased myelin

184 sheath formation (figure 5B-C), similarly to BQ3020. Second, we used zebrafish, due to their

185 amenability for rapid pharmacological treatment and assessment, to test the prediction that the

186 effects on myelination caused by the loss of EDNRB signalling would be rescued by activating the

187 downstream target PKCε in vivo. We obtained a zebrafish line containing a mutation termed rose

188 (*rse*) in one of the orthologues of *Ednrb (ednrba)*. Analysis of individual oligodendrocytes enabled by

189 sparse labelling of these cells in the fish larvae revealed the same phenotype as in the EDNRB cKO

190 mice; the average number of myelin sheaths formed per oligodendrocyte was reduced by 31% in *rse*

191 homozygous fish compared to wild type controls (figure 5F-G, figure 5 - figure supplement 2A),

192 while sheath length was unaffected (figure 5 -figure supplement 2B-C). As predicted, activation of

193 PKCε by FR236924 treatment from 3-4 days post fertilisation, rescued myelin sheath number in *rse*

194 homozygous fish (figure 5D-G). This result confirms a role for PKCε downstream of EDNRB in the

195 regulation of myelin sheath number in vivo.

196

8

197 ***Increasing EDNRB signalling rescues the reduction in myelin sheath number resulting from social***

198 ***isolation.***

199 Our results have shown that social isolation decreases the levels of EDN expression in the CNS

200 vasculature, and that EDNRB signalling in oligodendrocytes influences myelin sheath number. It

201 follows that the decreased EDN signalling is likely contributing to the reduction in myelin sheath

202 numbers seen in the prefrontal cortex following social isolation. If so, then we would predict that

203 activation of EDNRB in vivo in the prefrontal cortex would rescue the myelin sheath phenotype

204 associated with social isolation. To test this we used intranasal administration, a technique shown to

205 enable the delivery of peptides into the CNS (Scafidi et al., 2014, Crowe et al., 2018) to introduce the

206 EDNRB agonist BQ3020. Socially isolated mice were given 1µg BQ3020 twice daily intranasal

207 administration or saline for a period of 10 days from postnatal day 21 to 31 (during the period of

208 isolation), and perfused at postnatal day 35 for analysis of mPFC myelination as above. Importantly,

209 we confirmed that the daily handling involved in this protocol did not negate the effects of social

210 isolation by showing that the sheath numbers in the control isolated mice administered saline had

211 the same reduction in sheath numbers as completely isolated animals (figure 6B and D-E). Strikingly,

212 and in keeping with our prediction, 10 days of BQ3020 treatment to socially-isolated animals

213 rescued the myelination phenotype normally seen in these mice, with a 20% increase in the number

214 of myelin sheaths formed by mPFC oligodendrocytes in treated animals, meaning that the sheath

215 numbers were now not significantly different to wild-type animals housed in groups (figure 6D-E).

216

217 **Discussion**

218

219 Here we identify a novel signalling pathway in which the oligodendroglial G-protein coupled receptor

220 EDNRB regulates myelin sheath number and the response of oligodendrocytes to social experience

221 (figure 6 - figure supplement 21A). We studied mice following a previously established experimental

222 manipulation in which early postnatal social isolation leads to a reduction in the number of myelin

223 sheaths formed by individual oligodendrocytes in the medial prefrontal cortex (mPFC) of mice. We

224 showed that isolated animals have reduced EDN mRNA expression in the blood vessels of the mPFC.

225 We found that perturbing endothelin signalling in oligodendroglia by a conditional knockout of the

226 EDN receptor EDNRB phenocopies the reduced number of sheaths seen in the isolated animals. In

227 turn intranasal administration of an EDNRB agonist during the period of isolation rescues the effects

228 of social deprivation on myelination. Together with additional experiments in cell culture and

229 zebrafish, which identified the protein kinase C (PKC) epsilon isoform as being activated downstream

230 of EDNRB to regulate myelination by oligodendrocytes, our loss- and gain-of-function experiments

231 demonstrate a role for endothelin signalling in regulating the number of myelin sheaths formed by

232 individual oligodendrocytes.

233 Our work provides evidence for the importance of the interaction between the vasculature and

234 oligodendroglia during myelination per se, extending observations indicating that the vasculature

235 can influence earlier stages of the oligodendrocyte lineage. For example, during development,

236 oligodendrocyte progenitor cells use blood vessels as a scaffold to migrate along (Tsai et al., 2016).

237 Once in place, local oxygen levels influence their differentiation into oligodendrocytes, stalling the

238 process when levels are low. Only when an adequate oxygen supply is established are the cells able

239 to differentiate and begin the process of myelination (Yuen et al., 2014). Here, we demonstrate a

240 third role – the regulation of myelination itself through the control by EDN of myelin sheath number

241 by individual differentiated oligodendrocytes. Such a role is potentially important, as it provides an

242 indirect mechanism by which the regulation of myelination in the CNS might be linked to levels of

243 neuronal activity. A key function of myelin is the provision of metabolic substrates to the underlying

244 axon via specific transporters within the inner layer of the sheath (Fünfschilling et al., 2012, Lee et

245 al., 2012b, Meyer et al., 2018). These substrates drive ATP production within the axon, so providing a

246 source of energy at some distance from the cell body as required to sustain axonal activity. Prior

247   work has implicated that NMDA receptor activation in oligodendrocytes following glutamate release

248   by active axons in relaying the need for metabolic support of axons to oligodendrocytes (Saab et al.,

249   2016). We now propose that an increase in EDN production by endothelial cells, as predicted to

250   occur in the presence of increased blood flow or hypoxia, will increase myelin sheath generation by

251   local oligodendrocytes and so provide a further means of linking energy supply and demand.

252   Changes in blood flow and hypoxaemia are associated with increased cortical activity (forming the

253   basis of the BOLD signal detected by fMRI). Therefore, an EDN-mediated pathway driving an increase

254   in myelination is a plausible mechanism for ensuring that the metabolic demands of active axons are

255   met. Further work examining the effect of EDNRB loss in oligodendrocytes on axonal function and

256   viability in ageing are required to test this further.

257   A second important implication of our study relates to the emerging concept of adaptive

258   myelination. This concept argues that myelination in the CNS is plastic, and regulated by differing

259   codes of activity, each eliciting different effects on myelination that in turn alter circuit function. This

260   hypothesis therefore proposes that adaptive myelination represents a form of neural plasticity that

261   could, like synaptic plasticity, enable the brain to modify circuit function in response to experience-in

262   other words, to adapt. However, this hypothesis remains to be fully explored, and a key requirement

263   for these experiments is the identification of the mechanisms that link changes in activity patterns

264   following experience to the changes in oligodendrocyte number and/or sheath formation that could

265   alter circuit function. Prior work has identified a direct role for axon-derived signals in regulating

266   myelination. We now identify a mechanism by which the effects of activity could lead to an

267   increased number of myelin sheaths through indirect signalling via the vasculature. Our findings

268   showing that social isolation reduces endothelial EDN expression, that an oligodendroglial-specific

269   deletion of the relevant EDNRB receptor phenocopies the myelination defect caused by social

270   isolation, and that an EDNRB agonist rescues the myelination defect of individual oligodendrocytes

271   in the mPFC together argue strongly for a role of vascular EDN synthesis in mediating such indirect

272   effects. Our results do not however imply that changes in EDN signalling are solely responsible for

273   the effects of social isolation on myelination. It is clear, for example, that social isolation can affect

274   oligodendrocyte number, a feature that does not appear to be controlled by EDN in the healthy

275   nervous system. Other pathways could contribute to this aspect of adaptive myelination. Such

276   potential pathways are BDNF signalling via oligodendroglia TrkB (Geraghty et al., 2019, Gibson et al.,

277   2019) and glutaminergic signalling via AMPAR-mediated effects on newly differentiating

278   oligodendrocytes (Kougioumtzidou et al., 2017). Further work manipulating EDN expression

279   specifically in endothelial cells during social isolation is required to explore this.

280

281   Based on recent live imaging studies showing that myelin sheath numbers in cortical

282   oligodendrocytes remain largely stable throughout life (Hill et al., 2018, Hughes et al., 2018), we

283   propose that any endothelin-mediated regulation of sheath number is likely to occur around the

284   birth of each newly formed oligodendrocyte. The continuous generation of oligodendrocytes in the

285   adult CNS, itself increased by activity-related signals ensures that dynamic regulation of endothelin

286   signalling from the vasculature has the capacity to play a role in adaptive myelination throughout

287   life.

288   How though might new myelin sheaths formed by individual oligodendrocytes in response to EDN

289   signalling enhance circuit function the cortex? By linking metabolic demand to metabolic support for

290   axons in regions of high activity (as discussed above), EDN signalling in cortical oligodendrocytes

291   could play a central role in enhancing the ability of the axon to sustain higher levels of energy-

292   requiring conduction, so enabling changes in circuit function. An intriguing possibility suggested by a

293   comparison of gene expression in cortical oligodendroglia and spinal cord oligodendroglia showing

294   higher levels of expression of EDNRB in the former (Horiuchi et al., 2017, Marques et al., 2018), is

295   that these cortical oligodendrocytes are specialised for this role linking metabolic demand to

296   support. By contrast, oligodendrocytes in white matter might be more specialised for rapid axonal

297   conduction. Further work examining oligodendrocyte heterogeneity between grey matter and white

298   matter regions of the CNS will be required to test this. Another way in which additional myelin

299   sheaths might impact circuit function is the addition of sheaths to axons in the cortex that are

300   discontinuously and sparsely myelinated (Tomassy et al., 2014, Hill et al., 2018, Hughes et al., 2018).

301   The resulting effects on conduction velocity resulting from short stretches of axons now supporting

302   rapid saltatory conduction could, as has been suggested elsewhere, enable activity-dependent

303   myelination to have a role in signal synchrony (Seidl et al., 2010, Seidl et al., 2014, Freeman et al.,

304   2015, Baraban et al., 2016, Timmler and Simons, 2019). However, such a model requires a very

305   precise link between axonal selection and myelination, and the precision required may be difficult to

306   achieve with a diffusible vascular-derived signal.

307   Our findings on the role of EDN in myelination complement other investigations demonstrating a

308   role of astrocytic EDN hindering oligodendrocyte differentiation during remyelination (Hammond et

309   al., 2014, Hammond et al., 2015). In agreement with this observation, our global EDNRB mutant

310   zebrafish were found to have an increased number of oligodendrocytes (figure 5 - figure supplement

311   1). Together previous studies and ours suggest that EDN signalling plays two roles, indirectly

312   inhibiting oligodendrocyte formation, but, promoting myelin sheath formation once differentiation

313   has occurred. At apparent odds with this conclusion, conditional loss of EDNRB from

314   oligodendrocyte progenitors had no effect on remyelination; having deleted OPC EDNRB from adult

315   mice 3 days prior to demyelination of the external capsule, Hammond and colleagues observed that

316   the percentage of remyelinated axons, assessed by electron microscopy, was unchanged 14 days

317   after injury (Hammond et al., 2015). However, this divergence may result from the different

318   microenvironments of developmental myelination and remyelination or from intrinsic differences

319   between cortical and white matter oligodendrocytes, as discussed above, and further studies

320   examining grey matter remyelination are required to resolve the point.

321   The conclusion that EDN has contrasting effects on oligodendrocyte formation and on myelination

322   highlights that these two steps in oligodendrocyte development are regulated independently. This in

323   turn has important implications for the development of regenerative therapies for the progressive

324   neurodegenerative phase of the demyelinating disease Multiple Sclerosis (MS). Current strategies

325   seeking to re-purpose FDA approved drugs to enhance remyelination, and so restore the

326   neuroprotective effect of the myelin sheath to the axon, have focused largely on the differentiation

327   step from precursor cell to oligodendrocyte, with one screen using micropillar arrays to examine the

328   later stage of wrapping (Mei et al., 2014). None however specifically examine the key final step-

329   formation of the sheath itself. Neuropathological studies of MS lesions revealing pre-myelinating

330   oligodendrocytes unable to complete remyelination suggest for that failure of this final step may

331   contribute to pathology. These findings emphasise that the already differentiated oligodendrocyte

332   represents a possible target for remyelination strategies in MS, a conclusion reinforced by recent

333   papers using Carbon14 dating, electron microscopy or snRNA seq of post mortem human MS

334   material providing evidence that pre-existing adult oligodendrocytes contribute to repair (Duncan et

335   al., 2018, Jäkel et al., 2019, Yeung et al., 2019). Strategies to identify targets within pathways such as

336   that activated by EDNRB that promote sheath formation directly will therefore be an important

337   addition to the current approaches being taken in drug discovery for progressive multiple sclerosis.

338

346

347   **Competing interests**

348   No competing interests declared

349

350   **Figure legends**

351   **Figure 1 – Social isolation in mice reduces layer II/III medial prefrontal cortex oligodendrocyte**

352   **myelin sheath number.**

353   A Timeline for social isolation experiment. At postnatal day 21 male mice were housed in a social

354   environment containing 3-5 mice or on their own in isolation. Mice were analysed at P35.

355   B Schematic of social interaction assay. Mice were recorded for 5 minutes exploring an arena

356   containing 2 identical wire mesh containers: one container housed an unrelated male wild type

357   mouse (social zone), while the other remained empty (non-social zone).

358   C Time spent within 2.5cm of non-social container: Grouped 37.58 seconds ± 8.683 n=12, Isolated

359   45.59 ± 36.28 n=9 and social container: Grouped 110.7 seconds ± 21.71 n=12, Isolated 74.24 seconds

360   ± 37.07 n=9 (mean ± standard deviation). Unpaired T-test p=0.0107.

361   D Coronal section of mouse prefrontal cortex stained for CNPase and nuclei. Layers II/III of the

362   medial prefrontal cortex outlined by dashed box. Scale bars = 100 μm.

363   E Layer II/III oligodendrocyte stained for CNPase and MBP.

364   F Representative images of medial prefrontal cortex oligodendrocytes stained for CNPase.

365   G Mean number of myelin sheath formed by oligodendrocytes per mouse. Grouped 47.66 ± 1.015

366   n=7 mice, Isolated 37.11 ± 0.6425 n=4 mice (mean ± standard error). Mann-Whitney test, p=0.0106.

367 H Pooled data for number of myelin sheaths formed by layer II/III medial prefrontal cortex

368 oligodendrocytes. Grouped 47.80 ± 7.289 n=49 cells from 7 mice, Isolated 37.11 ± 5.202 n=28 cells

369 from 7 mice (mean ± standard deviation). Mann-Whitney test, p= <0.001.

370 **Figure 2 – Social isolation reduces vascular endothelin expression.**

371 A *Edn1* and *Edn3* mRNA expression in laminin positive and CD31 positive blood vessels as revealed

372 by RNAScope in situ hybridisation.

373 B Representative images of *Edn1* and *Pecam1* mRNA expression in the mPFC.

374 C Quantification of the number of *Edn1* expression *Pecam1* positive endothelial cells. Grouped

375 0.8033 ± 0.04411 n=4 mice, Isolated 0.5074 ± 0.05412 n=5 mice (mean ± standard error). Mann-

376 Whitney test, p=0.0159.

377 D Quantification of the mean *Edn1* mRNA molecules expressed by *Pecam1* positive cells per mouse.

378 Grouped 12.29 ± 3.312 n=5 mice, Isolated 4.673 ± 0.4059 n=4 mice (mean ± standard error). Mann-

379 Whitney test, p=0.0159.

380

381 **Figure 3 – Loss of oligodendroglial EDNRB reduces myelin sheath number and reduces sociability.**

382 A Quantification of CNP positive cells in medial prefrontal cortex layers II/III: Wild type 7709 ± 378.7

383 n=5 mice, EDNRB CKO 7288 ± 1054 n=4 mice (mean ± standard error) and layer V: Wild type 18879 ±

384 1559 n=9 mice, EDNRB CKO 21016 ± 2878 n=3 mice (mean ± standard error). Mann Whitney test,

385 layer II/III p= 0.9048, layer V p= 0.3527.

386 B Representative images of medial prefrontal cortex oligodendrocytes stained for CNPase. Scale bar

387 = 20 μm.

388   C Mean number of myelin sheath formed by oligodendrocytes per mouse. Wild type 47.66 ± 1.015

389   n=7 mice, EDNRB CKO 37.39 ± 1.099 n=4 mice (mean ± standard error). Mann-Whitney test,

390   p=0.0106.

391   D Pooled data for number of myelin sheaths formed by layer II/III medial prefrontal cortex

392   oligodendrocytes. Wild type 47.80 ± 7.289 n=49 cells from 7 mice, EDNRB CKO 37.39 ± 7.208 n=28

393   cells from 7 mice (mean ± standard deviation). Mann-Whitney test, p= <0.001.

394   E Time spent within 2.5cm of non-social container: Wild type 37.58 seconds ± 8.683 n=12, EDNRB

395   CKO 41.06 ± 21.29 n=14 and social container: Wild type 110.7 seconds ± 21.71 n=12, EDNRB CKO

396   88.39 seconds ± 25.79 n=14 (mean ± standard deviation). Unpaired T-test p=0.0267.

397

398   **Figure 4 – EDNRB enhances myelin sheath number in vitro.**

399   A Representative images of MBP positive oligodendrocytes in microfiber culture. Scale bar = 50μm.

400   B Mean number of myelin sheaths formed by rat oligodendrocytes on microfibers per independent

401   culture preparation. Control 7.253 ± 0.4258 n=8 independent cultures, BQ3020 11.21 ± 0.58635 n=7

402   independent cultures, BQ788 6.026 ± 0.7046 (mean ± standard error) n=4 independent cultures. 1-

403   way ANOVA with Tukey's post hoc test.

404   C Pooled data for number of myelin sheaths formed by rat oligodendrocytes on microfibers. Control

405   7.194 ± 3.544 n=160 cells from 8 independent cultures, BQ3020 11.25 ± 4.420 n=127 from 7

406   independent cultures, BQ788 6.024 ± 3.059 n=85 cells from 4 independent cultures (mean ±

407   standard deviation). Kruskal-Wallis test, with Dunns post hoc.

408   D Frequency distribution of myelin sheath lengths formed on microfibers.

409   E qPCR for EDNRB from mouse oligodendrocyte cultures. Wild type 1.292 ± 0.04933 n=4

410   independent cultures, EDNRB CKO 0.5958 ± 0.1117 n=4 independent cultures, BQ788 6.026 ± 0.7046

411   (mean ± standard error). Unpaired T-test p=0.0013.

412   F Mean number of myelin sheaths formed by mouse oligodendrocytes on microfibers per

413   independent culture preparation. Wild type 6.18 ± 0.2082 n=5 independent cultures, Wild type +

414   BQ3020 9.732 ± 0.07548 n=4 independent cultures, EDNRB CKO 5.380 ± 0.4181 n=5 independent

415   cultures, EDNRB CKO + BQ3020 5.989 ± 0.6125 n=4 independent cultures (mean ± standard error). 1-

416   way ANOVA.

417   G Pooled data for number of myelin sheaths formed by mouse oligodendrocytes on microfibers.

418   Wild type 6.138 ± 1.499 n=65 cells from 5 independent cultures, Wild type + BQ3020 9.705 ± 2.575

419   n=44 from 4independent cultures, EDNRB CKO 5.345 ± 1.824 n=65 cells from 5independent cultures,

420   EDNRB CKO + BQ3020 5.955 ± 2.632 n=44 cells from 4 independent cultures (mean ± standard

421   deviation). Kruskal-Wallis test, with Dunns post hoc.

422

423   **Figure 5 – Protein kinase C epsilon is downstream of EDNRB to regulate myelin sheath number.**

424   A Western blot images of rat oligodendrocytes treated with EDNRB agonist BQ3020 and PKCε

425   agonist FR 236924 for 15 minutes. Antibodies used: Phosphorylated PKCε S729 and loading control

426   GAPDH.

427   B Mean number of myelin sheaths formed by rat oligodendrocytes on microfibers per experiment.

428   Control 5.959 ± 0.4708 n=4, FR 236924 9.542 ± 0.3614 n=3 (mean ± standard error). Unpaired T-test

429   p=0.0024.

430   C Pooled data for number of myelin sheaths formed by rat oligodendrocytes on microfibers. Control

431   5.952 ± 2.525 n=62 cells from 4 experiments, FR 236924 9.542 ± 4.016 n=48 from 3 experiments

432   (mean ± standard deviation). Mann-Whitney test, p= <0.001.

18

433 D Schematic for zebrafish larvae treatment with FR 236924.

434 E Representative images of 4 dpf zebrafish larvae treated with DMSO control or FR 236924. Scale bar

435 = 500µm.

436 F Representative images of mbp:EGFP-CAAX oligodendrocytes in 4 dpf zebrafish larvae. Scale bar =

437 10µm.

438 G Pooled data for number of myelin sheaths formed by zebrafish oligodendrocytes. EDNRB Het

439 12.35 ± 3.746 n=20 cells, EDNRB Hom 9.073 ± 2.229 n=41 from 4 experiments, EDNRB Het + FR

440 236924 13.5 ± 3.098 n=22 cells from 5 experiments, EDNRB Hom + FR 236924 14 ± 4.807 n=19 cells

441 (mean ± standard deviation). 1-way ANOVA.

442

443 **Figure 6 – Intranasal administration of an EDNRB agonist rescues the myelin sheath number**

444 **reduction caused by social isolation.**

445 A Timeline for the intranasal experiment. At postnatal day 21 male mice were housed on their own

446 in isolation. Mice were given 2 daily administrations of saline or EDNRB agonist BQ3020 from P21-

447 P30. Mice were analysed at P35.

448 B Time spent within 2.5cm of non-social container: Saline 57.42 seconds ± 24.44 n=12, BQ3020 41.44

449 ± 17.43 n=17 and social container: Saline 75.82 seconds ± 13.26 n=12, BQ3020 84.96 seconds ±

450 37.20 n=17 (mean ± standard deviation). T-test.

451 C Quantification of CNP positive cells in medial prefrontal cortex layers II/III: Saline 7278 ± 1165 n=3

452 mice, BQ3020 9142 ± 713.3 n=3 mice (mean ± standard error) and layer V: Saline 17114 ± 2750 n=5

453 mice, BQ3020 16154 ± 1980 n=4 mice (mean ± standard error).

454 D Mean number of myelin sheath formed by oligodendrocytes per mouse. Saline 39.54 ± 0.4301 n=4

455 mice, BQ3020 47.29 ± 1.687 n=4 mice (mean ± standard error). Mann-Whitney test, p=0.0286.

456   E Pooled data for number of myelin sheaths formed by layer II/III medial prefrontal cortex

457   oligodendrocytes. Saline 39.54 ± 6.973 n=28 cells from 4 mice, BQ3020 47.29 ± 10.18 n=28 cells from

458   7 mice (mean ± standard deviation). Mann-Whitney test, p=0.0019.

459

460   **Figure 1- figure supplement 1 – Z-stack through a CNPase positive oligodendrocytes.**

461   A full maximum intensity projection of a complete a layer II/III oligodendrocytes in mouse cortex

462   stained for CNPase followed by 0.5 µm slices. Tracing the fine processes through each slice enables

463   assessment of the myelin sheaths formed by individual oligodendrocytes.

464

465   **Figure 1- figure supplement 2 – Social isolation reduces oligodendrocyte generation.**

466   A Distance travelled by mice during 5 minutes of exploration of the social interaction assay. Grouped

467   15.48 meters ± 3.326 n=12 mice, Isolated 16.15 meters ± 3.57 n=9 mice (mean ± standard deviation).

468   B Maximum speed travelled by mice during 5 minutes of exploration of the social interaction assay.

469   Grouped 0.2433 ± 0.04117 n=12 mice, Isolated 0.2619 meters ± 0.02792 n=9 mice (mean ± standard

470   deviation).

471   C Quantification of CNP positive cells in medial prefrontal cortex layers II/III: Grouped 7709 ± 378.7

472   n=5 mice, Isolated 4738 ± 385.7 n=4 mice (mean ± standard error).

473   D Quantification of CNP positive cells in medial prefrontal cortex layer V: Grouped 18879 ± 1559 n=9

474   mice, Isolated 13099 ± 1052 n=9 mice (mean ± standard error).

475   E Mean myelin sheath length formed by oligodendrocytes per mouse. Grouped 46.10 µm ± 2.485

476   n=7 mice, Isolated 51.48 µm ± 4.08 n=4 mice (mean ± standard error).

477   F Frequency distribution of myelin sheath lengths.

478

Figure 1- figure supplement 3 – Loss of oligodendroglial EDNRB reduces myelin sheath number in the visual cortex where social isolation does not affect myelination or *Edn1* expression.

A Mean number of myelin sheath formed by oligodendrocytes per mouse. Wild type 52.57 ± 1.270 n=4 mice, EDNRB CKO 42.39 ± 1.487 n=4 mice, Isolated 50.32 ± 0.8755 n=4 mice (mean ± standard error). Mann-Whitney test, p=0.0106. Kruskal-Wallis test, with Dunns post hoc.

B Pooled data for number of myelin sheaths formed by layer II/III visual cortex oligodendrocytes. Grouped 52.57 ± 8.779 n=28 cells from 4 mice, EDNRB CKO 42.39 ± 7.213 n=28 cells from 4 mice, Isolated 50.32 ± 5.716 n=28 cells from 4 mice (mean ± standard deviation). Kruskal-Wallis test, with Dunns post hoc.

C Quantification of the number of *Edn1* expressing *Pecam1* positive cells. Grouped 0.6449 ± 0.02814 n=4 mice, Isolated 0.63535 ± 0.02120 n=4 mice (mean ± standard error). Mann-Whitney test.

490

Figure 2- figure supplement 1 – EDN mRNA is not expressed in astrocytes and microglia.

Expression of *Edn1*, *Edn2* and *Edn3* in S100β positive astrocytes and IBA1 positive microglia. A RNAScope in situ hybridisation for *Edn1*, *Edn2* and *Edn3* mRNA in the mouse medial prefrontal cortex stained for S100β positive astrocytes. Note that S100β positive cells are negative for *Edn1* and *Edn3* mRNA while positive *Edn* signal can be seen in S100β negative cells.

B RNAScope in situ hybridisation for *Edn1*, *Edn2* and *Edn3* mRNA in the mouse medial prefrontal cortex stained for Iba1 positive microglia. Note that Iba1 positive cells are negative for *Edn1*and *Edn3*mRNA while positive *Edn* signal can be seen in Iba1 negative cells.

499

500 **Figure 2 - figure supplement 2 – Social isolation does not affect medial prefrontal cortex**

501 **vasculature.**

502 A Representative images of medial prefrontal cortex vasculature staining for PECAM1.

503 B Quantification of PECAM1 area in medial prefrontal cortex layer II/III: Grouped 2.468 % ± 0.3156

504 n=6 mice, Isolated 2.086 ± 0.13 n=6mice (mean ± standard error). Mann-Whitney test.

505 C Quantification of number of *Pecam1* mRNA expressing cells per field of view. Grouped 9 ± 0.5888

506 n=4 mice, Isolated 8.44 ± 1.162 n=4 mice (mean ± standard error). Mann-Whitney test.

507 D Quantification of the number of *Pecam1* mRNA molecules. Grouped 13.47 ± 6.204 n=77 cells from

508 4 mice, Isolated 10.89 ± 5.02 n=92 cells from 5 mice (mean ± standard deviation). Mann-Whitney

509 test, p= 0.002.

510 E Quantification of the number of *Pecam1* mRNA molecules per mouse. Grouped 13.53 ± 1.305 n=4

511 mice, Isolated 10.48 ± 1.018 n=5 mice (mean ± standard error). Mann-Whitney test.

512 F Quantification of the number of *Edn1* mRNA molecules in *Pecam1*positive endothelial cells.

513 Grouped 12.08 ± 9.759 n=77 cells from 4 mice, Isolated 4.837 ± 4.406 n=92 from 5 mice (mean ±

514 standard deviation). Mann-Whitney test, p< 0.0001.

515 G Quantification of the number of *Edn3* expressing *Pecam1* positive endothelial cells. Grouped

516 0.5463 ± 0.01586 n=5 mice, Isolated 0.3674 ± 0.02163 n=5 mice (mean ± standard error). Mann-

517 Whitney test, p=0.0079.

518

519 **Figure 3 - figure supplement 1 – Conditional EDNRB knock out does not affect oligodendrocyte**

520 **generation or myelin sheath length**.

521 A Representative Images of *Ednrb* mRNA expressing *Olig2* mRNA positive oligodendroglia.

522   B Ratio of CC1 positive oligodendrocytes over total population of Olig2 positive oligodendroglia. Wild

523   type 0.2086 ± 0.01087 n=5 mice, EDNRB CKO 0.2003 ± 0.0248 n=5 mice (mean ± standard error).

524   C Number of CC1 and Olig2 positive oligodendrocytes. Wild type 1626 ± 170.6 n=5 mice, EDNRB CKO

525   1419 ± 220.5 n=5 mice (mean ± standard error).

526   D Number Olig2 positive oligodendroglia. Wild type 7737 ± 903.8 n=5 mice, EDNRB CKO 7565 ± 1239

527   n=5 mice (mean ± standard error).

528   E Mean myelin sheath length formed by oligodendrocytes per mouse. Wild type 46.10 μm ± 2.485

529   n=7 mice, EDNRB CKO 49.64 μm ± 4.688 n=4 mice (mean ± standard error).

530   F Frequency distribution of myelin sheath lengths.

531   G Distance travelled by mice during 5 minutes of exploration of the social interaction assay. Wild

532   type 15.48 meters ± 3.326 n=12 mice, EDNRB CKO 17.62 meters ± 3.677 n=9 mice (mean ± standard

533   deviation).

534   H Maximum speed travelled by mice during 5 minutes of exploration of the social interaction assay.

535   Wild type 0.2433 ± 0.04117 n=12 mice, EDNRB CKO 0.2748 meters ± 0.05095 n=9 mice (mean ±

536   standard deviation).

537

538   **Figure 5 - figure supplement 1 – Global loss of EDNRB increases the number of oligodendrocytes in**

539   **the zebrafish spinal cord.**

540   A Representative images of MBP positive oligodendrocytes in the zebrafish spinal cord.

541   B Oligodendrocyte cell numbers in the dorsal tract: EDNRB Het 20.23 ± 5.639, EDNRB Hom 21.21 ±

542   7.138, ventral tract: EDNRB Het 39.09 ± 7.157, EDNRB Hom 45.47 ± 7.09 and total: EDNRB Het 59.32

543   ± 9.766, EDNRB Hom 66.79 ± 11.73. EDNRB Het n=22 , EDNRB Hom n=14. T-test, Ventral p=0.0119,

544   Total p=0.0463.

545

Figure 5 - figure supplement 2 - Global loss of EDNRB reduces the number of myelin sheath formed by zebrafish oligodendrocytes.

A Pooled data for number of myelin sheaths formed by zebrafish oligodendrocytes. Wild Type 11.9 ± 3.726 n=20 cells, EDNRB Het 11.44 ± 1.999 n=16 cells, EDNRB Hom 8.19 ± 2.994 n=21 cells from 4 experiments, (mean ± standard deviation). 1-way ANOVA.

B Pooled data for mean myelin sheath length formed by zebrafish oligodendrocytes. Wild Type 28.45 ± 6.8 n=20 cells, EDNRB Het 33.04 ± 8.629 n=16 cells, EDNRB Hom 32.65 ± 7.098 n=21 from 4 experiments, (mean ± standard deviation). 1-way ANOVA.

C Frequency distribution of myelin sheath length.

555

Figure 5 - figure supplement 3 – Protein kinase C epsilon activation does not affect myelin sheath length.

A Mean myelin sheath lengths formed by oligodendrocytes in microfiber cultures. Control 16.87 µm ± 0.7932, FR 16.42 µm ± 1.176 n=3-4 (mean ± standard error).

B Pooled data for number myelin sheath length formed by zebrafish oligodendrocytes. EDNRB Het 29.62 ± 8.685 n=20 cells, EDNRB Hom 28.27 ± 7.242 n=41 from, EDNRB Het + FR 236924 25.86 ± 10.25n=21 cells, EDNRB Hom + FR 236924 26.48 ± 8.951 n=19 cells (mean ± standard deviation).

563

Figure 6 - figure supplement 1 – Intranasal administration of EDNRB agonist BQ3020 does not affect myelin sheath length.

566   A Distance travelled by mice during 5 minutes of exploration of the social interaction assay. Saline

567   17.48 meters ± 2.697 n=12 mice, BQ3020 17.13 meters ± 35.209 n=17 mice (mean ± standard

568   deviation).

569   B Maximum speed travelled by mice during 5 minutes of exploration of the social interaction assay.

570   Saline 0.2769 ± 0.03276 n=12 mice, BQ3020 0.2773 meters ± 0.05085 n=17 mice (mean ± standard

571   deviation).

572   C Mean myelin sheath length formed by oligodendrocytes per mouse. Saline 59.38 µm ± 4.831 n=28

573   cells from 4 mice, BQ3020 53.98 µm ± 5.748 n=28 cells from 4 mice (mean ± standard deviation). D

574   Frequency distribution of myelin sheath lengths.

575

576   **Figure 6 - figure supplement 2 – Proposed model for how EDNRB regulates myelin sheath number.**

577   A Summary of results from paper. In a social environment, EDN from the vasculature increases

578   myelin sheath number in the medial prefrontal cortex, influencing sociability. In social isolation

579   vascular EDN production is reduced, leading to a decreased number of myelin sheaths formed by

580   oligodendrocytes and reduced sociability. Oligodendroglial knock out of EDNRB reduces the number

581   of myelin sheaths formed by oligodendrocytes and reduced sociability.

582   B Proposed hypothesis for how EDN signalling links neuronal activity to increased myelination and

583   thus support of active axons. 1. Increased local neuronal activity signals to the vasculature to

584   increase blood flow to the active area 2. The increased blood flow stimulates EDN production from

585   endothelial cells 3. Increased EDN levels leads to the formation of more myelin sheaths by

586   oligodendrocytes. Figures created with Biorender.

587

588   **Supplementary file 1 – Antibody array of phosphorylation events downstream of EDNRB.**

589   Wild type mouse oligodendrocytes were starved for 4-5 hours in media devoid of supplementation

590   and then treated for 15 minutes with either vehicle or BQ3020 (100 ng/mL). For each antibody the

591   background intensity was subtracted, dye signal normalised and an average calculated of the

592   duplicate spots. The ratio was calculated of binding to the phosphorylated amino acids vs the

593   binding to the non-modified regions of the protein for each molecule, calculating this for both

594   control and BQ3020 treated cells. The fold change in phosphorylation for each targeted amino acid

595   was generated by comparing BQ3020 to vehicle. For selection a fold change of greater than 2 and

596   less than 0.5 was set as the cut-off. Antibody array was performed once – one cell lysate per

597   condition.

598

599   **Key Resources Table**

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (M. musculus)	Ednrb flox/flox	The university of Edinburgh, Bagnall et al., 2006, Ge et al., 2006		
Genetic reagent (M. musculus)	Pdgfra-cre	Jackson labs	#013148	
Genetic reagent (D. rerio)	Rse Tlf802	Frohnhöfer et al., 2013, Krauss et al., 2014		

Genetic reagent (D. rerio)	Tg(mbp:EGFP)	The University of Edinburgh, Almeida et al., 2011		
Antibody	Mouse monoclonal anti CNPase	Atlas	AMAb91072	1:2000
Antibody	Rat monoclonal anti MBP	Serotec	MCA409S	1:250
Antibody	Rat monoclonal anti PECAM1	BD Pharmingen	550274	1:100
Antibody	Rabbit monoclonal anti S100β	Thermo	MA5-12969	1:100
Antibody	Rabbit monoclonal anti IBA1	Abcam	ab178846	1:500
Antibody	Rabbit polyclonal anti Laminin	Abcam	ab11575	1:300
Antibody	Rabbit polyclonal anti OLIG2	Millipore	ab9610	1:100
Antibody	Mouse monoclonal anti CC1	Abcam	ab16794	1:300

Antibody	Mouse monoclonal anti GAPDH	Millipore	MAB374	1:1000
Antibody	Mouse monoclonal anti Beta actin	Abcam	ab 8226	1:1000
Antibody	Rabbit polyclonal anti , Phospho-PKCε S729	Abcam	88241	1:1000
Commercial assay, kit	Phospho-explorer antibody array	Full Moon Biosystems,	Phospho-explorer array (PEX100)	
Sequence-based reagent	*Edn1*	Advanced Cell Diagnostics	435221	
Sequence-based reagent	*Edn2*	Advanced Cell Diagnostics	418221	
Sequence-based reagent	*Edn3*	Advanced Cell Diagnostics	Custom made	
Sequence-based reagent	*Pecam1*	Advanced Cell Diagnostics	316721-C3	
Sequence-based reagent	*Olig2*	Advanced Cell Diagnostics	447091	
Sequence-based reagent	*Ednrb*	Advanced Cell Diagnostics	473801	
Peptide, recombinant protein	BQ3020	Tocris	1189	100 ng/mL

Chemical compound, drug	BQ788	Tocris	1500	100 ng/mL
Chemical compound, drug	FR236924	Tocris	0373	25 µM
Other	Microfibers	The Electrospinning company		1-2 mcro diameter poly-l-lactic acid
Software, algorithm	Any-maze software	http://www.anymaze.co.uk/		
Software, algorithm	ImageJ	https://imagej.nih.gov/ij/		
Software, algorithm	Graphpad Prism	https://www.graphpad.com/scientific-software/prism/		

600

601

Materials and Methods

Mice

Animal husbandry and experiments were performed under UK Home Office project licenses issued

under the Animals (Scientific Procedures) Act. *Ednrb*flox/flox (Bagnall et al., 2006, Ge et al., 2006) were

generously provided by Professor David Webb and Professor Yuri Kotelevtsev (Edinburgh University)

where exons 3 and 4 are flanked with Cre-LoxP sites. Homozygous mice for the EDNRB floxed allele

were crossed to *Pdgfra*-cre mice obtained from Jackson laboratories (013148). Offspring were then

backcrossed to create mice Heterozygous for the floxed allele and carriers for the Cre transgene.

Experimental mice were obtained by crossing animals heterozygous for the floxed *Ednrb* allele and

carriers of the Cre transgene generating both *Ednrb* wild type control and *Ednrb* floxed homozygous

mice in the same litters. Mice were genotyped by transnetyx and confirmed as a CKO by performing

613   RNAScope for *Ednrb* (described below) Mice of each genotype were used at the ages and in the

614   numbers stated in the Results. For the social isolation experiments, postnatal day 21 male mice were

615   either group housed in a regular social environment, containing 3 – 5 mice, or in isolation, for 2

616   weeks.

617   **Sociability Test**

618   At postnatal day 35 mice were allowed to freely explore an open field arena for 5 minutes containing

619   two identical wire mesh containers. One container housed an unrelated male wild type mouse of a

620   similar age, while the other remained empty. Mice were allowed to explore the arena for 5 minutes

621   and the duration of time during which the mouse of interest came within 2.5 cm of either container

622   was automatically recorded using Any-maze software. Speed and distance travelled were also

623   recorded by the software. In all tests, mice were assessed with the experimenter blind to genotype.

624   **Intranasal Administration**

625   Mice were given brief isoflurane inhalation anaesthesia before being held upside down. Using a

626   pipette, 5µL of either saline or 1 µg BQ3020 (Tocris - 1189) in saline was placed on either nostril. This

627   was repeated twice daily (9am and 5pm) from postnatal day 21 to postnatal day 31.

628   **Antibodies**

629   CNPase Atlas AMAb91072 (1:2000), MBP Serotec MCA409S (1:250), PECAM1 BD Pharmingen 550274

630   (1:100), S100ß Thermo MA5-12969 (1:100), IBA1 Abcam ab178846 (1:500), Laminin Abcam ab11575

631   (1:300), Olig2 Millipore AB9610 (1:100), CC1 Abcam ab16794 (1:300).

632   **Immunofluorescence Staining - Cryosections**

633   Animals were intracardially perfused with 4% PFA (wt/vol; Sigma) in PBS, after which brains were

634   post-fixed overnight and then cryoprotected in sucrose prior to embedding in OCT and storage at

635   −80°C. Brains were cryosectioned coronally at a thickness of 16µm using a Thermo cryostat and

636   mounted onto Superfrost Plus slides. Sections were blocked for 1 hour at room temperature in 10%

637 goat serum, 0.1% triton in PBS. Primary antibodies were incubated in block solution at 4°C overnight.

638 Sections were washed with PBS for 3x15 minutes at room temperature and stained using species-

639 specific Alexa fluorophore-conjugated antibodies in block solution for 1 hour at room temperature.

640 Sections were washed in PBS for a further 3x15 minutes and stained with Hoechst for 5 minutes and

641 mounted onto slides with fluoromount G. Mouse medial prefrontal cortex was defined as the

642 infralimbic and prelimbic areas between bregma 1.7 mm and 2 mm and visual cortex bregma -3.5

643 mm and -4.5 mm.

644 **Immunofluorescence Staining - Vibratome Sections for Oligodendrocyte morphological analysis**

645 Animals were intracardially perfused with 4% PFA (wt/vol; Sigma) in PBS, after which brains were

646 post-fixed overnight and embedded in 2% low melting point agarose. Using a Leica vibratome, 100

647 µM coronal free-floating sections were cut. Sections underwent antigen retrieval in 0.05% Tween20,

648 10 mM tri-sodium citrate (pH 6.0) at 95 °C for 20 min. Sections were then blocked for 3 hours at

649 room temperature in 10% goat serum, 0.25% triton in PBS. Primary antibodies were incubated in

650 block solution at 4°C on a rocker for 24 hours. Sections were washed with PBS for 3 hours at room

651 temperature and stained using species-specific Alexa fluorophore-conjugated antibodies in block

652 solution for 4 hours at room temperature. Sections were washed in PBS for a further 3 hours and

653 stained with Hoechst for 20 minutes and mounted onto slides with fluoromount G. Sections were

654 analysed with the experimenter blind to experimental condition and/or genotype as below.

655 To analyse oligodendrocyte number 10 fields of 40x magnification were taken from 2 100 µm

656 sections per mouse of cortical layer II/III or layer V using a SP8 confocal microscope. CNP positive,

657 Olig2 and CC1 cells were counted.

658 To analyse oligodendrocyte morphology, random areas of the medial prefrontal cortex layer II/III

659 were imaged at 63x magnification using an SP8 confocal microscope. 7 individual CNP positive

660 oligodendrocytes per mouse, each with all myelin sheaths present within the 100 µm section as

661 assessed by following each process from the cell body and ensuring none exited the section, were

31

662   imaged using a z step size of 0.5 µm. Analysis was performed using an ImageJ plugin – simple neurite

663   tracer (Longair et al., 2011).

RNAScope In situ Hybridisation

665   Cryosections (cut as above) were processed as recommended by Advanced Cell Diagnostics. Briefly

666   sections were dried overnight at 60°C, after which they were incubated at 40°C with pre-treatment 4

667   for 30 minutes before incubation with RNAScope probes for 2 hours at 40°C. RNAScope probes used

668   were; *Edn1* (435221), *Edn2* (418221), *Edn3* (custom made), *Pecam1* (316721-C3), *Oolig2* (447091-

669   C2), *Ednrb* (473801). Following the RNAScope protocol, sections were stained as above.

670   For analysis, 5 random 63x areas of medial prefrontal cortex layer II/III were imaged using a SP8

671   confocal microscope. Cells positive for *Pecam1* and *Edn1/3* were counted. Sections were analysed

672   with the experimenter blind to experimental condition and/or genotype.

Rat Oligodendrocyte Precursor Cell Culture

674   Rat OPCs were prepared from mixed glial cultures as described previously (McCarthy and de Vellis,

675   1980, Bechler et al., 2015). Briefly, cortices of postnatal day 0-2 Sprague Dawley rats were dissected

676   out. The tissue was digested with 1.2 Units/mL papain, 0.1 mg/mL L-cysteine and 0.40 mg/mL DNase

677   for 1 hour at 37°C. Tissue was cultured in DMEM, 10% FCS, 1% P/S in T75 flasks, pre-coated with 5

678   µg/mL poly-D-lysine, at a density of 1.5 brains per flask. Cells were grown at 37°C in 7.5% CO_2 with

679   medium changes every 2-3 days. After 10-12 days cells were mechanically separated on an orbital

680   shaker at 250 rpm, 37°C. Loosely attached microglia were removed by shaking for 1 hour. Further

681   shaking for 16-18 hours detached OPCs. Cell yield was counted using a haemocytometer and plated

682   in assay-dependent conditions as below.

Microfiber Cultures

684   Custom parallel-aligned microfibers were purchased from the Electrospinning Company. 1-2 micron

685   diameter poly-l-lactic acid microfibers were synthesised and suspended over plastic scaffolds fitting

686   into 12-well tissue culture plates. Microfibers were washed with 70% EtOH for 10 minutes followed

687   by coating with PDL for 1 hour at 37°C in a 12 well tissue culture plate. Microfibers were washed

688   twice with sterile water and left in preheated myelination media. 35,000 rat OPCs and 50,000 mouse

689   OPCs in myelination media (50:50 DMEM:Neurobasal Media, B27 (Invitrogen), 5 µg/mL N-acetyl

690   cysteine, and 10 ng/mL D-biotin, ITS , and modified Sato (100 µg/mL BSA fraction V, 60 ng/ml

691   Progesterone, 16 µg/ml Putrecsine, 400 ng/mL Tri-iodothyroxine, 400 ng/mL L-Thyroxine; reagents

692   from Sigma-Aldrich)) were triturated to break up cell clumps and added dropwise to the microfibers.

693   Cells were left to recover for 3 days before media changing and addition of treatment, followed by

694   subsequent media changes every 3 days.

695   After 14 days of culture cells were fixed in 4% PFA for 15 minutes. To visualise myelination, cells

696   were permeabilised with 0.1% Triton-X for 10 minutes and stained with for MBP (1:250) overnight at

697   4°C followed by incubation with Alexa 488-conjugated goat ant-rat (1:1000) for 1 hour at room

698   temperature and Hoescht for 5 minutes to visualise nuclei.

699   To analyse myelination 15-30 individual myelinating oligodendrocytes from one coverslip were

700   imaged (with the experimenter blind to condition) using an SP8 confocal at 40x magnification with a

701   z-step of 0.35 µm. The same settings (laser power, gain, offset etc.) were used between coverslips.

702   ImageJ was used to analyse myelination, again with the experimenter blind to condition. A sheath

703   was defined as a continuous MBP positive wrap fully surrounding a microfiber as assessed using the

704   0.35 µm z-series. Concentric tubes were traced and the length measured. In addition the number of

705   concentric sheaths made per individual oligodendrocyte was recorded. Sheath lengths were grouped

706   into 5 µm bins and the frequency from one experiment calculated. Mean frequencies from at least 3

707   experiments were generated and plotted as a frequency distribution.

708   Drugs used: BQ3020 (100ng/mL, Tocris - 1189) in 0.03M sodium bicarbonate, BQ788 (100ng/mL,

709   Tocris - 1500) in DMSO, FR236924 (25 µM, Tocris - 0373) in DMSO.

710   **Mouse Oligodendrocyte Precursor Cell Culture**

711   Mouse OPCs were isolated from P6-P9 pups as described (Watkins et al., 2008). Ear clips were taken

712   for subsequent genotyping. Briefly, cerebral cortices were dissected, diced and dissociated into

713   single-cell suspensions gently using MACS Neural Tissue Dissociation Kit P (130-092-628). Cells were

714   resuspended in 0.2% BSA, Insulin, PBS and transferred to treated tissue culture dishes coated with

715   BSL1 (L-1100, Vector Labs) for 15 minutes twice. Cell solutions were then transferred to dishes

716   coated with anti-PDGFRα (CD140a) for 45 minutes. Solutions were aspirated and attached cells

717   washed twice with media and removed with a cell scraper. All collected cells were added to vented

718   T75 flasks and grown at 37°C 7.5 CO_2. Cells were grown in myelination media containing PDGF and

719   NT3 and changed every 2 days and supplemented daily with PDGF. After 7-9 days confluent flasks

720   were washed with PBS and then detached using TrypLE for 10 minutes at 37°C. Solutions were

721   centrifuged at 1000 rpm for 5 minutes, resuspended and counted using a haemocytometer.

722   **qPCR**

723   75,000 mouse OPCs were cultured on pre-coated PDL 6 well plates for 2 days. RNA was extracted

724   from cells using a Qiagen RNeasy mini kit. RNA was then converted to cDNA libraries using a

725   SuperScript® First-Strand Synthesis System. Sybr green qPCR was performed using primers either

726   bought from Qiagen or designed at 0.5 µM. qPCR was performed on a LightCycler 480 II. CT values

727   from designed primers were normalised against GAPDH purchased from Qiagen.

728   **Full Moon Phospho-Explorer Antibody Array**

729   Processing of the phospho-explorer array (PEX100) was performed following the Full Moon

730   Biosystems guidelines. OPCs were immunopanned from wildtype mice and expanded in the

731   presence of PDGF. Upon confluency, OPCs were differentiated through supplementation of CNTF,

732   NT3 and T3. After 2 days of differentiation cells were starved for 4-5 hours in media devoid of

733   supplementation to remove stimulation from the media and then treated for 15 minutes with either

734   vehicle or BQ3020 (100 ng/mL). Cells were lysed in RIPA buffer containing protease and phosphatase

735   inhibitors (Calbiochem – 539134 and 524621) and processed for the array as recommended by Full

736   Moon Biosystems.

737   Briefly, proteins present in the lysates were biotinylated through incubation with biotin. Chips were

738   blocked using milk and biotinylated protein lysates were washed over. Binding of the individual

739   proteins to each antibody spot on the array was assessed through fluorescent labelling with a dye-

740   labelled streptavidin read using an Innopsys 710-IR scanner and analysed using Mapix software.

741   For each antibody the background intensity was subtracted, dye signal normalised and an average

742   calculated of the duplicate spots. The ratio was calculated of binding to the phosphorylated amino

743   acids vs the binding to the non-modified regions of the protein for each molecule, calculating this for

744   both control and BQ3020 treated cells. The fold change in phosphorylation for each targeted amino

745   acid was generated by comparing BQ3020 to vehicle. For selection a fold change of greater than 2

746   and less than 0.5 was set as the cut-off.

747   Antibody array was performed once – one cell lysate per condition.

748   **Western Blot**

749   For western blot analysis, 9 cm treated plastic tissue culture dishes were coated with PDL for either

750   1 hour at 37°C or overnight at room temperature. 1 million OPCs were added in myelination media

751   to coated plates. After 3 days in culture, oligodendrocytes were starved from media

752   supplementation through incubation with DMEM and 1% P/S only for 4-5 hours. Fresh starvation

753   media was then added containing either vehicle control, BQ3020 (100 ng/mL) or FR236924 (25 µM).

754   After 10 minutes cells were lysed and scraped into RIPA buffer with protease and phosphatase

755   inhibitors (Calbiochem – 539134 and 524621) for 10 minutes on ice. Lysates were spun at 16,000 g

756   for 10 minutes and supernatants retained.

757   Protein concentration was estimated using a BCA assay kit and loaded into precast protein gels with

758   a protein marker of known molecular weights. Gels were ran at 60 volts for 30 minutes and then

759 increased to 100 volts for 1 hour. Protein was transferred from the gels to nitrocellulose membranes

760 pre-treated with methanol at 400 mA for 2 hours on ice. Membranes were blocked in 4% BSA in TBS-

761 0.1% Tween for 40 minutes and incubated with primary antibodies (GAPDH Millipore MAB374, Beta-

762 actin Abcam ab8226, Phospho-PKCε S729 Abcam 88241) overnight at 4^oC on an orbital shaker.

763 Membranes were washed in TBS-Tween for 30 minutes and incubated with species specific

764 secondary horseradish peroxide antibodies at room temperature for 1 hour. Membranes were

765 washed for a further 30 minutes and incubated with ECL2 for 5 minutes. Blots were detected using a

766 Licor scanner. Subsequent western blots were performed using the same membranes following

767 removal of bound antibodies through incubation with stripping buffer for 15 minutes.

768 **Zebrafish**

769 Animal husbandry and experiments were maintained in accordance with UK Home Office guideline.

770 The following zebrafish lines were used: Wild type WIK and AB, Tg(mbp:EGFP) and EDNRB Hom (Rse

771 tLF802) (Frohnhöfer et al., 2013, Krauss et al., 2014). Rse zebrafish contain a point mutation in the

772 *ednrba* gene at amino acid 163 where glutamine is substituted for lysine. This mutation creates a

773 restriction site for the enzyme PsiI. Touchdown PCR was performed using a light cycler. To

774 distinguish between wild type and homozygous mutants, the PCR product was incubated with

775 restriction enzyme PsiI for 3 hours at 37^oC and run on a 2% agarose TAE gel. Wild type DNA remains

776 uncut at 363 bp whereas homozygous DNA generates 2 similar sized bands of 187 and 176 bp.

777 Rse Homozygous mutants were crossed to the stable transgenic line Tg(mbp:EGFP) expressing EGFP

778 in all oligodendrocytes previously generated in the Lyons lab (Almeida et al., 2011). 5 dpf larval

779 zebrafish were embedded in 1.3% low melting point agarose with tricane. Lateral images of zebrafish

780 spinal cords were taken on an Apatome2 microscope at 20x and stitched together using ZEN imaging

781 software. Using ImageJ cell counter, GFP fluorescent oligodendrocytes were counted along the

782 entire length of the dorsal and ventral spinal cord. Fish were genotyped after imaging and analysis –

783 experimenters were therefore blinded to genotype during the data acquisition.

784 Offspring of the paired mating of either Rse heterozygotes or Rse heterozygotes to Rse homozygotes

785 were subject to single oligodendrocyte analysis. Single oligodendrocytes were labelled using the

786 mbp:EGFP-CAAX plasmid developed within the Lyons lab (Almeida et al., 2011). In brief, plasmid

787 DNA generated above for the mbp:EGFP transgenic line was adapted through the addition of the 4

788 amino acid CAAX motif therefore anchoring GFP to membranes of oligodendrocytes and myelin

789 sheaths. Mosaic fish were generated by injecting fertilised eggs with 1 nL of a solution of 12.5 ng/μL

790 mbp:EGFP-CAAX plasmid DNA and 12.5 ng/μL tol2 transposase mRNA between the 1-8 cell stage.

791 Transposase mRNA was produced using an Ambion message machine kit SP6 from pre-digested

792 DNA. Injections were performed using pulled glass needles using gas to force out a precise volume,

793 measured prior to injection in a drop of mineral oil on a calibration slide. 4 dpf larval zebrafish were

794 screened for fluorescent cells and embedded in 1.3% low melting point agarose with tricane. Lateral

795 images of individual cells were taken on a Zeiss LSM710 confocal. Analysis was performed using

796 ImageJ. GFP fluorescent sheaths longer than 5 μm were traced and the length measured. The

797 number of sheaths made by individual oligodendrocytes was recorded and the mean sheath length

798 was calculated per oligodendrocyte. Fish were genotyped after imaging and analysis - experimenters

799 were therefore blinded to genotype during the data acquisition.

800 For the experiments asking whether a protein kinase C agonist would rescue myelination defects in

801 the Rse Homozygous mutants, injected fish were treated with either 1%DMSO or 10μM FR236924 in

802 DMSO at 3 dpf for 24 hours before image acquisition.

803 **Statistics**

804 All analysis of cell counts and myelination and were performed on ImageJ blind to condition using a

805 filename randomiser macro. Data is presented showing standard deviations to show the variability,

806 or standard error of the mean when averaged data calculated as a mean per animal or per culture

807 was used for each n. Statistical analysis was performed using GraphPad Prism software. Data was

808 tested for normality using a Kolmogorov–Smirnov test. When data fitted a normal distribution

809   parametric t-tests and 1-way ANOVA, with Tukey's post hoc, were used. Where data did not meet

810   normal distribution non-parametric Mann-Whitney U tests and Kruskal-Wallis tests, with Dunns post

811   hoc, were used.

812

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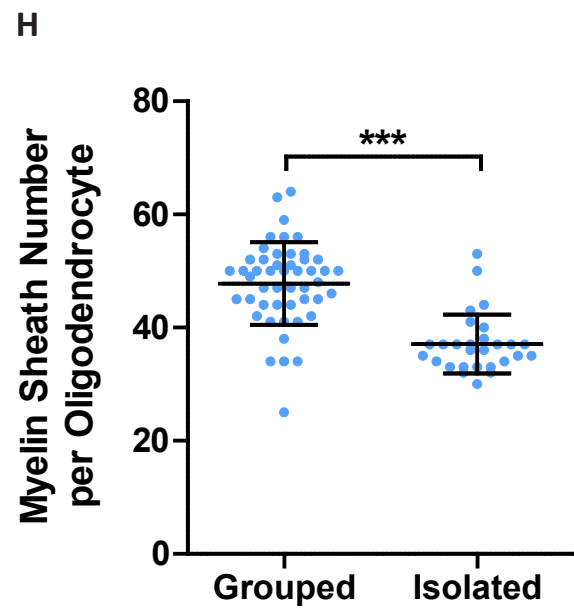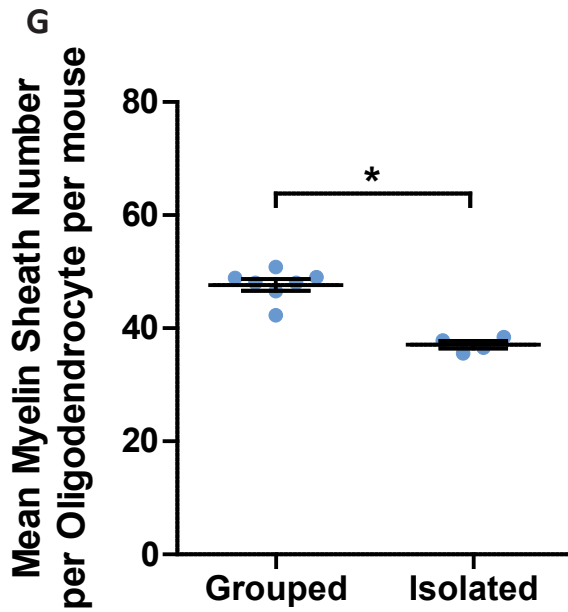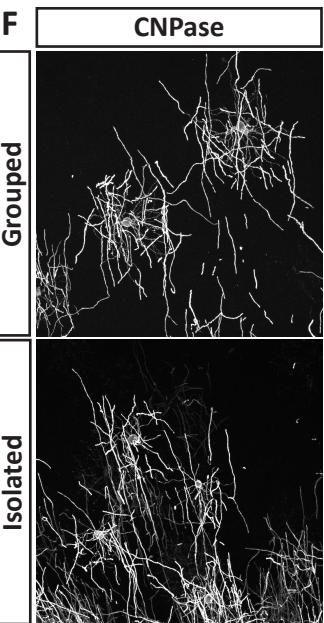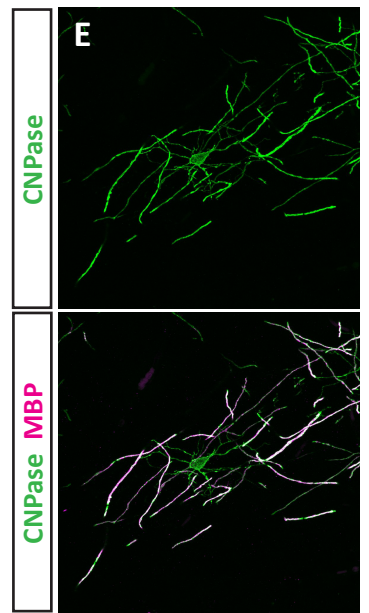998   cortex. *J Neurosci,* 34**,** 11929-47.
999

A

Grouped — Social Environment — P21 → P35

Isolated — Social Isolation — P21 → P35

B

Non-social Zone   Social Zone

C

Time Spent in Zones (s)

Grouped / Isolated — Non-social Zone
Grouped / Isolated — Social Zone

*

D

CNPase Hoechst

Postnatal day 21   Postnatal day 35

100 µm   100 µm

E

CNPase

CNPase MBP

F

CNPase

Grouped

Isolated

G

Mean Myelin Sheath Number per Oligodendrocyte per mouse

Grouped   Isolated

*

H

Myelin Sheath Number per Oligodendrocyte

Grouped   Isolated

***

Figure 2 – Social isolation reduces vascular endothelin expression.

A

CNP Cells/mm³ for Layer II/III and Layer V, comparing Wild Type and EDNRB CKO (ns, ns)

B CNPase: Wild Type and EDNRB CKO (20 μm)

C Mean Myelin Sheath Number per Oligodendrocyte per mouse: Wild Type vs EDNRB CKO (*)

D Myelin Sheath Number per Oligodendrocyte: Wild Type vs EDNRB CKO (***)

E Time Spent in Zones (s): Non-social Zone and Social Zone, comparing Wild Type and EDNRB CKO (*)

A

| Control Microfiber Culture | BQ3020 EDNRB Agonist | BQ788 EDNRB Antagonist |

MBP / Hoechst

50 μm

50 μm

B

Mean Myelin Sheath Number per Oligodendrocyte per Culture

*** *** ns

Control BQ3020 BQ788

Myelin Sheath Number per Oligodendrocyte

*** *** *

Control BQ3020 BQ788

D

Percent Frequency

Control
BQ3020
BQ788

Sheath Length μm

E

EDNRB mRNA

Relative Expression over GAPDH

**

Wild type EDNRB CKO

F

Mean Myelin Sheath Number per Oligodendrocyte per Culture

* * ns

Wild type   Wild type + BQ3020   EDNRB CKO   EDNRB CKO + BQ3020

G

Myelin Sheath Number per Oligodendrocyte

*** *** ns

Wild type   Wild type + BQ3020   EDNRB CKO   EDNRB CKO + BQ3020

A

Phospho-PKCε S729

GAPDH

Control | BQ3020 | Control | FR 236924

B

Mean Myelin Sheath Number per Oligodendrocyte per Culture

Control | FR 236924

*

C

Myelin Sheath Number per Oligodendrocyte

Control | FR 236924

***

D

0-1 hpf

Injected with mbp:EGFP-CAAX

3 dpf

DMSO or 10 μM FR 236924 added

4 dpf

EGFP positive cells Imaged

E

Control | FR 236924

500 μm

F

mbp:EGFP-CAAX

EDNRB Het

EDNRB Hom

10 μm

G

Myelin Sheath Number per Oligodendrocyte

EDNRB Het | EDNRB Hom | EDNRB Het | EDNRB Hom

FR 236924

** ***