The Conditioning Lesion Response in Dorsal Root Ganglion Neurons Is Inhibited in Oncomodulin Knock-Out Mice

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48

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75 **Abstract**

76 Regeneration can occur in peripheral neurons after injury, but the mechanisms involved
77 are not fully delineated. Macrophages in dorsal root ganglia (DRGs) are involved in the
78 enhanced regeneration that occurs after a conditioning lesion (CL), but how macro-
79 phages stimulate this response is not known. Oncomodulin (Ocm) has been proposed
80 as a pro-regenerative molecule secreted by macrophages and neutrophils, is expressed
81 in the DRG after axotomy, and stimulates neurite outgrowth by DRG neurons in culture.
82 Wild type (WT) and *Ocm* knockout (KO) mice were used to investigate whether Ocm
83 plays a role in the CL response in DRG neurons after sciatic nerve transection. Neurite
84 outgrowth was measured after 24 and 48 h in explant culture 7 d after a CL. Sciatic
85 nerve regeneration was also measured *in vivo* 7 d after a CL and 2 d after a subsequent
86 sciatic nerve crush. The magnitude of the increased neurite outgrowth following a CL
87 was significantly smaller in explants from *Ocm* KO mice than in explants from WT mice.
88 *In vivo* after a CL, increased regeneration was found in WT animals but not in KO ani-
89 mals. Macrophage accumulation and levels of *interleukin-6 (IL-6)* mRNA were meas-
90 ured in axotomized DRG from WT and *Ocm* KO animals, and both were significantly
91 higher than in ganglia from naïve animals. Our data support the hypothesis that Ocm
92 plays a necessary role in producing a normal CL response and that its effects possibly
93 result in part from stimulation of the expression of pro-regenerative macrophage cyto-
94 kines such as IL-6.

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100 **Significance Statement**

101 Peripheral neurons are capable of regeneration after axotomy. Regeneration is en-
102 hanced if a conditioning lesion (CL) precedes a test lesion. This CL effect seen in ex-
103 plant cultures does not occur if macrophage accumulation after injury is blocked; how-
104 ever, the mechanism underlying this macrophage effect is not known. To determine
105 whether the macrophage cytokine oncomodulin (Ocm) is involved in this effect, WT and
106 *Ocm* knockout mice were examined. The CL effect was inhibited in explants from *Ocm*
107 knockout animals. Experiments performed *in vivo* in these two genotypes showed a
108 clear CL effect in the WT mice but none in the *Ocm* knockout animals.

109

110

111 Introduction

112 Neurons in the peripheral nervous system can regenerate after injury; however, much
113 remains to be clarified about the mechanisms involved. Regeneration is not a neuron
114 autonomous process but instead is influenced by interactions between neurons and
115 both glial cells and immune cells. After axotomy, macrophages play a well-known phag-
116 ocytic role in the distal nerve segment, clearing discarded myelin and axonal debris
117 (Gaudet et al., 2011; Rotshenker, 2011). In addition, macrophages accumulate around
118 axotomized neuronal cell bodies, which has been shown to play a crucial role in nerve
119 regeneration (Kwon et al., 2013; Niemi et al., 2013) and possibly in neuropathic pain
120 (Yu et al., 2020).

121 A CL has been shown to enhance outgrowth both in explant culture (Edstrom et al.,
122 1996) and *in vivo* (McQuarrie and Grafstein, 1973). Subsequent studies have sought to
123 define the neuron-macrophage interaction and identify critical molecules that are in-
124 volved in the CL response. The chemokine CCL2, and perhaps other macrophage
125 chemokines, bring CCR2+ monocytes from the bloodstream into the axotomized DRG,
126 where they differentiate into macrophages, and promote the CL response (Niemi et al.,
127 2013). What the exact interaction is between the accumulating macrophages and neu-
128 rons that results in the promotion of regeneration is unclear, although there are many
129 candidate molecules expressed by macrophages that could be involved (Benowitz and
130 Popovich, 2011).

131 One candidate is Ocm, an EF-hand Ca^{2+} buffer protein, which was originally detected
132 in rat hepatoma cells (MacManus et al., 1983; Climer et al., 2019). Ocm has since been

133 found in outer hair cells of the ear (Sakaguchi et al., 1998; Yang et al., 2004), in macro-
134 phages (Yin et al., 2006; Yin et al., 2009), and in neutrophils (Kurimoto et al., 2013).
135 The idea that Ocm might promote regeneration arose from studies on the regeneration
136 of axons in the optic nerve in response to an inflammatory reaction in the eye. In this
137 context, Ocm was identified as a macrophage-derived growth factor (Yin et al., 2006).
138 When retinal ganglion cells were exposed in culture to Ocm and forskolin, neurite out-
139 growth was stimulated (Yin et al., 2006). Within a day after initiating inflammation in the
140 eye with zymosan (a yeast cell wall protein-carbohydrate complex), the infiltrating im-
141 mune cells showed high levels of *Ocm* mRNA and protein, the latter of which was se-
142 creted and bound to retinal ganglion cells. Inflammation-induced stimulation of axon
143 growth following optic nerve crush was significantly reduced by two Ocm-blocking rea-
144 gents. In addition to macrophages, neutrophils express Ocm, and they are the first re-
145 sponders during inflammation and after injury (Kurimoto et al., 2013; Fine et al., 2020).

146 However, the importance of Ocm in the context of optic nerve regeneration has been
147 contested. Hauk and colleagues found that Ocm did not increase after lens injury or
148 zymosan treatment and that substantial depletion of macrophages in the eye during in-
149 flammation did not prevent regeneration (Hauk et al., 2008). Additionally, they reported
150 that gp130 cytokines such as ciliary neurotrophic factor (CNTF), leukemia inhibitory fac-
151 tor (LIF) and IL-6 secreted by glial cells in the retina are more important in stimulating
152 regeneration than Ocm (Leibinger et al., 2009; Fischer and Leibinger, 2012; Leibinger et
153 al., 2013). Thus, questions have been raised about the importance of Ocm in nerve re-
154 generation.

155 Three studies examined a role for Ocm in the context of injury to DRG neurons. Yin

156 et al. (2006) found that addition of Ocm to DRG neurons in dissociated cultures pro-
157 duced an increase in neurite outgrowth. Harel et al. (2012) reported what they described
158 as a “meager” effect of Ocm plus dibutyryl cAMP on the growth of DRG neurons across
159 the dorsal root entry zone after a dorsal root nerve crush. Kwon et al. (2013) found an
160 increase in *Ocm* mRNA in the DRG 7 d after a sciatic nerve transection and found that a
161 neutralizing antibody to Ocm blocked stimulation of outgrowth by DRG neurons follow-
162 ing co-culture with macrophages.

163 Surprisingly there have been no studies of nerve regeneration in *Ocm* KO mice. In
164 addition, there have been no experiments published on Ocm’s role in regeneration of
165 the peripheral process of DRG neurons *in vivo*. Here, an *Ocm* KO mouse is used to an-
166 alyze the importance of this protein in the CL response of DRG neurons.

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170 **Materials and Methods**

171 **Animals and surgeries.**

172 Eight to twelve week old mice with a targeted deletion of exons 2-4 of the *Ocm* cod-
 173 ing sequence were used for this investigation. These *Ocm* KO mice are designated
 174 $\text{Actb}^{\text{cre}}\text{Ocm}^{\text{flox/flox}}$ (Tong et al., 2016). Briefly (as described in Tong et. al., 2016), *Ocm*
 175 KO mice were generated by inserting a LoxP site 5' of exon 2 and Flp-neo-Flp-LoxP
 176 cassette was inserted 3' of exon 4 in BAC DNA. The vector was electroporated into ES
 177 cells and clones resistant to G418 were isolated and checked for homologous recombina-
 178 tion by southern blot analysis. Two male clones were injected into C57BL/6J blasto-
 179 cysts. High percentage chimeras were crossed with CBA/CaJ mice, and the pups were
 180 checked for germ-line transmission using southern blots. PCR primers used for geno-
 181 typing were made from the deleted region (5'-CTC CAC ACT TCA CCA AGC AG-3' and
 182 5'-GCT TGG GGA CCC CCT GTC TTC A-3') and from the targeting vector (5'-CTC
 183 CAC ACT TCA CCA AGC AG-3' and 5'-TTT CAT GTT CAG GGA TCA AGT G-3'). The
 184 neo gene was removed when generating the *Ocm* heterozygote to avoid any possible
 185 interference. $\text{Ocm}^{\text{flox/flox}}$ mice were generated and crossed with β -actin Cre mice (strain
 186 003376; The Jackson Laboratory) to generate β -actin^{Cre} $\text{Ocm}^{\text{flox/flox}}$ mice (*Ocmtm1.1Ddsi*,
 187 MGI:97401), referred to as *Ocm* KO mice. For these studies, the *Ocm* KO mice were
 188 backcrossed onto the CBA/CaJ strain (99% congenicity), and WT and mutant *Ocm* KO
 189 littermates were used. Additional age matched WT mice were acquired from Jackson
 190 Laboratory (Bar Harbor, ME, USA; CBA/CaJ) when needed. The animals were housed
 191 3-5 per cage under a 12 h:12 h light:dark cycle with ad libitum access to food and water.
 192 In the mouse, neurons in lumbar DRG L3, L4, and L5 project into the sciatic nerve

(Rigaud et al., 2008). Two lesion protocols were followed. In one, the sciatic nerve was transected unilaterally under isoflurane anesthesia, and a 2 mm piece of the distal nerve segment was removed to prevent regeneration. The contralateral nerve was exposed but not transected, and the corresponding ganglia served as sham-operated controls. The animals were sacrificed by CO₂ inhalation 7 d later, and L3 and L4 DRGs and sciatic nerves were removed for neurite outgrowth studies, flow cytometry, and molecular biology. In other experiments, the sciatic nerve was transected unilaterally anterior to its trifurcation, and 7 d later the nerve was crushed at the level of the sciatic notch. Two days after the second lesion (i.e., the test lesion), the ipsilateral DRG and the sciatic nerve distal to the crush site were analyzed. The contralateral sciatic nerve was exposed and then 7 d later received a crush lesion. Authors' university's Institutional Animal Care and Use Committee approved all surgical procedures.

DRG explants.

To assess the outgrowth in response to injury of sensory neurons in culture while maintaining the ganglion's *in vivo* tissue architecture, neurite outgrowth was evaluated in explanted ganglia from WT and *Ocm* KO mice after a CL. Seven days after unilateral sciatic nerve transection, axotomized and sham-operated L5 DRGs were removed, desheathed, placed on coverslips, and overlaid with 7.5 μ l Matrigel (Becton Dickinson; Franklin Lakes, NJ, USA). Culture plates were placed in a 37°C incubator for 5 min to allow gelling of the Matrigel before adding 1 ml F12 medium with the following additives: 0.5% bovine serum albumin (Jackson ImmunoResearch), 1% penicillin/streptomycin (Thermo Fisher, Gibco), 5 μ g/ml insulin (Millipore Sigma, Burlington, MA, USA), 630 ng/ml progesterone (Millipore Sigma), 5 ng/ml selenium (Millipore Sigma), 9 μ g/ml pu-

216 tresscine (Millipore Sigma), and 100 µg/ml transferrin (BD Biosciences). Phase-contrast
217 images of neurite outgrowth from each DRG were captured at 24 and 48 h after explant-
218 tion using an Axiovert 405 M microscope at 10x magnification. Neurite outgrowth was
219 assessed using MetaMorph by measuring the distance between the edge of the gangli-
220 on and the leading tip of the longest 20 processes in each explant. The length of these
221 20 neurites were averaged for each ganglion. Five sham-operated and five axotomized
222 ganglia were analyzed for each genotype. At 48 h, explants were fixed and labeled with
223 an antibody against β III tubulin (1:500; Promega; RRID:AB_430874) and the outgrowth
224 was photographed.

225

226 **In vivo CL studies and regeneration analysis.**

227 Seven days after a distal unilateral sciatic nerve transection, both sciatic nerves were
228 crushed more proximal to the DRG than the initial nerve transection site. The nerves
229 were crushed for 45 sec with ultra-fine hemostats (Fine Science Tools, Foster City, CA,
230 USA) at the level of the hip. Two days after nerve crush, the animals were sacrificed by
231 CO₂ inhalation, and sciatic nerves and L4 DRG were removed for immunohistochemical
232 analysis. Sciatic nerves were removed, cleaned, pinned down straight in a 35 mm dish,
233 and fixed by immersion in 4% paraformaldehyde (PFA). Nerves were cryoprotected in
234 30% sucrose, embedded in Tissue-Tek O.C.T. (Electron Microscopy Sciences), and
235 sectioned. After blocking, 60 µm sections were incubated with an antibody to SCG10
236 (1:4000; Novus Biologicals; Littleton, CO, USA; RRID:AB_10011569) overnight at 4°C
237 and then incubated in AlexaFluor 555 secondary antibodies (1:400; Thermo Fisher Sci-
238 entific, RRID:AB_162543). Nerves were imaged on a Leica SP8 confocal. The images

239 underwent despeckling in ImageJ prior to SCG10 quantification. The regeneration index
240 was measured based on the method of Shin et al. (2014). Briefly, the amount of fluores-
241 cence was assessed using MetaMorph in a 100 pixel wide rectangle spanning the width
242 of the nerve where it had been crushed, which was identified by transferring fluorescent
243 microspheres from the tip of the hemostat at the time of the crush (not shown). Another
244 rectangle was placed where the amount of fluorescence was 50% of that at the crush
245 site. The distance between these two rectangles was measured and expressed as the
246 regeneration index. One section from a crush only animal and one from a conditioned
247 animal, and six to eight animals for each genotype were analyzed. Crush only indicates
248 a nerve harvested 2 d after a crush injury, and conditioned indicates a nerve harvested
249 9 d after a CL and 2 d after a crush injury. An additional measure of regeneration was
250 also quantified by measuring the percent area stained by an antibody to SCG10 at 500
251 μm intervals distal to the crush site and normalizing to the measured percent area
252 stained at the crush site as performed by Weng et al. (2017). These data were repre-
253 sented as a regenerative ratio at each distance.

254 **Macrophage accumulation *in vivo*.**

255 The accumulation of macrophages in the sciatic nerve and L5 DRG after an *in vivo*
256 CL followed by a nerve crush versus a nerve crush alone was determined. Sciatic nerve
257 sections (20 μm) and DRG sections (10 μm) from WT and *Ocm* KO mice were incubat-
258 ed overnight at 4°C in an antibody to CD68 (1:200; Bio-Rad; Hercules, CA, USA;
259 RRID:AB_322219) and then incubated in Cy3 secondary antibodies (1:400; Jackson
260 ImmunoResearch; West Grove, PA, USA; RRID:AB_2340619) for 1 h followed by 4',6-
261 diamidino-2-phenylindole (DAPI). Images were captured at 10x (sciatic) or 25x (DRG)

262 magnification using HCLImage (Hamamatsu Corporation; Bridgewater, NJ, USA) then
 263 quantified using MetaMorph. Macrophage cell counts were acquired by counting the
 264 number of CD68+ cells containing DAPI using the ImageJ cell counter. For macrophage
 265 quantification in the DRG, only areas of tissue containing neuronal cell bodies were
 266 analyzed. The total number of cells across three images for each sample was calculat-
 267 ed and then averaged for each experimental group. One section from each crush only
 268 and each conditioned animal from five (sciatic) or three (DRG) animals for each geno-
 269 type were analyzed.

270 **Flow cytometry.**

271 Flow cytometry was performed on pooled L3 and L4 DRG or L3, L4, and L5 DRG af-
 272 ter explant culture or after a test lesion *in vivo*, respectively. Explant and *in vivo* CL
 273 DRGs were enzymatically digested in 0.125% collagenase for 1 h at 37°C. Mechanical
 274 dissociation using a 23-gauge needle attached to a 1 ml syringe produced single-cell
 275 suspensions, which were filtered through a 35 µm cell strainer. For all cell suspensions,
 276 dead cells were labeled using Live Dead Fixable Blue Dead Cell Stain kit (Invitrogen,
 277 Cat#L23105) for 30 min at 4°C. Cells were then washed in FACS buffer (PBS, 1% BSA)
 278 and blocked with a monoclonal antibody to CD16/CD32 (1:500; eBioscience,
 279 RRID:AB_467133) for 10 min at 4°C. Cells were incubated with fluorophore-conjugated
 280 antibodies against CD11b (1:400, Biolegend, San Diego, CA, USA; RRID:AB_312789)
 281 and F4/80 (1:400, Biolegend, RRID:AB_2293450) and Ly6G (1:400, Biolegend,
 282 RRID:AB_1134159) for 1 h at 4°C. Cells were washed and resuspended on a shaker in
 283 fixation buffer (2% PFA in PBS) for 15 min at room temperature. For flow experiments in
 284 which neurons were examined, the cells were then washed in PBS and resuspended in

285 permeabilization buffer (0.7% Tween-20 in PBS) on a shaker for 15 min at room tem-
 286 perature. Cells were then incubated with a fluorophore-conjugated antibody against β -
 287 III-tubulin to label neurons (1:80, Biolegend, RRID:AB_2563609) for 30 min at room
 288 temperature in the dark. Cells were subsequently washed in FACS buffer and then run
 289 on a BD FACS Aria (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo
 290 (Tree Star, RRID:SCR_008520). All events were gated based on viable single cells.
 291 Compensation and gating were performed using negative, single-stained and isotype
 292 controls. Cell populations were gated as follows: F4/80⁺CD11b⁺ (macrophages); β -
 293 tubulin⁺CD11b⁻ (neurons), and Ly6G⁺CD11b⁺ (neutrophils).

294 **Myelin visualization by luxol fast blue.**

295 To assess myelin clearance in the distal sciatic nerve segment, axotomized and
 296 sham-operated sciatic nerves from WT and *Ocm* KO mice were removed 7 d after tran-
 297 section and fixed by immersion in 4% paraformaldehyde. The tissues were cryoprotect-
 298 ed in 30% sucrose and embedded in Tissue-Tek O.C.T. compound. Staining with luxol
 299 fast blue (Electron Microscopy Sciences) was performed on 20 μ m cryostat sections.
 300 Briefly, nerves were incubated in H₂O, 35% and 70% ethanol for 5 min each, followed
 301 by incubation in 0.1% Luxol fast blue overnight at 60°C. Nerves were subsequently des-
 302 tained in 0.05% lithium carbonate and incubated for 5 min each in increasing concentra-
 303 tions of ethanol (70%/95%/100%), followed by xylenes before images were captured
 304 using a light microscope. Positive myelin staining is expressed as a percentage of the
 305 total area examined. Images were captured at 20x magnification using Volocity software
 306 (PerkinElmer; Waltham, MA, USA) and then quantified using MetaMorph. Three images
 307 per section were analyzed and averaged for each sample. Five samples per genotype

308 were analyzed.

309

310 Real-time polymerase chain reaction (PCR).

311 The expression of three gp130 cytokines (*Lif*, *Cntf*, and *Il-6*) was analyzed by quanti-
 312 tative real-time PCR. Six hours and 9 d after unilateral sciatic nerve transection axoto-
 313 mized and sham-operated L3 and L4 DRG from WT and *Ocm* KO mice were removed,
 314 desheathed, and placed in RNAlater (Thermo Fisher Scientific). Two ganglia were
 315 pooled per sample. Three animals were included for each group. The tissue was ho-
 316 mogenized, RNA was isolated, total RNA was measured, and 331 ng were reverse
 317 transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Sci-
 318 entific). Real-time PCR was performed in an ABI Step-One, using prevalidated TaqMan
 319 expression assays (Thermo Fisher Scientific) for *Lif* (Mm00434762), *Cntf*
 320 (Mm00446373), and *Il-6* (Mm00446190). Samples were assayed in triplicate and rela-
 321 tive expression was determined using the comparative Ct method. mRNA values were
 322 normalized to the mRNA values for the internal control glyceraldehyde-3-phosphate de-
 323 hydrogenase (GAPDH; Mm99999915) of the respective sham group for each genotype.

324 Statistics.

325 Experimenter's performing data analysis were blinded to genotype. Data are ex-
 326 pressed as means $\pm$ S.E.M. Statistically significant differences were determine by a two-
 327 way ANOVA with a Tukey post-hoc test using GraphPad Prism 9.2.0. Groups are con-
 328 sidered statistically different if $p < 0.05$. The number of samples per group is indicated in
 329 each figure legend.

331 Results

332 Effects of Ocm on the CL response examined in explant culture.

333 The increased growth capacity of sensory neurons as the result of a CL can be
334 demonstrated in explant culture and *in vivo* (McQuarrie and Grafstein, 1973; McQuarrie
335 et al., 1977; Edstrom et al., 1996; Shoemaker et al., 2005). To determine if Ocm is in-
336 volved in the CL response of DRG neurons, we began by examining the CL response
337 measured in explant culture from WT and *Ocm* KO mice. Explant cultures enable the *in*
338 *vitro* study of neurons while maintaining much of their *in vivo* tissue environment, includ-
339 ing the presence of macrophages as shown in Fig. 1a, e. The number of neurons was
340 also determined for each sample (Fig. 1b, f), and the number of macrophages was also
341 expressed per neuron (Fig. 1c).

342 After both 24 and 48 h in culture, DRG explants from both WT and *Ocm* KO mice ex-
343 hibited a CL response (Fig. 2a, b): however, the outgrowth from *Ocm* KO DRG in re-
344 sponse to a CL was significantly smaller than that from WT DRG after 48 h in culture
345 (Fig. 2b-d).

346 Effects of Ocm on the CL response *in vivo*.

347 We next looked at the CL response *in vivo* in WT and KO mice. Nine days after a CL
348 (unilateral sciatic nerve transection) and 2 d after a test lesion (bilateral sciatic nerve
349 crush at a site proximal to the site of transection), sciatic nerves were harvested, sec-
350 tioned, and labeled with an antibody to SCG10. SCG10 is preferentially expressed in
351 sensory axons, is rapidly down-regulated distal to an injury site, and is highly expressed
352 in regenerating fibers (Shin et al., 2012; Shin et al., 2014). The regeneration index iden-

353 tifies the distance from the crush site to the location where levels of SCG10 are half of
354 their levels at the crush site, thus identifying the length to which approximately half of
355 the axons have regenerated (Shin et al., 2014). Under these conditions, we found that
356 sciatic nerves from WT mice exhibited a CL response while those from *Ocm* KO mice
357 did not (Fig. 3a, c-f).

358 To examine further the distance the regenerating axons grew *in vivo* after a CL, a re-
359 generative ratio was also determined by measuring the percent area stained from the
360 site of the crush injury in 0.5 mm increments distally out to 3 mm (Fig. 3b). The data
361 were displayed as a ratio of the percent area stained at each individual distance divided
362 by the percent area stained at the crush site. WT conditioned nerves showed signifi-
363 cantly more SCG10 staining at 0.5, 1, 1.5, and 2 mm distal to the crush site compared
364 to WT crush only nerves, indicating that a CL increases the length of the axons regen-
365 erating *in vivo* (Fig. 3b). Nerves from *Ocm* KO mice did not show a significant difference
366 in SCG10 staining in response to a CL at any distance distal to the crush site (Fig. 3b).
367 Additionally, WT conditioned nerves showed significantly more SCG10 staining than
368 *Ocm* KO conditioned nerves at 2 mm distal to the crush site (Fig. 3b, d, f). These data
369 indicate that *Ocm* plays a prominent role *in vivo* in the CL response of DRG neurons.
370 This *in vivo* experiment was repeated a second time, and similar results were obtained.

371 **Macrophage accumulation in the absence of *Ocm*.**

372 In an attempt to explore how *Ocm* might facilitate nerve regeneration *in vivo*, we ex-
373 amined three factors known to influence regeneration: macrophage accumulation, Wal-
374 lerian degeneration, and the induction of gp130 cytokines. The accumulation of macro-

phages in the distal nerve segment of the sciatic nerve after injury has been correlated with nerve regeneration (Bisby and Chen, 1990; Chen and Bisby, 1993; Dailey et al., 1998; Barrette et al., 2008). Therefore, we sought to determine whether *Ocm* might play a role in the accumulation of macrophages in the sciatic nerve after injury. Using immunohistochemistry, the number of CD68+ macrophages in the nerve was determined either 9 d after a transection (Conditioned) and 2 d after a crush injury, or just 2 d after a crush injury (Crush Only). We found that the CL produced a similar increase in CD68+ cells in the sciatic nerve from both WT and *Ocm* KO mice (Fig. 4a-e). These data indicate that *Ocm* does not influence macrophage accumulation in the distal nerve.

We also assessed the number of CD68+ macrophages in the DRG under the same conditions since macrophage accumulation in the ganglion is correlated with the CL response (Kwon et al., 2013; Niemi et al., 2013). In this tissue, there was a significant increase in CD68+ cells in conditioned DRG from WT mice but not in DRG from conditioned *Ocm* KO mice although this may result in part from the high standard error for this group (Fig. 4f-j).

To obtain a more quantitative measure of macrophage accumulation, CD11b+F4/80+ macrophages were examined in the sciatic nerve and DRG using flow cytometry. The results obtained were similar to those obtained with immunohistochemistry. No difference in macrophage presence in the sciatic nerve was found between genotypes after a CL (Fig. 5a, c), but an increase in macrophage accumulation in the DRG was found after a CL in WT but not in *Ocm* KO mice (Fig. 5d, f).

Neutrophils were identified by being positive for CD11b and Ly6G. Such cells are un-

397 detectable in the sciatic nerve from sham-operated mice (Lindborg et al., 2017). Follow-
 398 ing axotomy, no significant differences were found in the sciatic nerve between geno-
 399 types or between crush only and a CL followed by crush (Fig. 5b, c). As reported previ-
 400 ously (Lindborg et al., 2018), no significant number of neutrophils were found in the
 401 DRG from sham-operated or lesioned animals (Fig. 5e, f).

402 **Clearance of myelin in the absence of Ocm.**

403 Myelin clearance from the distal nerve after axotomy is important in the peripheral
 404 nervous system for subsequent regeneration (e.g., Barrette et al., 2008). Therefore, we
 405 examined myelin clearance in WT and *Ocm* KO mice. The clearance of myelin in the
 406 distal sciatic nerve was assessed by staining with luxol fast blue. At 7 d after sciatic
 407 nerve transection, luxol fast blue staining was reduced to the same extent in nerves
 408 from WT and *Ocm* KO mice (Fig. 6). These results indicate that Ocm is not exerting its
 409 pro-regenerative effects by influencing myelin clearance.

410 **Effects of Ocm on expression of gp130 cytokines.**

411 It has been shown that axotomy influences expression of gp130 cytokines in periph-
 412 eral ganglia (for review see Zigmond, 2012). Although LIF is expressed in the superior
 413 cervical ganglion after axotomy, it is not expressed in the axotomized DRG at times ex-
 414 amined thus far (Sun and Zigmond, 1996; Thompson et al., 1997). CNTF is expressed
 415 in myelinating Schwann cells in the intact sciatic nerve (Dobrea et al., 1992; Rende et
 416 al., 1992), but expression actually decreases after nerve transection (Friedman et al.,
 417 1992; Sendtner et al., 1992; Seniuk et al., 1992). CNTF was not found in neurons in the
 418 DRG *in vivo* though it is expressed in cultured neurons (Sango et al., 2007). *Il-6* mRNA

419 expression is increased in DRG after axotomy (Murphy et al., 1995). There is a disa-
420 greement in the literature as to whether there is a CL response in DRG neurons from *Il-*
421 *6* KO mice (Cafferty et al., 2004; Cao et al., 2006). We looked at mRNA expression of
422 these three gp130 cytokines in the DRG 6 h after a sciatic nerve transection (Fig. 7a)
423 and 7 d after a conditioning lesion followed by 2 d after a nerve crush (Fig. 7b). Only *Il-6*
424 expression was affected differentially in WT and *Ocm* KO animals at the 6 h time point.
425 Although *Il-6* increased after axotomy in ganglia from both genotypes, the increase was
426 significantly less in DRG from *Ocm* KO mice compared to that from WT mice (Fig. 7).

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430 Discussion

431 Our study is the first to look at nerve regeneration in *Ocm* KO mice. *Ocm* was pro-
432 posed as a promoter of nerve regeneration based on studies on regeneration of retinal
433 ganglion cell axons following induced ocular inflammation in male Fischer rats (Yin et
434 al., 2006). Benowitz and colleagues reported *Ocm* protein in the eye and showed that
435 the stimulation of regeneration caused by inflammation could be blocked by reagents
436 that antagonize the action of *Ocm* (Yin et al., 2009). Fischer and colleagues have pre-
437 sented data supporting a different view of how inflammation leads to the growth of reti-
438 nal ganglion axons. Working with female Sprague-Dawley rats and female C57BL/6
439 mice, they reported that the stimulation of regeneration by inflammation is mediated by
440 the release of gp130 cytokines (i.e., CNTF, LIF, and IL-6) from retinal astrocytes (Hauk
441 et al., 2008; Leibinger et al., 2009; Leibinger et al., 2013).

442 We find that the CL effect measured in sensory neurons *in vivo* is diminished in *Ocm*
443 KO mice. In addition, the CL effect measured in explanted DRG was inhibited in KO
444 mice, though it was not completely blocked. Obviously, the *in vivo* experiment is the
445 most physiologically relevant and makes no assumptions as to the cellular site of action
446 of *Ocm*. Thus, *Ocm* might act in the ganglion or in the proximal or distal transected
447 nerve.

448 It should be noted that in our experiments, we have performed a unilateral condition-
449 ing lesion and then compared the effects in the ipsilateral to those in the contralateral
450 DRGs. This comparison may underestimate the magnitude of the effect on the ipsilat-
451 eral ganglia, as in recent studies in the rat, it has been shown that, after spinal nerve

452 transection, some stimulation of gene expression and neurite outgrowth occurs not only
453 in the ipsilateral DRGs but also, though to a lesser extent, in the contralateral DRGs
454 (Hasmatali et al., 2019; Hasmatali et al., 2020; Verge et al., 2020).

455 Ocm binds to retinal ganglion cells *in vivo*; however, whether this is also true for cells
456 in the DRG, either neuronal or non-neuronal, is not known. Additionally, an Ocm recep-
457 tor and an exact signaling pathway in the nervous system have not been defined. The
458 growth effect of Ocm on cultured retinal ganglion cells is completely blocked by the
459 transcriptional inhibitor actinomycin D (Yin et al., 2006). In an earlier study, the CL effect
460 on DRG neurons was similarly shown to be blocked by a different transcriptional inhibi-
461 tor, 5,6-dichlorobenzimidazole riboside (Smith and Skene, 1997). Our results raise the
462 possibility that Ocm promotes regeneration of sensory neurons in part by altering neu-
463 ronal gene expression, specifically the expression of the gp130 cytokine IL-6. *IL-6*
464 mRNA has been shown to increase in DRG neurons after axotomy (Murphy et al.,
465 1995). Cafferty et al. (2004) showed that addition of IL-6 to DRG cultures increased
466 neurite outgrowth, and Dubový et al. (2019) showed that intrathecal injection of IL-6
467 produced a CL-like response after peripheral nerve injury. In the present study, we
468 found that *IL-6* mRNA was also increased in DRG from *Ocm* KO mice at 6 h, but to a
469 significantly lesser extent than that from WT animals (Fig. 7). LIF and CNTF, two other
470 members of this cytokine family, were neither induced in the DRG by axotomy nor al-
471 tered by the absence of Ocm (Fig. 7). Although the time point analyzed here (6 h) is be-
472 fore hematogenous macrophage accumulation in the DRG occurs, it is possible that
473 resident macrophages also express Ocm. In addition, neutrophils, which can express
474 Ocm, have been shown to accumulate already in the sciatic nerve when examined 8 h

475 after partial nerve injury (Perkins and Tracey, 2000). The possibility that Ocm alters the
476 expression of IL-6 offers a partial resolution of the conflicting views proposed by the Be-
477 nowitz and Fischer groups described above on whether Ocm or gp130 cytokines medi-
478 ate the effects of inflammation on optic nerve regeneration.

479 A finding that was quite unexpected in our study was that accumulation of macro-
480 phages in the DRG, though not in the sciatic nerve, was inhibited in the *Ocm* KO ani-
481 mals (Figs. 4 and 5). This finding raises the possibility that Ocm has chemotactic activity
482 or that it can modulate the expression of monocyte chemokines (e.g., CCL2) or their
483 signaling within the DRG. Under conditions in which macrophage accumulation in the
484 DRG is inhibited, the CL effect is blocked (Niemi et al., 2013).

485 Often a neuron's ability to initiate axonal growth after an injury is distinguished from
486 its ability to elongate that axon in order to reach its target tissues. Here we observed
487 significantly less outgrowth *in vitro* from explants of *Ocm* KO compared to WT mice at
488 48 h, but not at 24 h, indicating that Ocm may be involved in elongation of regenerating
489 axons rather than initiation of outgrowth.

490 To promote regeneration and functional recovery, it is likely that a combinatorial ap-
491 proach will be most effective (Benowitz and Popovich, 2011). Prior studies of Ocm have
492 indicated that Ocm can exert its growth-promoting effects in coordination with other
493 agents. For example, it has been shown that increasing retinal levels of Ocm and de-
494 creasing PTEN expression together can be used to promote regeneration after optic
495 nerve crush (de Lima et al., 2012). In addition, the fact that elevated cAMP levels are
496 required for the pro-regenerative effects of Ocm indicates that there may be other play-
497 ers involved.

498 In summary, using the *Ocm* KO mouse, we have demonstrated that this cytokine is
499 necessary for the normal CL response to occur and that this effect could be due to a
500 decrease in the axotomy-induced expression of IL-6 or another macrophage cytokine.

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507 **Figure legends**

508 **Figure 1.** Relative macrophage content of DRGs *in vivo* and in explant culture after a
 509 CL. For *in vivo* studies, seven days after a unilateral sciatic nerve transection or
 510 contralateral sham surgery, the nerves were crushed bilaterally and nerve regeneration
 511 was assayed 2 d later. For explant studies, DRGs were placed in explant culture for 2 d
 512 after unilateral sciatic nerve transection or contralateral sham surgery. The macrophage
 513 (a) and neuronal (b) content of each sample was determined by flow cytometry using
 514 two macrophage markers (CD11b and F4/80), one neuronal marker (β III tubulin), and a
 515 live/dead cell stain. The ratio of macrophages to neurons is also given (c). Cell counts
 516 are also displayed for live cells (d) CD11b⁺ F4/80⁺ macrophages (e), and β -Tubulin⁺
 517 CD11b⁻ neurons (f). Representative heat maps are shown for CD11b and F4/80 (f) and
 518 CD11b and β III tubulin (g). Numbers in plots correspond to the percentage of total
 519 events in each quadrant. Events in quadrants outlined with a red box correspond to
 520 cells that are CD11b⁺F4/80⁺ (f, macrophages) or CD11b⁻ β III tubulin⁺ (g, neurons). N =
 521 5/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

522 **Figure 2.** L5 DRG explants from both WT and *Ocm* KO mice exhibited a CL response
 523 at 24 h (a) and 48 h (b), although ganglia from *Ocm* KO mice showed a significantly
 524 smaller response than those from WT ganglia after 48 h in culture (b). Micrographs
 525 represent WT (c) and *Ocm* KO (d) ganglia following a CL and 48 h in culture. N =
 526 5/group. Scale bars = 250 μ m. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

527 **Figure 3.** DRG from *Ocm* KO mice do not exhibit a CL response *in vivo*. Nine days after
 528 a CL and 2 d after a crush injury, axons from WT mice exhibit enhanced growth
 529 response *in vivo*, whereas axons from *Ocm* KO mice did not (a). The nerves were

530 immunostained for SCG10. The regeneration index is the distance from the crush site to
 531 the site where the staining for SCG10 is half that seen at the crush site (**a, c-f**). The
 532 regeneration ratio is the ratio of staining at a site to that at the crush site. This ratio was
 533 determined for distances from 0.5 mm to 3.0 mm distal to the crush site (**b**). Images
 534 represent WT crush only (**c**) and conditioned plus crush (**d**) and *Ocm* KO crush only (**e**)
 535 and conditioned plus crush (**f**). Asterisks in the images indicate the crush site for each
 536 nerve. Dashed rectangles indicate where the immunostaining for SCG10 is reduced by
 537 50% compared to the staining at the crush site. N = 8/group. Scale bars = 500 μ m. In
 538 the line graph, * $p < 0.05$, ** $p < 0.01$ comparing WT conditioned versus WT crush only. #
 539 $p < 0.01$ comparing WT conditioned versus *Ocm* KO conditioned.

540 **Figure 4.** Macrophage accumulation in the distal sciatic nerve and DRG 2 d after a
 541 nerve crush with and without a prior CL in WT and *Ocm* KO mice. Macrophage
 542 accumulation observed by CD68 immunostaining in the distal sciatic nerve was similar
 543 after a CL in both genotypes (**a**). Macrophage accumulation was also increased in WT
 544 DRG after a CL but not in the DRG from *Ocm* KO mice (**f**). Three adjacent fields from
 545 each sample were counted and summed. Images of WT crush only (**b, g**) and
 546 conditioned plus crush (**d, i**) and *Ocm* KO crush only (**c, h**) and conditioned plus crush
 547 (**e, j**). N = 5-6/group. Scale bars = 100 μ m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

548 **Figure 5.** Flow cytometry studies on macrophage and neutrophil accumulation in the
 549 distal sciatic nerve and DRG in WT and *Ocm* KO mice after a nerve crush with and
 550 without a prior CL. Macrophages were determined by double staining with antibodies
 551 against CD11b and F4/80. Neutrophils were determined by double staining with
 552 antibodies against CD11b and Ly6G. For animals receiving a CL, the sciatic nerve was

transected unilaterally 7 d before the nerve was crushed (samples labeled “CL”). Forty-eight hours later the ipsilateral distal sciatic nerve and the ipsilateral DRG were taken for flow cytometry. The contralateral sciatic nerve received a crush only (samples labeled “Crush”). Representative dot plots for macrophages (**a, d**) and neutrophils (**b, e**) in the sciatic nerve (**a, b**) and DRG (**d, e**). Numbers in plots correspond to the percentage of total events in each quadrant. Events in quadrant outlined with a red box correspond to cells that are CD11b⁺F4/80⁺ (**a, d** macrophages) or CD11b⁺Ly6G⁺ (**b, e** neutrophils). Bar graphs indicate mean percent CD11b⁺F4/80⁺ and CD11b⁺Ly6G⁺ events in the sciatic nerve (**c**) and DRG (**f**). n = 4 animals per condition per genotype. * p<0.05, ** p<0.01.

Figure 6. Myelin clearance from the distal sciatic nerve from WT and *Ocm* KO mice. Seven days after unilateral sciatic nerve transection changes in myelin clearance were determined by staining with luxol fast blue. Transected distal nerve segments from WT and *Ocm* KO mice showed significantly less staining than the sham-operated contralateral nerves, and no difference was seen between genotypes (**a**). The micrographs represent sections of WT sham-operated nerves (**b**) and transected distal nerves (**d**) and sections of *Ocm* KO sham-operated nerves (**c**) and transected distal segments (**e**). N = 5/group. Scale bar = 100 μm. **** p<0.0001.

Figure 7. *Il-6* mRNA expression is upregulated in the DRG at various timepoints after sciatic nerve injury. Although an increase was seen in DRG from both WT and *Ocm* KO mice 6 hours post-sciatic nerve transection, the increase was significantly larger in the former (**a**). *Il-6* mRNA expression was not different between WT and *Ocm* KO mice following a conditioning lesion compared to sham-operated contralateral DRG (**b**). *Lif*

576 and *Cntf* mRNA was not found to increase with injury in pooled lumbar DRG 6h after a
577 sciatic nerve transection (**a**) or a conditioning lesion (**b**). N =3-5/group. * $p<0.05$, ***
578 $p<0.001$, **** $p<0.0001$.

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