

1 **VEGF-A/VEGFR-1: a painful astrocyte-mediated signaling blocked by the anti-VEGFR-1**  
2 **mAb D16F7**

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35 **Abstract**

36 Chemotherapy-induced neuropathic pain is a clinically relevant adverse effect of several anticancer  
37 drugs leading to dose reduction or therapy discontinuation. The lack of knowledge about the  
38 mechanisms of neuropathy development and pain chronicization makes chemotherapy-induced  
39 neuropathic pain treatment an unmet medical need. In this context, the vascular endothelial growth  
40 factor A (VEGF-A) has emerged as a neurotoxicity biomarker in a model of chemotherapy-induced  
41 neuropathy, and its decrease has been related to pain relief. Aim of this study was to clarify the  
42 VEGF-A-dependent pain signaling in the CNS for individuating new targeted therapeutic  
43 approaches. In mice, the intrathecal infusion of VEGF-A induced a dose-dependent noxious  
44 hypersensitivity mediated by the VEGF receptor 1 (VEGFR-1) as demonstrated by pharmacological  
45 and genetic tools. In electrophysiological study, VEGF-A stimulated the spinal nociceptive neurons  
46 activity through VEGFR-1. In the dorsal horn of the spinal cord, VEGF-A increased in astrocytes of  
47 animals affected by neuropathy suggesting this cell population as a source of the potent pain  
48 mediator. Accordingly, the selective knockdown of astrocytic VEGF-A, by shRNAmir, blocked the  
49 development of oxaliplatin-induced neuropathic pain. Besides, the anti-VEGFR-1 mAb D16F7  
50 (previously described as anticancer) effectively relieved neuropathic pain induced by  
51 chemotherapeutic agents. In conclusion, astrocyte-released VEGF-A is a new player in the complex  
52 neuron-glia network that oversees physiological and pathological pain and D16F7 mAb rises as a  
53 potent pain killer strategy.

54

55 **Running title:** VEGF-A/VEGFR-1 block for pain relief

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57 **Keywords:** VEGF-A, VEGFR-1, chemotherapy-induced neuropathic pain, astrocytes, D16F7 mAb,  
58 biomarker

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## 69 **Introduction**

70 Persistent pain induced by the neurotoxic effects of anticancer chemotherapy (hereafter referred to  
71 as chemotherapy-induced neuropathic pain, CINP) is a kind of pure neuropathic hypersensitivity.  
72 Direct nervous tissue damages and a complex maladaptive response of peripheral and central  
73 nervous systems orchestrate a painful syndrome that keeps on after therapy discontinuation and  
74 beyond cancer resolution (Di Cesare Mannelli *et al*, 2013; Miltenburg & Boogerd, 2014; Ibrahim &  
75 Ehrlich, 2020).

76 Studying the role of stem cells in relieving neuropathic pain, we recently showed that the plasma  
77 concentration of vascular endothelial growth factor A (VEGF-A) was enhanced in rats repeatedly  
78 treated with oxaliplatin. Stem cells were able to control pain and normalize VEGF-A suggesting a  
79 possible role of the growth factor as pain mediator (Di Cesare Mannelli *et al*, 2018).

80 VEGF-A is a large anti-parallel homodimeric peptide that belongs to the “Cys-loop” superfamily of  
81 proteins. It is mainly known as a pro-angiogenic factor mediating blood vessel formation, vascular  
82 permeability, endothelial cell proliferation, differentiation, leakage, migration, survival, and  
83 motility (Iyer & Acharya, 2011). Alternative splicing of the *Vegfa* gene selectively removes intron  
84 regions and joins specific combinations of exons to generate distinct VEGF-A isoforms. Differing  
85 in respect to their length, isoforms are designated as VEGF<sub>xxx</sub> where xxx represents the number of  
86 amino acids present in the final protein sequence (the prototypical transcript VEGF<sub>165</sub> as well as the  
87 other commonly represented VEGF<sub>111</sub>, VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>; (Peach *et al*,  
88 2018). A major site of alternative splicing occurs at exon 8, whereby proximal splicing results in the  
89 VEGF<sub>xxx</sub>a forms and distal splicing generates the VEGF<sub>xxx</sub>b isoforms containing exon 8b (Stevens  
90 & Oltean, 2018). VEGF<sub>xxx</sub>a isoforms (that only differ from VEGF<sub>xxx</sub>b in the six amino acids found  
91 at their C termini) are considered to be “pro-angiogenic”, whereas VEGF<sub>xxx</sub>b isoforms have been  
92 reported to have “anti-angiogenic” properties (Peach *et al*, 2018), although this description does not  
93 reflect the whole functions of VEGF-A<sub>xxx</sub>b’s (Ved *et al*, 2018). Interestingly, in quiescent vessels, a  
94 higher proportion of total VEGF-A is represented by VEGF<sub>165</sub>b (Woolard *et al*, 2004).

95 Despite its discovery as an angiogenic factor, from an evolutionary perspective VEGF-A emerged  
96 in the CNS of primitive organisms that lacked an established vasculature, suggesting a vessel-  
97 independent activity (Ruiz de Almodovar *et al*, 2009; Ponnambalam & Alberghina, 2011). Indeed,  
98 growing evidence indicates a diverse range of effects of VEGF-A on neural cells during  
99 development and in adulthood (Ruiz de Almodovar *et al*, 2009; Lange *et al*, 2016). It promotes  
100 CNS perfusion and induces direct neurotrophic effects in normal and pathological conditions and,  
101 as a permeability factor, VEGF-A modulates blood-brain barrier (BBB) functionality (Argaw *et al*,  
102 2012; Licht & Keshet, 2013).

103 Cellular responses to VEGF-A are mainly driven by their cognate receptors, VEGFR-1 and -2,  
104 belonging to the class IV receptor tyrosine kinase family. The well-known VEGFR-2 plays essential  
105 roles in angiogenesis (Nakayama *et al*, 2013), as well as it mediates the neuroprotective effects of  
106 VEGF-A (Taiana *et al*, 2014; Verheyen *et al*, 2013). VEGFR-1 has a higher affinity for VEGF-A  
107 than VEGFR-2 but it shows decreased tyrosine kinase activity. VEGFR-1 is widely expressed also  
108 in non-endothelial cells and its soluble forms exhibit a negative modulatory activity on VEGFR-2  
109 effects (Failla *et al*, 2018; Peach *et al*, 2018; Seki *et al*, 2018); nevertheless, its biological functions  
110 remain largely undefined.

111 The role of VEGF-A in pain signalling is debated as conflicting literature data suggest both algescic  
112 (Lin *et al*, 2010; Selvaraj *et al*, 2015; Hamilton *et al*, 2016; Lee *et al*, 2019) and analgesic effects  
113 (Verheyen *et al*, 2012; Hulse *et al*, 2014; Ved *et al*, 2018; Hu *et al*, 2019; Verheyen *et al*, 2013).  
114 The present work intends to dissect the pain modulatory properties of VEGF-A in the CNS in  
115 physiological and neuropathic conditions by using preclinical *in vivo* models of CINP. Moreover,  
116 the role of the different receptor subtypes in pain signalling and the potential of the VEGF-  
117 A/VEGFRs system as target for relieving pain was explored.

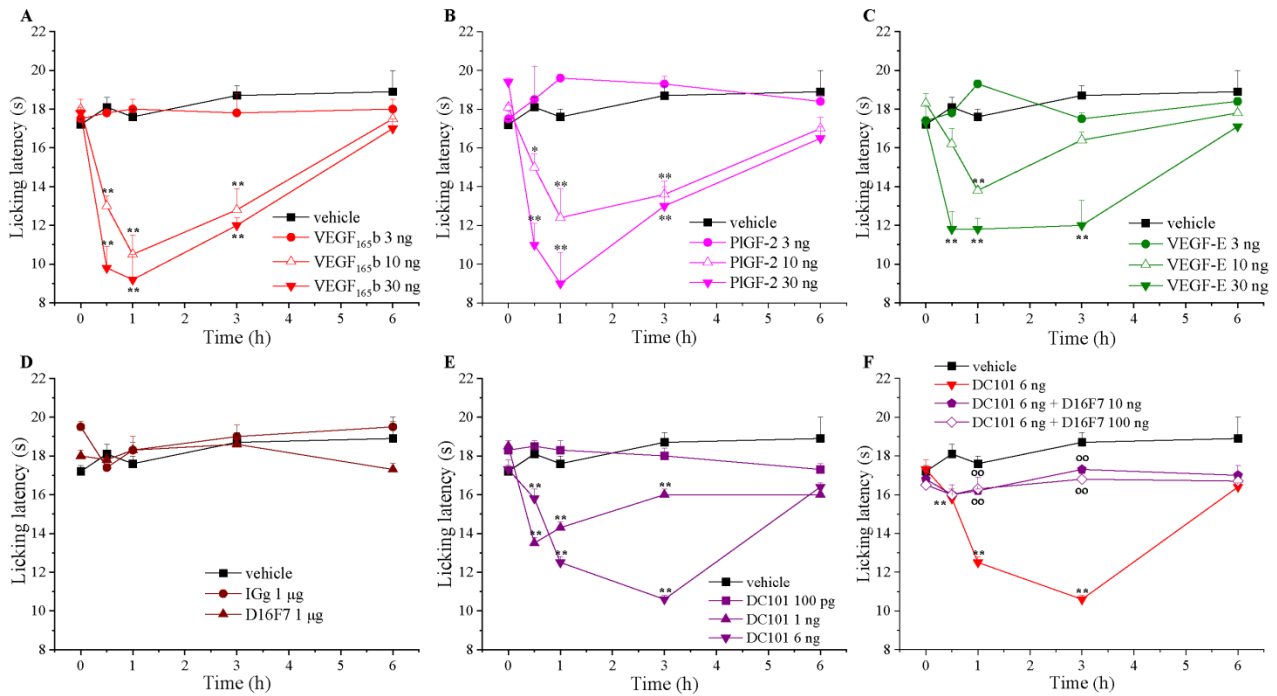
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## 119 **Results**

### 120 *Nociceptive effect of VEGFRs selective ligands infused in spinal cord*

121 To study the spinal impact of VEGF-A signalling modulation on pain threshold in mice, we firstly  
122 evaluated the effect of the most expressed isoform VEGF<sub>165b</sub>. After i.t. administration, pain  
123 sensitivity was measured as latency response to a cold stimulus (Cold plate test). As shown in Fig.  
124 1A, VEGF<sub>165b</sub> (3, 10 and 30 ng, in bolus in a total volume of 5 µl) dose-dependently reduced pain  
125 threshold with a long-lasting effect starting 30 min after injection that completely disappeared only  
126 after 6 h, resembling its effect observed in rats (Di Cesare Mannelli *et al*, 2018). To note, VEGF<sub>165a</sub>  
127 (1, 3 and 30 ng, i.t.) evoked similar dose-dependent nociceptive effects (Supplementary Fig. S1).  
128 Since VEGF<sub>165</sub> isoforms may interact with both VEGFR-1 and VEGFR-2, in order to explore the  
129 implications of the receptor types in pain modulation, we also tested the effect of placental growth  
130 factor 2 (PlGF-2) and VEGF-E, which are specific VEGFR-1 and VEGFR-2 agonists, respectively  
131 (Cudmore *et al*, 2012). As shown in Fig. 1B and 1C, both PlGF-2 and VEGF-E (3, 10 and 30 ng,  
132 i.t.) significantly reduced the licking latency of animals challenged on a cold surface (Cold Plate  
133 test), even if PlGF-2 showed a profile similar to VEGF<sub>165b</sub> while VEGF-E exhibited a lower  
134 efficacy. Interestingly, the selective VEGFR-1 blockade by the anti-VEGFR-1 mAb D16F7 (1 µg,  
135 i.t.), in the absence of VEGF<sub>165b</sub>, did not significantly alter pain threshold at microgram dose (Fig.  
136 1D). On the contrary, nanogram dose of the anti-murine VEGFR-2 mAb DC101 (1 and 6 ng, i.t.)

137 induced hypersensitivity (Fig. 1E) and this effect was blocked by D16F7 mAb (10 and 100 ng; Fig.  
 138 1F). In this test, non-specific mouse IgG (1  $\mu$ g), used as control, was inactive. These findings  
 139 suggested that the nociceptive effects evoked by VEGF<sub>165</sub>b were the result of VEGFR-1  
 140 stimulation. Furthermore, algescic effects induced by the DC101 mAb were likely due to the  
 141 antibody-dependent displacement of the endogenous VEGF-A from VEGFR-2, thus making it  
 142 available for binding to VEGFR-1; this hypothesis was further demonstrated by the loss of the  
 143 effect when the anti-VEGFR-1 mAb D16F7, was administered together with DC101.



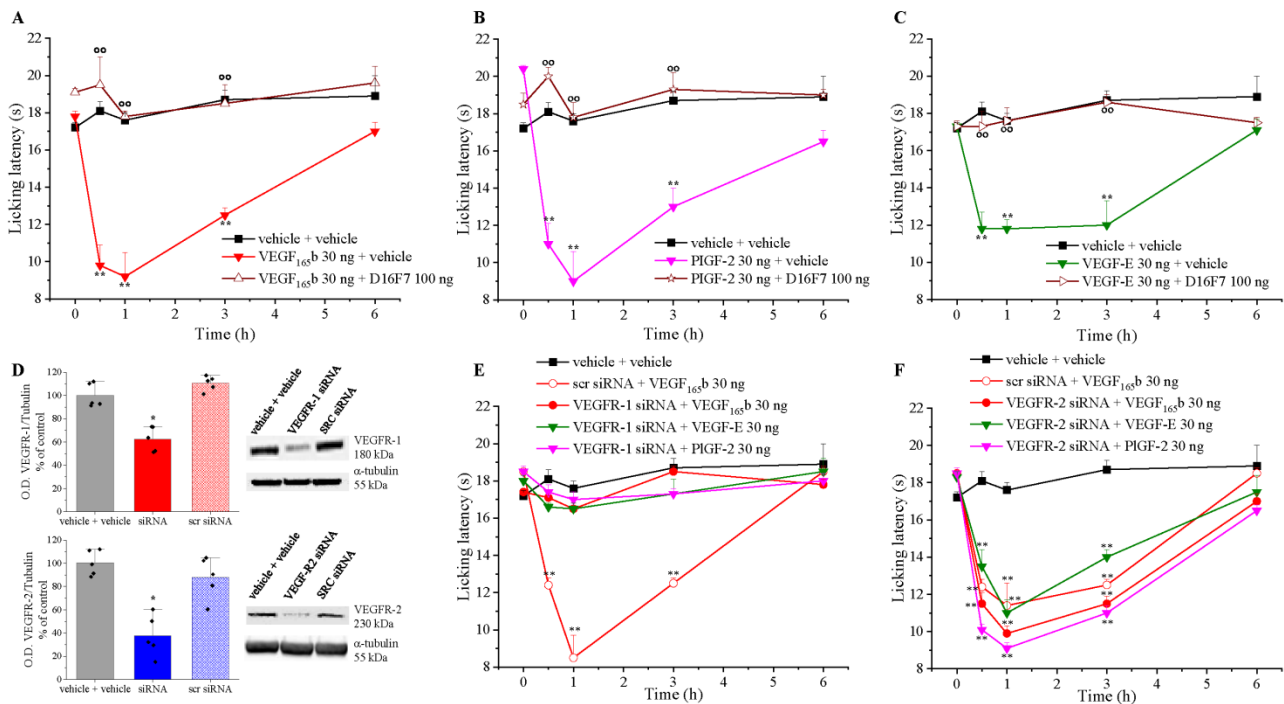
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 145 **Figure 1. Nociceptive effect of VEGFRs selective ligands infused in spinal cord.**

146 The pain threshold was measured by the Cold plate test over time after the i.t. injection of compounds. Effect of (A)  
 147 VEGF<sub>165</sub>b (n=7), (B) the selective VEGFR-1 agonist PIGF-2 (n=5), (C) the selective VEGFR-2 agonist VEGF-E  
 148 (n=5), (D) the selective anti-VEGFR-1 antibody D16F7 (n=7) or a murine control IgG (n=5) and (E) the selective anti-  
 149 VEGFR-2 antibody DC101 (n=5). (F) Effect of DC101 in mice pre-treated (15 min before) with D16F7. Each value  
 150 represents the mean  $\pm$  SEM. \*P < 0.05 and \*\*P < 0.01 vs vehicle-treated animals; °°P < 0.01 vs DC101 6 ng treated  
 151 animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure  
 152 was used as post hoc comparison.

153  
 154 **Hypersensitivity-induced by VEGF-A signalling modulators is due to VEGFR-1 activation**

155 The hypothesis that VEGFR-1 activation is required for VEGF<sub>165</sub>b-mediated nociception was  
 156 demonstrated by crossing the combinations of receptor agonists and antagonists. Both selective  
 157 agonists, PIGF-2 and VEGF-E (Cai *et al*, 2017; Park *et al*, 1994; Persico *et al*, 1999; Meyer, 1999),  
 158 share the same binding sites of VEGF-A on the corresponding receptors. At variance with DC101  
 159 mAb which is a competitive antagonist of VEGF-A and VEGF-E for VEGFR-2 binding (Falcon *et al*,  
 160 2016), D16F7 mAb is a non-competitive antagonist since it interacts with VEGFR-1 at a site

161 different from that used by the receptor ligands (Graziani *et al*, 2016; Lacial & Graziani, 2018).  
 162 Consistently with our hypothesis, the algescic effects of VEGF<sub>165b</sub> are blocked by D16F7 mAb (Fig.  
 163 2A). A similar profile was obtained also for the VEGFR-1 ligand PIGF-2 (Fig. 2B) as well as for  
 164 the VEGFR-2 ligand VEGF-E (Fig. 2C). DC101 mAb used at the highest non-algescic dose (but able  
 165 to selectively block VEGFR-2) (Falcon *et al*, 2016) did not block the effect of both VEGF<sub>165b</sub> and  
 166 PIGF-2, but further exacerbated VEGF-E hypersensitivity (Supplementary Fig. S2). These findings  
 167 confirmed the pivotal role of VEGFR-1 in pain signalling which is directly activated by the  
 168 selective agonist PIGF-2 or by the exogenously added (Fig. 2A) or endogenously present VEGF-A  
 169 (Fig. 2C) displaced from VEGFR-2. Moreover, the selective knockdown of VEGFR-1 or VEGFR-2  
 170 by siRNA further validated the specificity of the VEGFR-1-mediated mechanism (Fig. 2D-F). The  
 171 silencing of VEGFR-1 completely blocked the effects of VEGF<sub>165b</sub>, PIGF-2 and VEGF-E (Fig. 2E)  
 172 whereas the silencing of VEGFR-2 did not alter the algescic properties of the ligands (Fig. 2F).



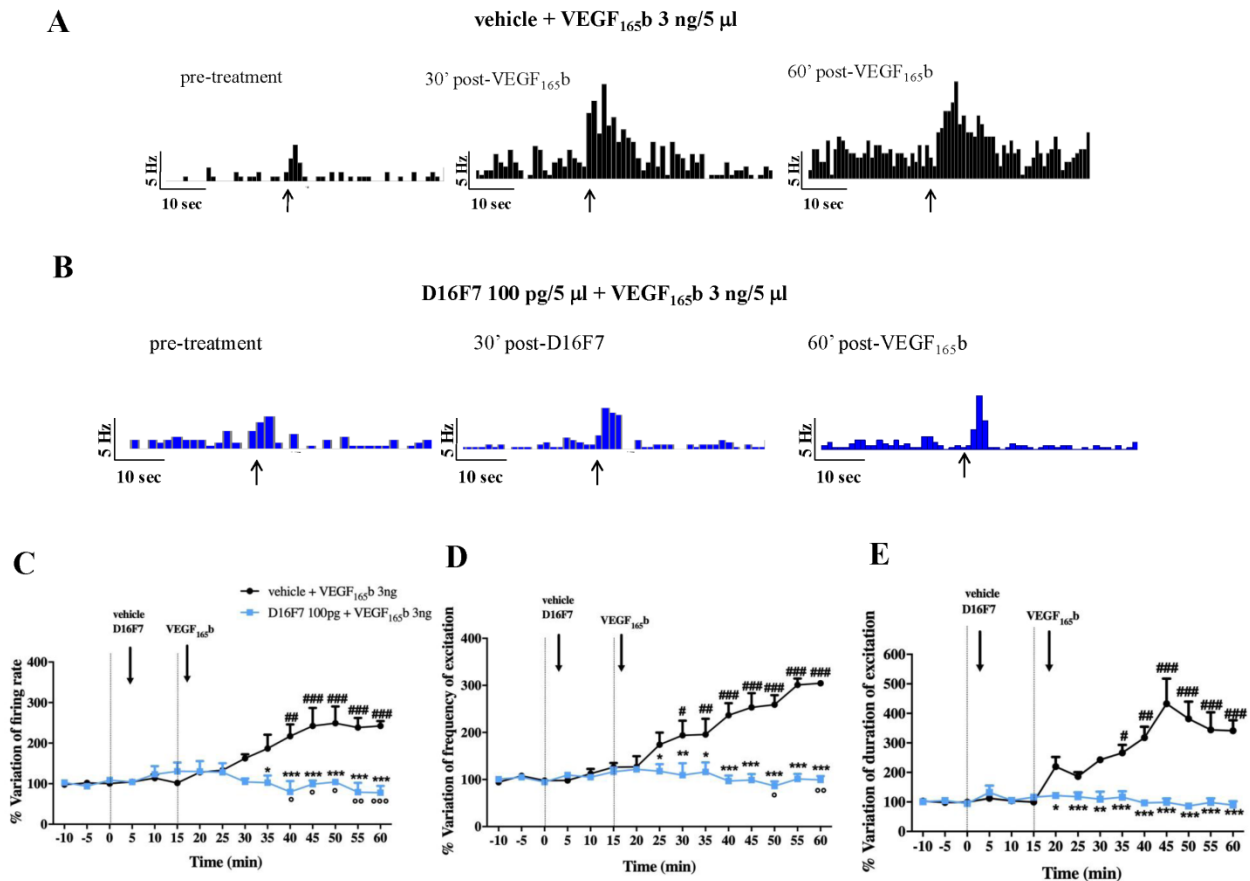
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 174 **Figure 2. Hypersensitivity-induced by VEGF-A signalling modulators is due to its interaction with VEGFR-1.**  
 175 The response to a thermal stimulus (Cold plate test) was recorded after *i.t.* infusion of different VEGFR ligands (30 ng)  
 176 preceded (15 min before) or not by the anti-VEGFR-1 mAb D16F7 (100 ng) or vehicle: (A) VEGF<sub>165b</sub> ± D16F7 (n=5),  
 177 (B) PIGF-2 ± D16F7 (n=5), (C) VEGF-E ± D16F7 (n=5). (E - F) Effects of VEGFRs ligands (*i.t.*) in mice undergone a  
 178 selective knockdown of VEGFR-1 (D, n=5) or VEGFR-2 (E, n=5) at the lumbar level of the spinal cord by siRNA. (D)  
 179 Representative western blot images and densitometric analysis showing the expression of VEGFR-1 or VEGFR-2 in the  
 180 lumbar section of the spinal cord after the siRNAs administration (n=5). Each value represents the mean ± SEM.  
 181 \*\*P<0.01 vs vehicle + vehicle-treated animals; °°P<0.01 vs vehicle + VEGFRs ligands-treated animals. The analysis  
 182 of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc  
 183 comparison.

184



185 *VEGF<sub>165b</sub> increases the activity of spinal nociceptive neurons by VEGFR-1 activation*

186 To investigate the effect of the spinal application of VEGF<sub>165b</sub> on the hyperexcitability of spinal  
187 nociceptive specific (NS) neurons, *in vivo* electrophysiological experiments were performed. The  
188 results relate to NS neurons (one cell recorded from each animal per treatment) localized at a depth  
189 of 0.7-1.0 mm from the surface of the spinal cord. This cell population was characterized by a mean  
190 rate of basal firing of  $0.015 \pm 0.002$  spikes/sec and only cells showing this pattern were chosen for  
191 the experiment. Spontaneous and noxious-evoked (mechanical stimulation) activity of NS neurons  
192 was measured after spinal application of VEGF<sub>165b</sub> preceded or not by treatment with the anti-  
193 VEGFR-1 mAb D16F7 to investigate the involvement of VEGF-A receptor subtype. Representative  
194 ratematers of the results obtained with VEGF<sub>165b</sub> in the absence or presence of D16F7 mAb are  
195 shown in Fig. 3A and 3B, respectively. In mice pre-treated with vehicle (DMSO in 0.9% NaCl),  
196 VEGF<sub>165b</sub> (3 ng/5  $\mu$ l) spinal application induced an increase in spinal electrical activity as  
197 compared to baseline levels (100%). In particular, NS neurons showed a variation of spontaneous  
198 activity compared to baseline of  $217.05 \pm 29.2\%$  as well as a noxious-evoked activity with  
199 frequency of  $234 \pm 30.9\%$  and duration of  $316.2 \pm 27.2\%$ , starting from 25 min post VEGF<sub>165b</sub> (Fig.  
200 3A, C-E). The spinal VEGF<sub>165b</sub>-induced hypersensitivity was mainly mediated by VEGFR-1 rather  
201 than VEGFR-2 activation. Indeed, electrophysiological recordings revealed that spinal pre-  
202 application of D16F7 mAb (100 pg/5  $\mu$ l) significantly prevented the increase of spontaneous and  
203 noxious-induced activity of NS neurons resulting in a pattern similar to basal (Fig. 3B, C-E). D16F7  
204 (100 pg/5  $\mu$ l) alone was not able to affect either spontaneous or evoked activity of NS neurons (Fig.  
205 3B). On the contrary, DC101 at 30 and 100 pg, showed a pro-nociceptive effect on NS spinal  
206 activity *per se* (Supplementary Fig. S3, representative ratematers). In fact, post-injection level of  
207 either spontaneous ( $187.3 \pm 17.7\%$  at 100 pg and  $151.1 \pm 6.9\%$  at 30 pg) or noxious pressure-  
208 evoked firing rates (frequency:  $212.6 \pm 27\%$  at 100 pg and  $152.9 \pm 6.9\%$  at 30 pg; duration:  $235.7 \pm$   
209  $25.3\%$  at 100 pg and  $119.7 \pm 8.6\%$  at 30 pg) were significantly higher respect to the baseline, in a  
210 dose-dependent manner. Overall, these results further confirmed the involvement of VEGFR-1 in  
211 VEGF-A-induced electrophysiological changes of NS.



212

213 **Figure 3. VEGF165b increases spontaneous and noxious-evoked activity of NS neurons by VEGFR-1.**

214 Representative ratemeters showing spontaneous and noxious-evoked activity of NS neurons after spinal application of  
 215 VEGF165b alone or in combination with D16F7 mAbs (A and B, respectively); black arrows indicate the noxious  
 216 stimulation on the mouse hind-paw. Mean ± S.E.M population data of spinal cord application of VEGF165b (3 ng/5 μl)  
 217 in the presence of vehicle (DMSO in 0.9% NaCl), or D16F7 (100 pg/5 μl) on firing rate of spontaneous activity (C),  
 218 frequency (D) and duration of evoked activity (E) of NS neurons in CD1 mice. Black arrows indicate vehicle, D16F7 or  
 219 VEGF165b spinal application. Each point represents the mean of 5 different mice per group (one neuron recorded per  
 220 each mouse). #P<0.05, ##P<0.01 and ###P<0.001 indicate statistically difference vs baseline; \*P<0.05, \*\*P<0.01  
 221 and \*\*\*P<0.001 indicate statistically difference vs vehicle + VEGF165b. One-way ANOVA followed by Dunnet's  
 222 multiple comparison post-hoc test was performed for statistical significance within groups. Two-way ANOVA followed  
 223 by Bonferroni post-hoc test was used for comparison between groups.

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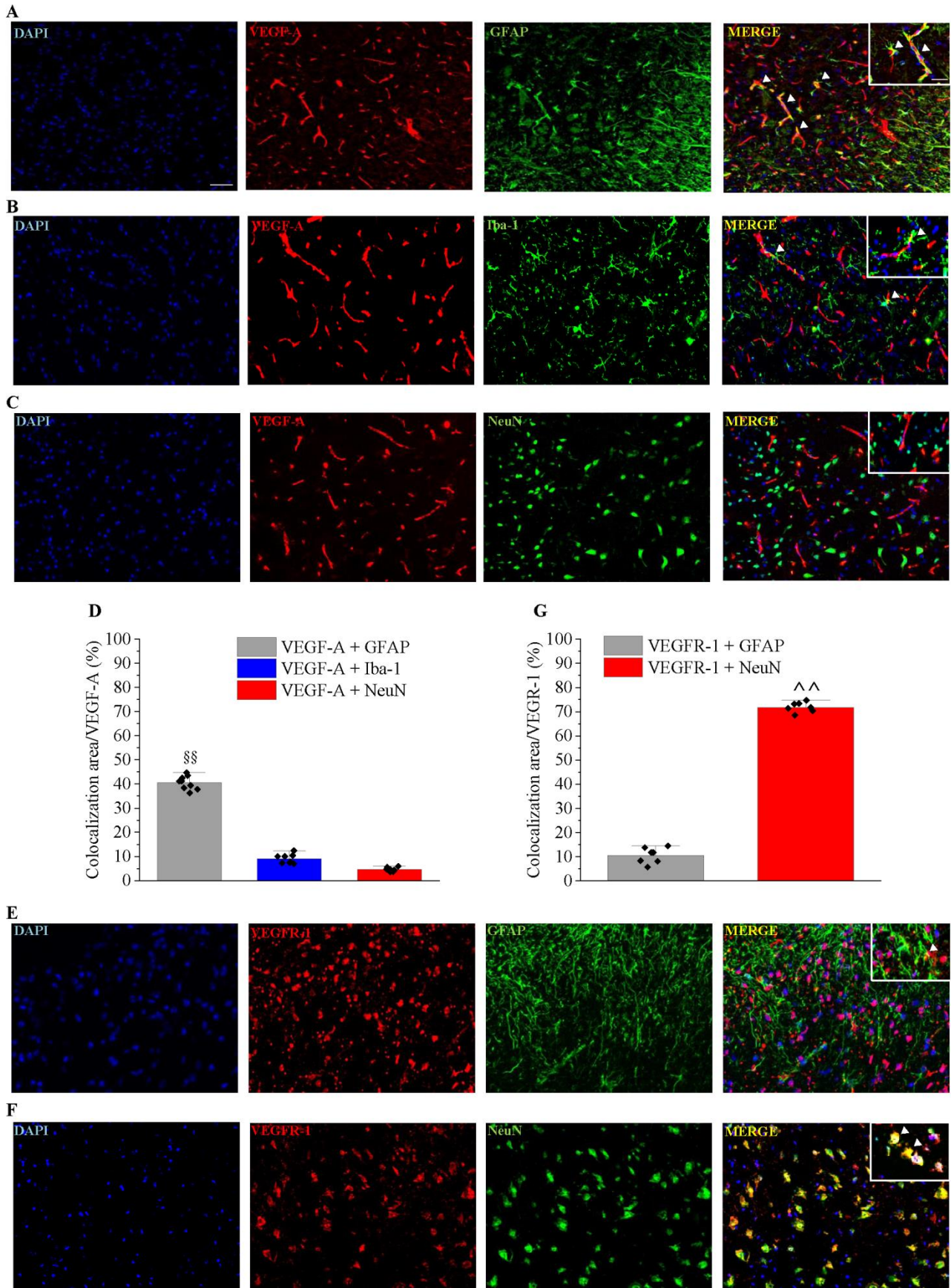
225 **VEGF-A and VEGFR-1 localization in the spinal cord of naïve mice**

226 Immunofluorescence analysis was performed in the dorsal horn of the spinal cord to study VEGF-A  
 227 and VEGFR-1 expression profile in the nervous cells. VEGF-A immunoreactivity in astrocytes (as  
 228 colocalization with GFAP; Fig. 4A and 4D) was significantly higher in comparison to microglia  
 229 (Iba1 positive cells) and neurons (NeuN positive cells) (Fig. 4B, 4C and 4D). As expected, VEGF-  
 230 A staining is strictly related to vessel structure (Fig. 4) since its expression was observed both on



231 endothelial cells and astrocyte endfeet (Lange *et al*, 2016). To better investigate this aspect, we  
232 compared the co-localization of VEGF-A with GFAP and RECA-1, a marker of endothelial cells.  
233 As shown in Fig. 5A, it is possible to identify separate areas of VEGF-A/GFAP and VEGF-  
234 A/RECA-1 colocalization. Furthermore, VEGF-A expression in astrocytes was also confirmed by  
235 confocal microscopy. Results shown in Fig. 5B and 5C confirm the colocalization of VEGF-A with  
236 GFAP and Aquaporin 4 (AQP4, a marker of astrocytic endfeet). Indeed, the Van Steensel's cross-  
237 correlation function (CCF) clearly shows that VEGF-A co-localizes with GFAP and AQP4 in  
238 cellular structures with an estimated diameter of  $1.00 \pm 0.11 \mu\text{m}$  and  $1.28 \pm 0.12 \mu\text{m}$  (CCF at  
239 FWHM, mean  $\pm$  SD, Supplementary Figure S4C and S4F), respectively, which are compatible with  
240 the size of astrocytic processes. Collectively, these analyses demonstrate the presence of a VEGF-A  
241 pool in astrocytes.

242 As regards the expression of VEGFR-1, it was more prominent in neurons than in astrocytes (Fig.  
243 4E, 4F and 4G).



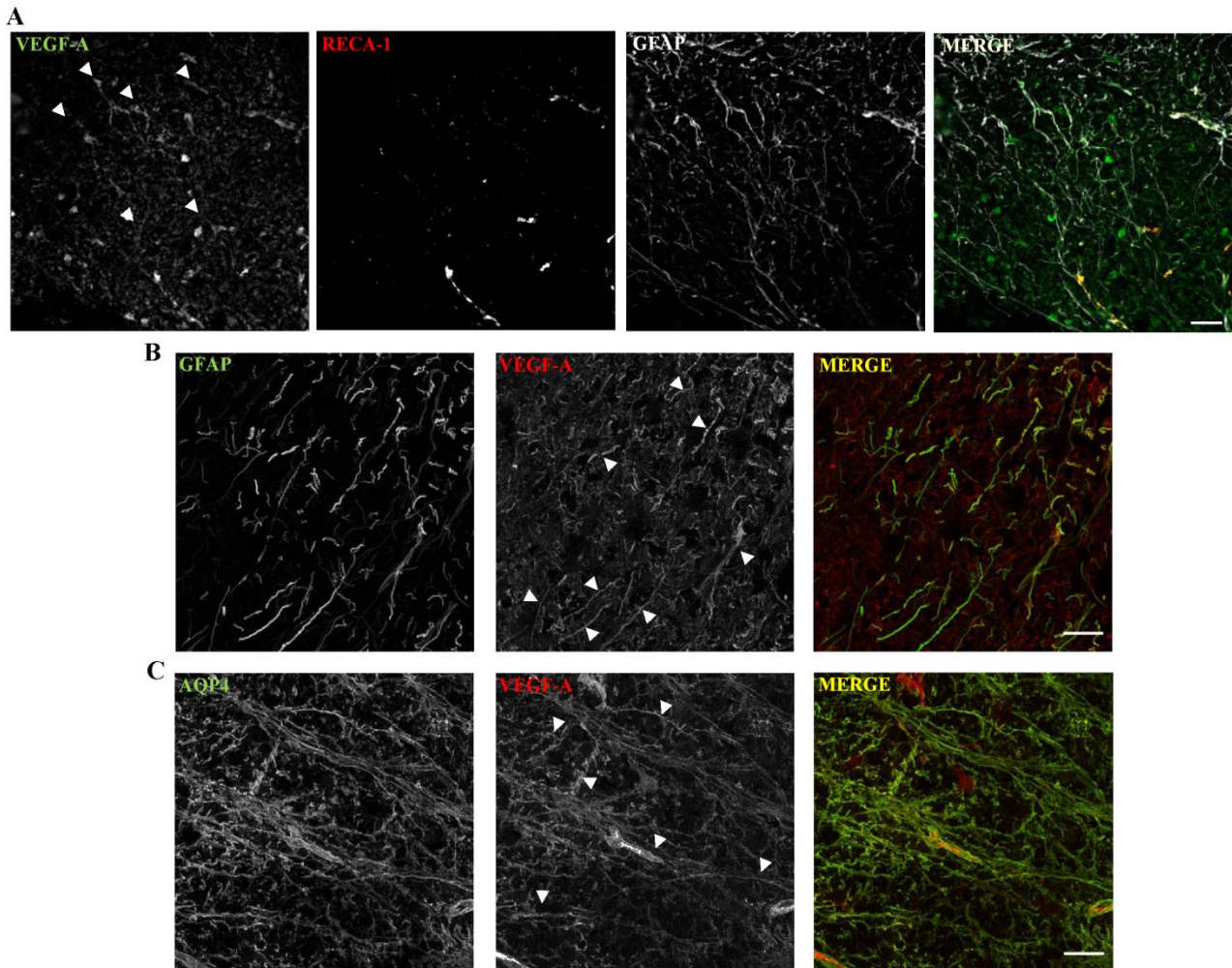
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Figure 4. Cellular localization of VEGF-A and VEGFR-1 in the spinal cord of naïve mice.



246 VEGF-A immunoreactivity was analysed in the spinal cord dorsal horn of naïve mice. Colocalization with GFAP-  
 247 positive astrocytes (A, n=9), Iba1 positive microglia (B, n=8) and NeuN-positive neurons (C, n=7) was evaluated and  
 248 quantified (D). Immunofluorescence co-staining of VEGFR-1 in the dorsal horn with GFAP (E, n=7) or NeuN (F, n=8)  
 249 positive cells, and quantitative analysis (G). Scale bar: 100  $\mu$ m. Each value represents the mean  $\pm$  SEM. §§P<0.01 vs  
 250 VEGF-A + Iba1 and VEGF-A + NeuN. ^P<0.01 vs VEGFR1 + GFAP.  
 251



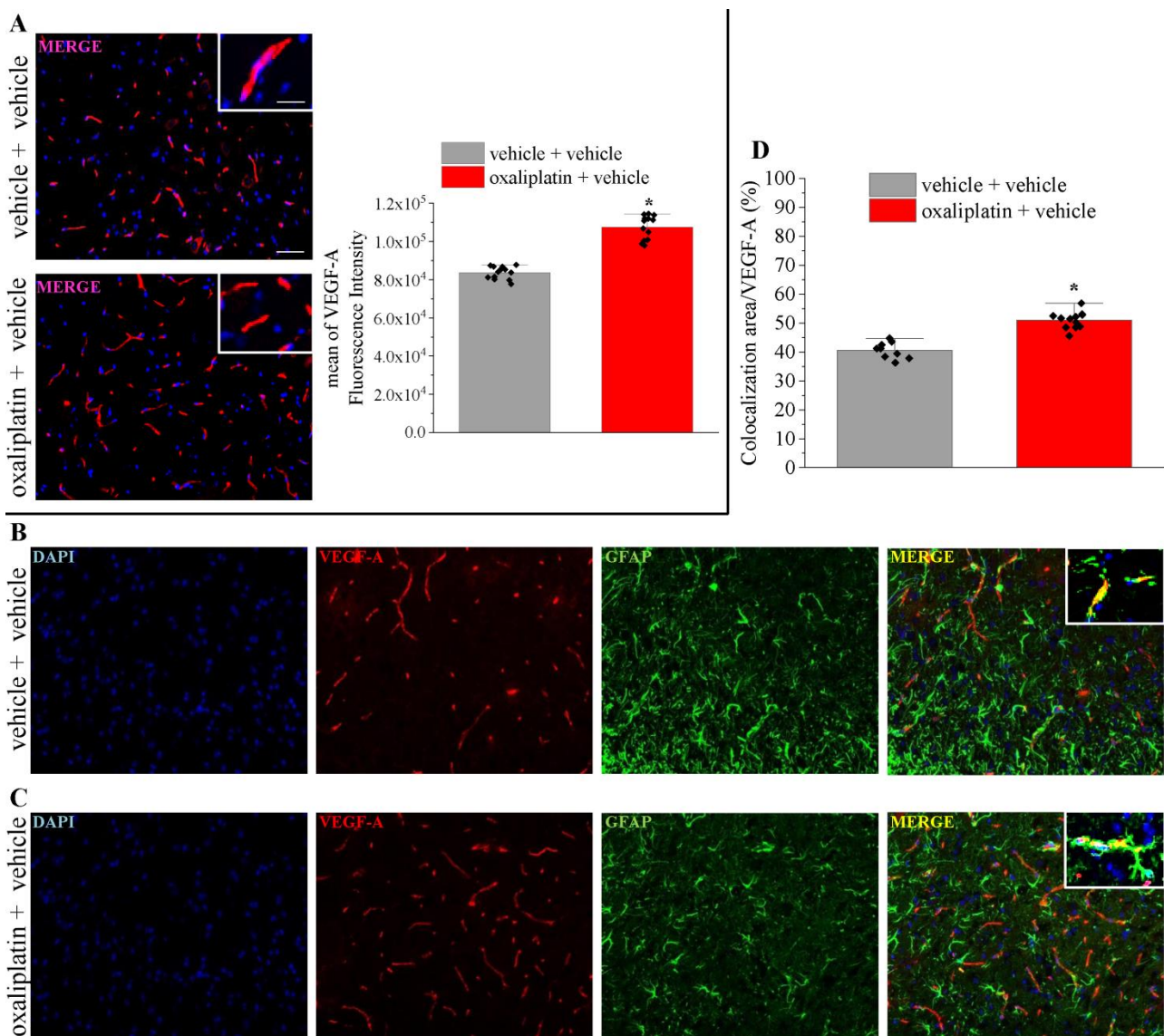
Staining	PCC	M1	M2	Li's ICQ
GFAP/VEGF-A	0.38 $\pm$ 0.04	0.42 $\pm$ 0.06	0.33 $\pm$ 0.04	0.20 $\pm$ 0.01
AQP4/VEGF-A	0.78 $\pm$ 0.01	0.62 $\pm$ 0.08	0.87 $\pm$ 0.03	0.35 $\pm$ 0.01

252  
 253 **Figure 5. Colocalization analysis of VEGF-A and RECA-1, GFAP or AQP4 in the spinal cord of naïve mice.**  
 254 A) VEGF-A immunoreactivity was analysed in the spinal cord dorsal horn of naïve mice in comparison to RECA-  
 255 1-positive endothelial cells and GFAP-positive astrocytes; arrows indicate the presence of VEGF-A in astrocytes; scale  
 256 bar: 100  $\mu$ m. B and C). Deconvolved confocal z-stacks shown as maximum intensity projection. Arrows indicate points  
 257 of interest. B) Representative GFAP and VEGF-A z-stack. C) Representative GFAP and Aquaporin-4 z-stack. Table).  
 258 Colocalization parameters are given as mean  $\pm$  SEM (n=8), PCC= Pearson's Correlation Coefficient; M1=Mander's  
 259 M1; M2= Mander's M2; Li's ICQ= Li's Intensity Correlation Quotient. Colocalization graph are shown in  
 260 Supplementary Figure S4.  
 261

262 *VEGF-A is increased in spinal astrocytes of mice with oxaliplatin-induced neuropathy*

263 A painful neuropathy was reproduced in mice by a repeated treatment with oxaliplatin (Cavaletti *et*  
264 *al*, 2001; Di Cesare Mannelli *et al*, 2017). After 2 weeks of treatment, when hypersensitivity was  
265 developed, VEGF-A immunoreactivity significantly increased in dorsal horns of the spinal cord in  
266 comparison to control animals (Fig. 6A and Supplementary Fig. S5). The increment was  
267 specifically confirmed in astrocytes when colocalization of VEGF-A expression in GFAP-positive  
268 cells was measured (Fig. 6B, 6C and 6D). As regards VEGFRs, VEGFR-2 expression increased in  
269 the spinal cord of oxaliplatin-treated mice as revealed by western blot, on the contrary VEGFR-1  
270 was unaffected by chemotherapy (Supplementary Fig. S6).

271



272

273 **Figure 6. VEGF-A is increased in spinal astrocytes of mice with oxaliplatin-induced neuropathy.**

274 (A) Representative images and quantitative analysis of mean VEGF-A fluorescence intensity in the dorsal horn of  
275 oxaliplatin-treated mice in comparison to control (n=13). (B-D) Colocalization analysis of VEGF-A and GFAP in the  
276 different groups, a quantitative analysis was reported (D) (vehicle + vehicle, n=13; oxaliplatin + vehicle, n=12). Scale

277 *bar: 100  $\mu$ m; insert: 50  $\mu$ m. Each value represents the mean  $\pm$  SEM. \* $P$ <0.05 vs vehicle + vehicle group. The analysis*  
278 *of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc*  
279 *comparison.*

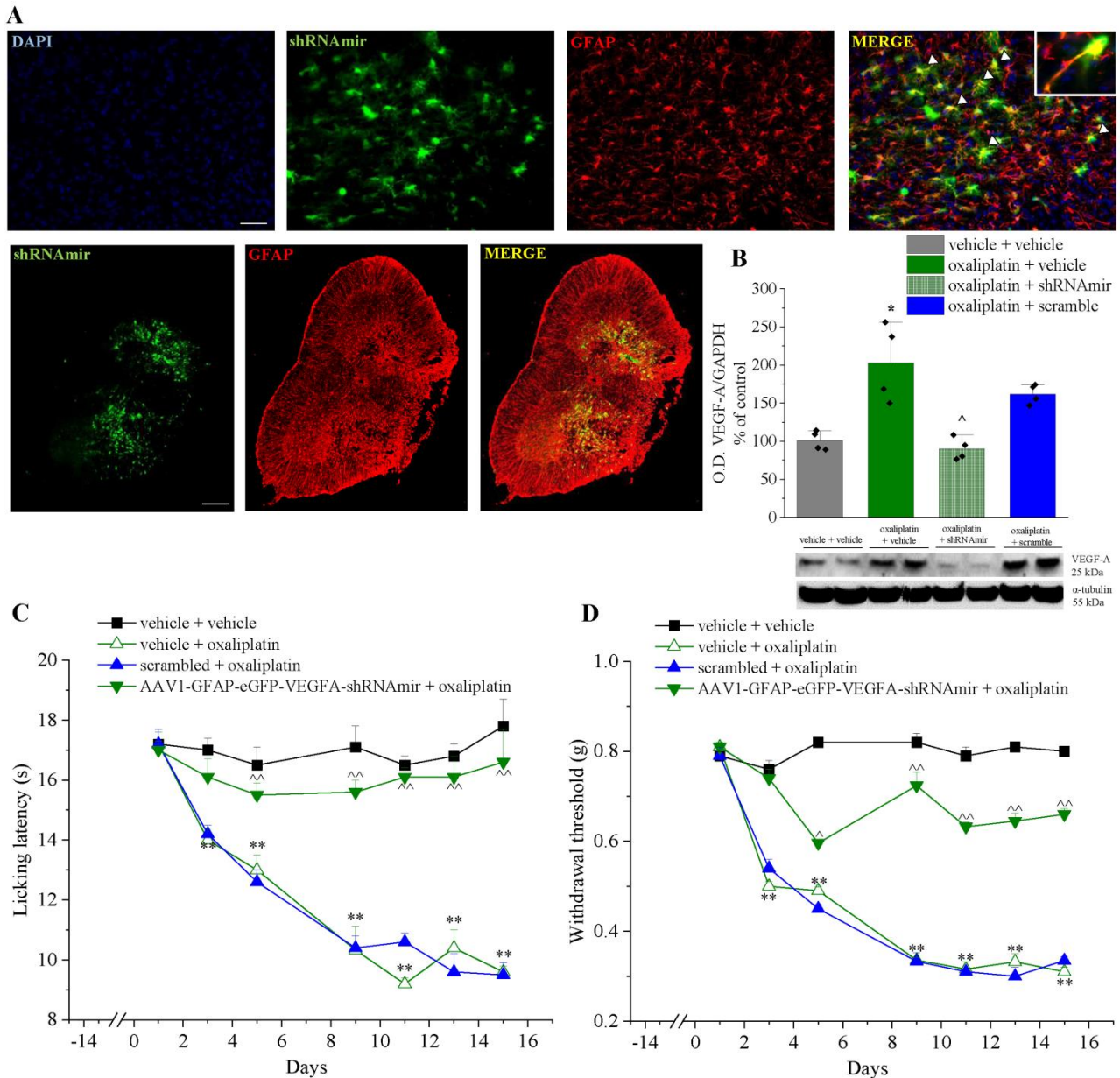
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### 281 *VEGF-A silencing in astrocytes prevents neuropathic pain*

282 To study the influence of astrocytic VEGF-A modulation on pain signaling, we selectively silenced  
283 VEGF-A in spinal astrocytes by injecting an AAV1-GFAP-eGFP-VEGFA-shRNAmir. The vector  
284 was bilaterally injected at the lumbar and thoracic levels of the spinal cord 2 weeks before the first  
285 oxaliplatin treatment. As shown in Fig. 7A, 4 weeks after injection, the vector fluorescence  
286 colocalized with GFAP-positive cells inducing a significant decrease of VEGF-A expression (Fig.  
287 7B). The pain threshold measurements by employing thermal (Cold plate test) and mechanical (Von  
288 Frey test) non-noxious stimuli over time showed a significant prevention of hypersensitivity  
289 development during the 2 weeks of oxaliplatin treatment in the group that received the VEGF-A  
290 specific shRNAmir in comparison to scrambled- and vehicle-treated mice (Fig. 7C and 7D). To  
291 verify the lack of neurological and motor alterations which could interfere with pain behavior  
292 recordings, VEGF-A shRNAmir and scrambled-treated mice motor functionality and exploratory  
293 activity were evaluated by the Hole board test. No alterations were highlighted with the exception  
294 of a higher exploratory activity on day 3 of oxaliplatin protocol (Supplementary Table S2).

295





296

297 **Figure 7. VEGF-A silencing in astrocytes prevents neuropathic pain.**

298 AAV1-GFAP-eGFP-VEGFA-shRNAmir was injected in the spinal cord to decrease VEGF-A expression in astrocytes.

299 (A) Representative image of eGFP and GFAP fluorescence in a whole section at lumbar level, scale bar: 100  $\mu$ m.

300 Higher magnifications were reported to visualize the colocalization, scale bar: 50  $\mu$ m (n=4).

301 Representative western blot images and densitometric analysis showing the expression of VEGF-A in the lumbar section of the spinal cord after the vector administration (n=4, blot of samples obtained from 2 animals of each group are shown).

302 Pain threshold was evaluated by (C) Cold plate and (D) Paw pressure test (n=5). Each value represents the mean  $\pm$  SEM.

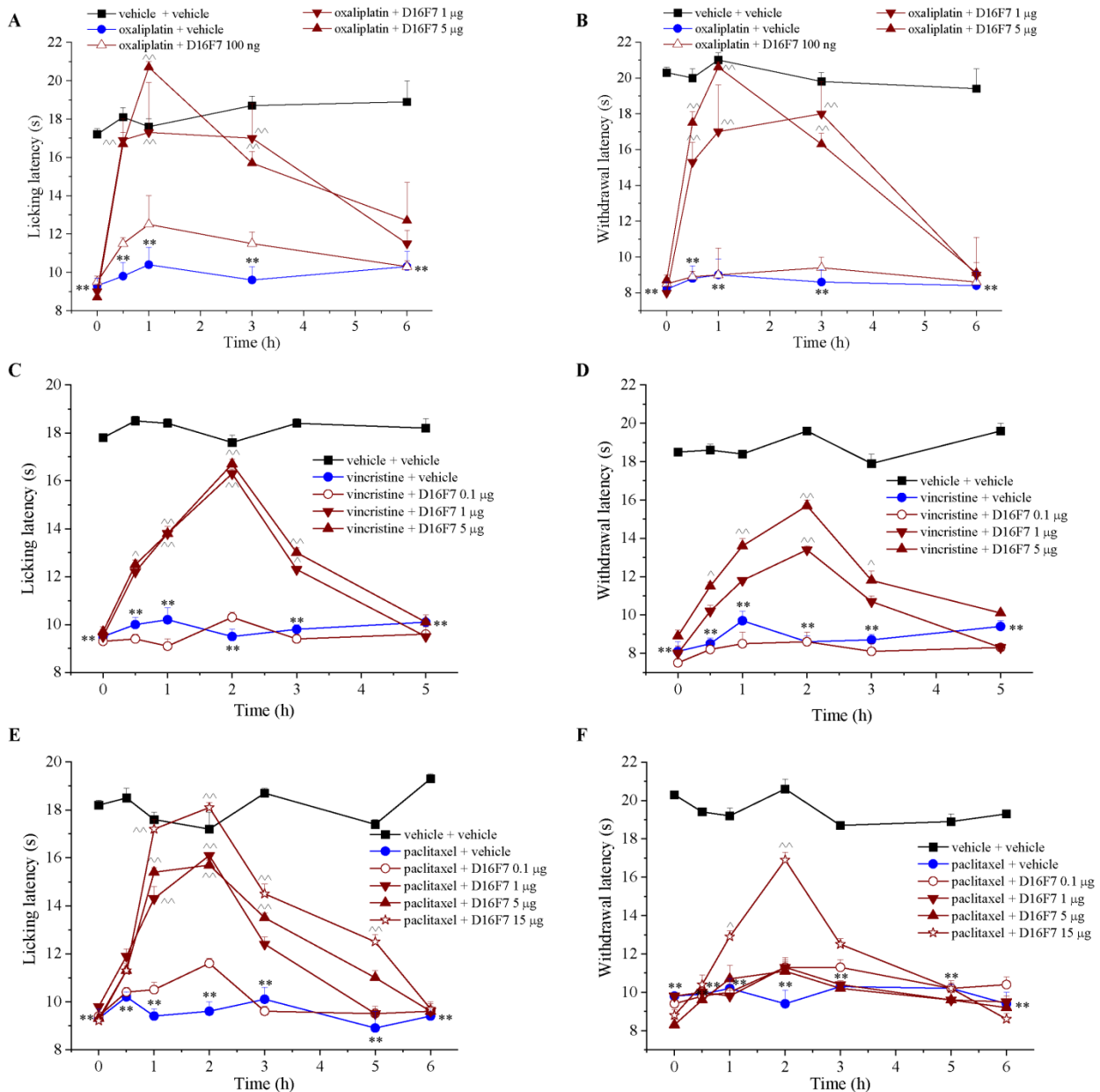
303 \* $P < 0.05$  and \*\* $P < 0.01$  vs vehicle + vehicle; ^ $P < 0.05$  and ^^ $P < 0.01$  vs scrambled + oxaliplatin group. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

307

308 The anti VEGFR1 mAb D16F7 relieves pain in different models of CINP



309 To evaluate the anti-hypersensitivity potential of D16F7, we tested its activity against neuropathic  
310 pain induced by anticancer drugs. In the already described oxaliplatin model, D16F7 was infused i.t.  
311 (100 ng, 1  $\mu\text{g}$  and 5  $\mu\text{g}$ ) showing a significant, dose-dependent, increase of the pain threshold both  
312 after thermal and mechanical non-noxious and noxious stimulation, respectively. Hypersensitivity  
313 was fully counteracted (up to control values) from 30 min to 3 h after treatment (Fig. 8A and 8B).  
314 On the contrary, the anti VEGFR-2 antibody DC101 (100 pg i.t.) was ineffective (data not shown).  
315 Interestingly, D16F7 mAb maintained its efficacy also when systemically injected by the i.p. route  
316 (1, 5, 15 and 25 mg  $\text{kg}^{-1}$ ). It was active starting from the dose 5 mg  $\text{kg}^{-1}$ , the onset of the analgesic  
317 effect was observed at 60 min and efficacy maintained up to 120 min (Supplementary Fig. S7A and  
318 S7B). The pain relieving properties of D16F7 mAb seems to be not limited to the oxaliplatin  
319 neurotoxicity since it was also effective in mice become hypersensitive after treatment with the  
320 neurotoxic anticancer drugs vincristine and paclitaxel. In both models, D16F7 mAb (1 and 5  $\mu\text{g}$ ,  
321 i.t.) was active between 30 min and 3 h (Fig. 8C-F) in the Cold plate and Paw pressure tests with a  
322 particular efficacy when the pain response was evoked by thermal stimuli (Fig. 8C and 8E). In  
323 paclitaxel-treated mice 15  $\mu\text{g}$  D16F7 mAb dosed i.t. was effective till to 5 h (Fig. 8E).  
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**Figure 8. D16F7 mAb reduces pain in different models of chemotherapy-induced neuropathy.** Effect of D16F7 mAb evaluated by (A) Cold plate and (B) Paw pressure tests in a mouse model of oxaliplatin-induced neuropathy after *i.t.* injection (A, B,  $n=6$ ). (C, D) Effect of D16F7 mAb after *i.t.* administration in vincristine-treated mice stimulated with thermal (C) or mechanical (D) stimuli ( $n=6$ ). (E, F) Effect of D16F7 after *i.t.* administration in paclitaxel-treated mice stimulated with thermal (E) or mechanical (F) stimuli ( $n=6$ ). Each value represents the mean  $\pm$  SEM. \*\* $P<0.01$  vs vehicle + vehicle-treated animals;  $\wedge P<0.05$  and  $\wedge\wedge P<0.01$  vs chemotherapeutic drugs + vehicle-treated animals. The analysis of variance was performed by One-way ANOVA. A Bonferroni's significant difference procedure was used as post hoc comparison.

## Discussion

336 Our data indicate that VEGF-A evokes pain through VEGFR-1 activation at the CNS site in  
337 physiological and pathological conditions. In particular, CINP is sustained by a spinal VEGF-A  
338 release from astrocytes that can be counteracted by the anti-VEGFR-1 mAb D16F7.

339 Using recombinant VEGF<sub>165b</sub>, the most represented VEGF-A isoform, we showed an increase of  
340 the electrophysiological activity of nociceptive neurons in the spinal cord with a consequent,  
341 significant decrease of the pain threshold (hypersensitivity was similarly induced by the VEGF<sub>165a</sub>  
342 isoform). These data are in agreement with our previous results (Di Cesare Mannelli *et al*, 2018)  
343 and with the peripheral pro-nociceptive effect of VEGF-A demonstrated by Selvaraj and colleagues  
344 (Selvaraj *et al*, 2015) after an intraplantar injection as well as with the VEGF-A increase in synovial  
345 fluid of subjects afflicted by osteoarticular pain (Hamilton *et al*, 2016). Furthermore, in peripheral  
346 nervous system, anti-VEGF-A mAb treatment was found to alleviate neuropathic pain induced by  
347 the chronic constriction injury of the sciatic nerve (Lin *et al*, 2010). In our hands, both the selective  
348 VEGFR-1 agonist PIGF-2 and the selective VEGFR-2 agonist VEGF-E induced nociception after  
349 i.t. infusion. In addition, the selective anti-VEGFR-2 mAb DC101 induced hypersensitivity whereas  
350 the selective anti-VEGFR-1 mAb D16F7 did not. Based on these data, it could be hypothesized that  
351 VEGFR-1 selectively mediates pain, either directly stimulated by the exogenously added PIGF-2 or  
352 by the endogenously produced VEGF-A displaced from VEGFR-2 by VEGF-E or by the  
353 competitive DC101 mAb. In this context, it is worth noting that D16F7 mAb is a non-competitive  
354 inhibitor that hampers VEGFR-1 activation without affecting ligand binding (Graziani *et al*, 2016;  
355 Lacal & Graziani, 2018). The involvement of VEGFR-1 in algisia induced by VEGF-A was  
356 confirmed by the ability of D16F7 mAb to block the nociceptive effects of all the agonists as well  
357 as of DC101. Furthermore, the knockdown of VEGFR-1 prevented VEGF<sub>165b</sub>, PIGF-2 and VEGF-E  
358 effects, strongly indicating the pivotal role of this receptor in the spinal pain pathway. These data  
359 are in agreement with those described by Selvaraj and colleagues (Selvaraj *et al*, 2015) in the  
360 peripheral nervous system where VEGF-A induced nociceptive sensitization via VEGFR-1.

361 Consistently with behavioural data, electrophysiological experiments revealed that VEGF<sub>165b</sub> spinal  
362 application, caused a strongly increase of both spontaneous and evoked activity of NS neurons in  
363 naïve animals. In particular, the increased responsiveness to mechanical noxious stimuli of NS  
364 neurons induced by VEGF<sub>165b</sub> spinal microinjection suggests that low doses of this compound were  
365 able to induce a central sensitisation, similarly to the neuropathic pain condition induced by nerve  
366 injury. In this context, the pre-application of D16F7 prevented the VEGF-A-induced neuronal  
367 hyperexcitability, ruling out the contribution of this receptor in VEGF-A-mediated painful effects.

368 In the normal healthy CNS, VEGF-A regulates microvascular density, vessel permeability, and  
369 maintains endothelial cell fenestration in the choroid plexus, stimulates neural stem cell

370 proliferation and promotes neurogenesis (Lange *et al*, 2016). In pathological conditions (besides  
371 beneficial vascular effects), it safeguards stressed neurons, induces axon extension and branching,  
372 and promotes synaptic plasticity; furthermore, VEGF-A triggers proliferation, survival and  
373 migration of astrocytes and stimulates expression of trophic factors by astrocytes and microglia  
374 (Ruiz de Almodovar *et al*, 2009; Lange *et al*, 2016). Glia cells play a crucial role in the maladaptive  
375 plasticity of the nervous system in chronic pain and, particularly, in neuropathies (Scholz & Woolf,  
376 2007). Activated by neuronal damage or by signals from periphery, glia participate in pain  
377 development and chronicization, amplifying the excitatory synaptic microenvironment (Stockstill *et*  
378 *al*, 2018; Di Cesare Mannelli *et al*, 2015). Released soluble factors, like cytokines and growth  
379 factors, possess a direct nociceptive effect (Sommer *et al*, 2018). Among the latter, NGF, BDNF,  
380 GDNF, etc, seem unable to separate the neuroprotective effect from the algic one probably  
381 following the evolutionary positive alarm role of physiological pain (Alles & Smith, 2018; Nencini  
382 *et al*, 2018; Garraway & Huie, 2016). In this context, the nociceptive effect of VEGF-A is not  
383 surprising. Neuropathies induced by trauma to a peripheral nerve (Hulse *et al*, 2014) or by  
384 chemotherapy (Di Cesare Mannelli *et al*, 2018) are characterized by enhanced spinal concentration  
385 of VEGF-A. The present results show, as expected (Lange *et al*, 2016), a relevant spinal VEGF-A  
386 concentration in the vessel structure; nevertheless, the existence of an *extra*-endothelial component  
387 was verified and confirmed. In comparison to microglia and neurons, astrocytes of healthy mice  
388 showed the highest amount of VEGF-A, which was clearly distinguishable from the vascular  
389 component. The repeated treatment with oxaliplatin up to the development of painful neuropathy  
390 significantly enhanced the presence of the growth factor in astroglia. The selective VEGF-A  
391 knockdown in dorsal horn astrocytes at the lumbar and thoracic levels of the spinal cord strongly  
392 reduced oxaliplatin-dependent neuropathic pain suggesting astrocytic VEGF-A as a relevant  
393 component of the pain signalling orchestrated by glia. Furthermore, enhanced concentrations of  
394 VEGF-A can also lead to other pathological alterations related to neuropathies like an increased  
395 BBB permeability (Di Cesare Mannelli *et al*, 2014; Branca *et al*, 2018; Montague-Cardoso *et al*,  
396 2020). As low maintenance levels of VEGF-A are necessary for the integrity of the BBB, high  
397 levels can alter permeability and compromise CNS (Argaw *et al*, 2012; Licht & Keshet, 2013). The  
398 hypoxia inducible factor-1 driven by IL-1 promotes VEGF-A release from astrocytes that induces  
399 down-regulation or loss of endothelial tight proteins claudin-5 and occludin, determining a loss of  
400 BBB function (Argaw *et al*, 2012; Chapouly *et al*, 2015) by mechanisms involving VEGFR-1 (Lee  
401 *et al*, 2019). On the other hand, the increase in VEGF-A levels in neurotoxic conditions is generally  
402 related to hypoxia, clearly demonstrated in diabetic- as well as in chemotherapy-induced  
403 neuropathies (Ved *et al*, 2018; Rojas *et al*, 2018; Di Cesare Mannelli *et al*, 2018), suggesting the

404 need of improving vascular functions (Lange *et al*, 2016). The rescue role of VEGF-A is also based  
405 on its extra-vascular neuroprotective and neurodegenerative properties mainly due to the activation  
406 of the VEGFR-2. VEGF-A stimulates the migration and survival of Schwann cells (Schratzberger *et*  
407 *al*, 2000), it protects neurons against chemotherapy-induced cytotoxicity via activation of VEGFR-2  
408 and MEK1/2 and inhibition of caspase-3 (Beazley-Long *et al*, 2013). VEGF-A-signalling through  
409 VEGFR-2 leads to the protection of dorsal root ganglion sensory neurons in models of drug  
410 (paclitaxel) or hyperglycaemia-induced neuropathies, through induction of Heat Shock Protein 90  
411 deacetylation and increase of Bcl-2 (Verheyen *et al*, 2012, 2013). The loss of endothelial VEGFR-2  
412 signalling leads to tissue alteration in the dorsal horn and the development of hyperalgesia whereas  
413 neuronal overexpression of VEGFR-2 in mice reduced the sensitivity to paclitaxel-induced  
414 peripheral neuropathy (Verheyen *et al*, 2012). This outcome seems to be related to neuroprotective  
415 effects and, accordingly, we showed an increase of VEGFR-2 spinal expression in oxaliplatin-  
416 treated mice that could be considered an adaptive response to the damage. On the contrary, the  
417 acute stimulation of VEGFR-2 does not directly interfere with pain.

418 Our data show that VEGF-A induces pain by selectively activating the VEGFR-1, which is  
419 expressed on spinal sensory neurons. A dichotomy between the pro-algesic VEGFR-1-signaling and  
420 the protective VEGFR-2-signaling is suggested, offering the possibility to relieve pain through a  
421 target that conserves the neuroprotective effects of the endogenous VEGF-A. In this view, the  
422 selective anti-VEGFR-1 mAb D16F7 induced a potent pain-relieving effect against nociception  
423 triggered by VEGF-A or PIGF-2 as well as against neuropathic pain evoked by the neurotoxic  
424 adverse reactions of different anticancer drugs like oxaliplatin, paclitaxel and vincristine. In  
425 addition, the pain-relieving effect of D16F7 was demonstrated after local (i.t.) and systemic (i.p.)  
426 administration. D16F7 mAb is able to inhibit VEGFR-1 homodimerization, auto-phosphorylation  
427 and downstream signal transduction (Graziani *et al*, 2016; Atzori *et al*, 2017, 2018) and down-  
428 modulates membrane receptor signaling without affecting VEGF-A or PIGF binding (Graziani *et al*,  
429 2016; Lacal & Graziani, 2018). Indeed, D16F7 mAb interacts with a receptor site corresponding to  
430 amino acids 149-161 of human VEGFR-1, which is different from that involved in VEGF-A or  
431 PIGF binding (Davis-Smyth *et al*, 1998; Christinger *et al*, 2004; Graziani *et al*, 2016).

432 CINP is one of the most common adverse events of several first-line chemotherapeutic agents,  
433 affecting several million patients worldwide each year and reducing the benefits of effective  
434 anticancer therapies in the long-term outcome. It is not possible to predict which patients will  
435 develop symptoms and when during the chemotherapy course. Moreover, pain and sensory  
436 abnormalities may persist for months, or even years after the cessation of chemotherapy (Argyriou  
437 *et al*, 2012; Kolb *et al*, 2016). The management of chemotherapy-induced neuropathy is a

438 significant challenge and there are no drugs approved to prevent or alleviate CINP (Ibrahim &  
439 Ehrlich, 2020). In this scenario, VEGF-A is candidate to be a possible plasmatic biomarker (Di  
440 Cesare Mannelli *et al*, 2018) strictly related to pain and the selective blockade of VEGFR-1 may  
441 offer the safety qualities requested for the pharmacological treatment of cancer patients in the  
442 presence of a possible co-treatment with chemotherapy. In fact, VEGFR-1 is mostly involved in  
443 pathological processes rather than in physiological conditions (reviewed in Lacal and Graziani)  
444 (Lacal & Graziani, 2018) and in preclinical *in vivo* studies repeated dosing schedules did not cause  
445 significant adverse effects, both as single agent or in combination with immune checkpoint  
446 inhibitors (Graziani *et al*, 2016; Atzori *et al*, 2018; Ceci *et al*, 2020; Lacal, Pedro Miguel, Atzori,  
447 MG; Ruffini, F; Scimeca, M; Bonanno, E; Cicconi, R; Mattei, M; Bernardini, R; D'Atri, S; Tentori,  
448 L; Graziani, G, 2020). Interestingly, D16F7 mAb has shown activity in orthotopic *in vivo* models of  
449 two highly aggressive tumors: melanoma and glioblastoma (Graziani *et al*, 2016; Atzori *et al*, 2017,  
450 2018). D16F7 efficacy against glioblastoma indicates that therapeutic concentrations of the mAb  
451 are reached at the CNS level after systemic administration (Atzori *et al*, 2018). Since VEGFR-1 is  
452 expressed not only in tumor cells but also in cell subsets of tumor microenvironment, its blockade  
453 by D16F7 results in: a) inhibition of tumor-associated angiogenesis; b) reduction of myeloid  
454 progenitor mobilization and tumor infiltration by M2 macrophages/microglia; c) inhibition of  
455 invasiveness and vasculogenic mimicry of VEGFR-1 positive tumor cells (Graziani *et al*, 2016;  
456 Atzori *et al*, 2018; Lacal, Pedro Miguel, Atzori, MG; Ruffini, F; Scimeca, M; Bonanno, E; Cicconi,  
457 R; Mattei, M; Bernardini, R; D'Atri, S; Tentori, L; Graziani, G, 2020).

458 In conclusion, VEGF-A is a pro-nociceptive molecule that in the CNS activates neuronal firing and  
459 induces pain by VEGFR-1 stimulation. Actually, VEGF-A increases during chemotherapy-induced  
460 neuropathy, and its release from astrocytes plays a decisive role in CINP development. Moreover,  
461 the anti-VEGFR-1 mAb D16F7 is suggested as a promising candidate for the treatment of CINP,  
462 adding this property to its previously described anti-tumour efficacy. The results of this proof of  
463 concept study encourage further investigation on the most effective therapeutic schedule for a long-  
464 term pain control.

465

## 466 **Materials and Methods**

### 467 *Study approval*

468 All animal manipulations were carried out according to the Directive 2010/63/EU of the European  
469 parliament and of the European Union council (22 September 2010) on the protection of animals  
470 used for scientific purposes and with IASP. The ethical policy of the University of Florence  
471 complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes



472 of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number:  
473 A5278-01). Formal approval to conduct the experiments described was obtained from the Italian  
474 Ministry of Health (No. 171/2018-PR) and from the Animal Subjects Review Board of the  
475 University of Florence and from the Animal Ethics Committee of University of Campania of  
476 Naples. Experiments involving animals have been reported according to ARRIVE guidelines  
477 (McGrath & Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the  
478 number of animals used.

479

#### 480 *Animals*

481 Eight week old male CD-1 mice (Envigo, Varese, Italy) weighing 20-25 g at the beginning of the  
482 experimental procedure were used. Animals were housed in the Centro Stabulazione Animali da  
483 Laboratorio (University of Florence, Italy) and in Stabulario Centralizzato di Ateneo (University of  
484 Campania “Luigi Vanvitelli”, Naples, Italy) and used at least 1 week after their arrival. Ten mice  
485 were housed per cage (size 26 cm x 41 cm); animals were fed a standard laboratory diet and tap  
486 water ad libitum and kept at  $23 \pm 1$  °C with a 12 h light/dark cycle (light at 7 am).

487

#### 488 *Treatments*

489 VEGF<sub>165b</sub> (3 – 30 ng; 5 µl; cat. #3045-VE-025, R&D System, USA), PIGF-2 (3 – 30 ng; 5 µl; cat.  
490 465-PL/CF, R&D System, USA), VEGF-E (3 – 30 ng; 5 µl; cat. #CYT-263, Prospec, Israel),  
491 D16F7 (10 - 100 ng, 5 µg; 5 µl) (Graziani *et al.*, 2016) and DC101 (100 pg, 1 – 6 ng; 5 µl; catalog.  
492 #BE0060 BioCell, Boston, MA, USA) or vehicle (0.9% NaCl 5 µl) were injected i.t in conscious  
493 mice as previously described (Hylden & Wilcox, 1980). Briefly, a 25-µl Hamilton syringe  
494 connected to a 30-gauge needle was intervertebral inserted between the L4 and L5 region, and  
495 advanced 6 mm into the lumbar enlargement of the spinal cord. Behavioural measurements were  
496 performed before and 30 min, 1 h, 3 h and 6 h after the administration of compounds. DC101 or  
497 D16F7 were injected 15 min before the VEGFR-1/2 agonists when administered in the co-treatment  
498 experiments.

499 The scrambled siRNA or the specific VEGFR siRNA (VEGFR-1-VEGFR-2 siRNA, Ambion Life  
500 Technologies, Monza, Italy) were intrathecally (i.t.) injected twice spaced 24 h apart (3.3 µg/5 µl  
501 per mouse) at the lumbar level of the mice spinal cord. On the third day, behavioural measurements  
502 were conducted after VEGFRs agonists administration. Mice were sacrificed between the 4<sup>th</sup> and  
503 the 5<sup>th</sup> days for the western blot analysis. The target sequences of the anti-mouse VEGFRs siRNAs  
504 were: VEGFR-1, sense strand 5'-GCAUCUAUAAGGCAGCGGAtt-3' and antisense strand  
505 UCCGCUGCCUUAUAGAUGCtc-3'; VEGFR-2, sense strand 5'-

506 CCGGUAUGCUUGUAAAGAAAtt-3' and antisense strand 5'-UUCUUUACAAGCAUACGGGct-  
507 3'.

508

#### 509 *AAV virus infection*

510 An AAV1-GFAP-eGFP-mVEGFA-shRNAmir (1.6 X 10<sup>13</sup> GC/ml, Vector Biosystem Inc,  
511 Malvern, PA, USA) or scrambled were used. Mice were deeply anaesthetized by intraperitoneal  
512 (i.p.) injection of ketamine (100 mg kg<sup>-1</sup>) (Ketavet, MSD Animal Health, Milan, Italy) and xylazine  
513 (10 mg kg<sup>-1</sup>) (Rompum, 20 mg/ml, Bayer, Milan, Italy) and then were placed in a stereotaxic frame  
514 using the mouse spinal adaptor (Stoelting, Wood Dale, IL, USA). The skin was incised at Th12–L5  
515 and the mouse muscles around the left side of the interspace between Th12 - L1 and L4 - L5  
516 vertebrae were removed and the dura mater and the arachnoid membrane were carefully incised  
517 using the tip of a 30G needle to make a small window to allow vector infusion. Intraspinal  
518 injections were done using a 5- $\mu$ l Hamilton syringe connected to a 34G needle. The needle was  
519 placed 0.5 mm lateral to the spinal midline at a depth of 0.4 mm from the dorsal surface of the  
520 spinal cord and 1  $\mu$ l of vector or scrambled was bilaterally injected at 0.25  $\mu$ l/min with a digital  
521 microinjector (Stoelting). The needle was left on place for another 3 min to prevent backflow. The  
522 surgical site was then sutured with 3-0 silk and mice were kept on a heating pad until recovery.

523

#### 524 *CINP in vivo models*

525 Mice treated with oxaliplatin (Carbosynth, Pangbourne, UK; 2.4 mg kg<sup>-1</sup>) were administered i.p. for  
526 two weeks (Cavaletti *et al*, 2001; Di Cesare Mannelli *et al*, 2017). Oxaliplatin was dissolved in a  
527 5% glucose solution. Control animals received an equivalent volume of vehicle. Behavioural tests  
528 were performed on day 15 for the acute treatments. In mice injected spinally with the viral vector or  
529 with the scrambled, oxaliplatin was administered for two weeks (10 total injections) starting 14  
530 days after surgery for the viral vector administration. Control animals received an equivalent  
531 volume of vehicle. Behavioural measurements were performed on days 3, 5, 9, 11, 13 and 15.

532 Mice treated with paclitaxel (Carbosynth; 2.0 mg kg<sup>-1</sup>) were injected i.p. on four alternate days  
533 (days 1, 3, 5 and 8) (Polomano *et al*, 2001; Di Cesare Mannelli *et al*, 2017). Paclitaxel was  
534 dissolved in a mixture of 10% saline solution and Cremophor EL, a derivative of castor oil and  
535 ethylene oxide that is clinically used as paclitaxel vehicle. Control animals received an equivalent  
536 volume of vehicle. Behavioural measurements started on day 10.

537 Mice treated with vincristine (Carbosynth; 0.1 mg kg<sup>-1</sup>) were injected i.p. for five consecutive days  
538 (Weng *et al*, 2003). Vincristine was dissolved in saline solution and control animals received an  
539 equivalent volume of vehicle. Behavioural measurements started on day 8.

540 *Assessment of mechanical hyperalgesia (Paw pressure test)*

541 Mechanical hyperalgesia was determined by measuring the latency in seconds to withdraw the paw  
542 away from a constant mechanical pressure exerted onto the dorsal surface (Russo *et al*, 2012;  
543 Lucarini *et al*, 2019). A 15 g calibrated glass cylindrical rod (10 mm diameter) chamfered to a  
544 conical point (3 mm diameter) was used to exert the mechanical force. The weight was suspended  
545 vertically between two rings attached to a stand and was free to move vertically. A single measure  
546 was made per animal. A cut-off time of 40 s was used.

547

548 *Assessment of thermal allodynia (Cold plate test)*

549 Thermal allodynia was assessed using the Cold-plate test. With minimal animal-handler interaction,  
550 mice were taken from home-cages, and placed onto the surface of the cold-plate (Ugo Basile,  
551 Varese, Italy) maintained at a constant temperature of  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Ambulation was restricted by a  
552 cylindrical plexiglas chamber (diameter: 10 cm, height: 15 cm), with open top. A timer controlled  
553 by foot peddle began timing response latency from the moment the mouse was placed onto the cold-  
554 plate. Pain-related behaviour (licking of the hind paw) was observed, and the time (seconds) of the  
555 first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 30 s  
556 (Baptista-de-Souza *et al*, 2014).

557

558 *Assessment of mechanical allodynia (Von Frey test)*

559 Mechanical allodynia was measured with the dynamic plantar aesthesiometer (von Frey instrument)  
560 (Ugo Basile) as described by Di Cesare Mannelli and colleagues (Di Cesare Mannelli *et al*, 2017)  
561 with minor modifications. Briefly, the mice were placed in individual Plexiglas cubicles ( $8.5 \times 3.4$   
562  $\times 3.4$  cm) on a wire mesh platform. After approximately 30 min accommodation period, during  
563 which exploratory and grooming activity ended, the mechanical paw withdrawal threshold was  
564 measured as the hind paw withdrawal responded to von Frey hair stimulation. The mechanical  
565 stimulus was delivered to the plantar surface of the hind paw of the mouse from below the floor of  
566 the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronic  
567 ascending force (0–5 g in 35 s). When the animal withdrew its hind paw, the mechanical stimulus  
568 was automatically withdrawn, and the force recorded to the nearest 0.1 g. Nociceptive response for  
569 mechanical sensitivity was expressed as mechanical withdrawal threshold in grams. The mean was  
570 calculated from six consecutive trials and averaged for each group of mice.

571

572 *Assessment of locomotor activity (Hole-Board test)*

573 The locomotor activity was evaluated by using the hole-board test. The apparatus consisted of a 40  
574 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4×4 in an  
575 equidistant, grid-like manner. Mice were placed on the centre of the board one by one and allowed  
576 to move about freely for a period of 5 min each. Two photobeams, crossing the plane from mid-  
577 point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically  
578 signalled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor  
579 activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the  
580 exploration of the holes (exploratory activity) by the mice (Ghelardini *et al*, 2002).

581  
582 *Electrophysiological recordings of nociceptive specific (NS) neurons*

583 On the day of electrophysiological recordings, mice were initially anesthetized with  
584 tribromoethanol (Avertin, Winthrop laboratories, New York, NY, USA; 1.25%). After tracheal  
585 cannulation, a catheter was placed into the right external jugular vein, to allow continuous infusion  
586 of propofol (5–10 mg/kg/h, i.v.) and spinal cord segments L4-L6 were exposed by laminectomy,  
587 near the dorsal root entry zone, up to a depth of 1 mm (McGaraughty *et al*, 2010). An elliptic rubber  
588 ring (about 3×5 mm), sealed with silicone gel onto the surface of the cord, was used for topical  
589 spinal drug application and to gain access to spinal neurons. Animals were fixed in a stereotaxic  
590 apparatus (David Kopf Instruments, Tujunga, CA, USA) through clamps attached to the vertebral  
591 processes. Single unit extracellular activity of dorsal horn NS neurons was performed by using a  
592 glass-insulated tungsten filament electrode (3–5 MΩ) (FHC Frederick Haer & Co., ME, USA).  
593 Spinal neurons were defined as NS neurons, when they were responding only to high intensity  
594 (noxious) stimulation (Telleria-Diaz *et al*, 2010). In particular, to confirm NS response patterns,  
595 each neuron was characterized by applying a mechanical stimulation to the ipsilateral hind paw  
596 using a von Frey filament with 97.8 mN bending force (noxious stimulation) for 2 s until it buckled  
597 slightly (Boccella *et al*, 2015; Simone *et al*, 2008). Only neurons that specifically responded to  
598 noxious hind paw stimulation were considered for recordings. The recorded signals were visualized  
599 into a window discriminator, whose output was processed by an interface CED 1401 (Cambridge  
600 Electronic Design Ltd., UK) connected to iOS 5 PC. Spike2 software (CED, version 5) was used to  
601 create peristimulus rate histograms on-line and to store and analyze digital records of single unit  
602 activity off-line. The spontaneous and noxious-evoked neuronal activity was expressed as  
603 spikes/sec (Hz) and the effect of drugs was analyzed as % variation of firing rate, frequency and  
604 duration of excitation. After recording a stable basal activity (15 min), topical spinal application of  
605 vehicle or drugs was performed, and each extracellular recording was monitored until 45-60 min  
606 post-injection. In particular, groups of animals were divided as following: 1) VEGF<sub>165b</sub> (3 ng/5 μl,

607 pro-nociceptive dose on NS neurons), 2) VEGF<sub>165</sub>b + DC101 (10 pg/5  $\mu$ l, the highest non pro-  
608 nociceptive dose) and 3) VEGF<sub>165</sub>b + D16F7 (100 pg/5  $\mu$ l). At the end of the experiment, animals  
609 were killed with a lethal dose of urethane.

610

#### 611 *Western blot analysis*

612 The lumbar spinal cord of mice was explanted and immediately frozen with liquid nitrogen. The  
613 frozen tissues were homogenized with lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM  
614 NaCl, 1 mM EDTA, 0.5% Triton X-100 and complete protease inhibitors (Roche, Milan, Italy). The  
615 suspensions were sonicated on ice using three high intensity 10s bursts with a cooling period of 10s  
616 each burst and centrifuged at 13.000xg for 10 min at 4°C. Protein concentrations were quantified by  
617 bicinchoninic acid test. Fifty  $\mu$ g of tissue homogenate were resolved with prefabricated  
618 polyacrylamide gel (BOLT 4-12% Bis-Tris Plus gel; Thermo Fisher Scientific, Monza, Italy) before  
619 electrophoretic transfer to nitrocellulose membranes (Bio-Rad, Milan, Italy). The membranes were  
620 blocked with 1% BSA and 5% fat-free powdered milk in PBS containing 0.1% Tween 20 (PBST)  
621 and then probed overnight at 4°C with primary antibodies specific for VEGFR-1, VEGFR-2,  
622 VEGF-A, GAPDH or  $\alpha$ -Tubulin (Supplementary Table S1). The membranes were then incubated  
623 for 1 h in PBST containing the appropriate secondary anti-rabbit or anti-mouse antibody  
624 (Supplementary Table S1). ECL (Enhanced Chemiluminescence Pierce, Rockford, IL, USA) was  
625 used to visualize peroxidase-coated bands. Densitometric analysis was performed using the  
626 "ImageJ" analysis software (ImageJ, NIH, Bethesda, MD, USA). Normalization for  $\alpha$ -tubulin or  
627 GAPDH content was performed. The values were reported as percentages of controls arbitrarily set  
628 at 100%.

629

#### 630 *Immunofluorescence staining and confocal imaging*

631 Mice were sacrificed, the L4/L5 segments of the spinal cord were exposed from the lumbovertebral  
632 column via laminectomy and identified by tracing the dorsal roots from their respective DRG.  
633 Formalin-fixed (and no-fixed, used for VEGFR-1 primary antibody) cryostat sections (7  $\mu$ m) were  
634 washed 3x phosphate-buffered saline (PBS) and then were incubated, at room temperature for 1 h,  
635 in blocking solution (PBS, 0.3% Triton X-100, 5% albumin bovine serum; PBST). The sections  
636 were subsequently incubated with primary antibody, anti-VEGFR-1, anti-VEGF-A, or anti-AQP-4,  
637 overnight at 4°C (Supplementary Table S1). The following day, slides were washed 3 $\times$  with PBS  
638 and then sections were incubated in the dark with secondary antibody, goat anti-rabbit or anti-  
639 mouse IgG labeled with Alexa Fluor 568, in PBST at room temperature for 2 h. After 3 $\times$  PBS 0.3%  
640 Triton X-100 wash for 10 min, the sections were incubated with DAPI, a nuclei-marker, at room

641 temperature for 5 min and then the slides were mounted using Fluoromount™ (Life Technologies-  
642 Thermo scientific, Rockford, IL, USA) as a mounting medium.

643 For double immunofluorescence, on the first day, anti-Iba1 was added and the slides incubated  
644 overnight at 4°C conditions. While, the sections to be labelled for GFAP or NeuN were incubated  
645 the second day for 2 h in the dark with mouse anti-GFAP Alexa Fluor 488-conjugated or mouse  
646 anti-NeuN Alexa Fluor 488-conjugated antibodies (Supplementary Table S1). For triple  
647 immunofluorescence, on the first day, anti-RECA-1 was added and the slides incubated overnight at  
648 4°C conditions, then sections were incubated with the anti-mouse IgG labeled with Alexa Fluor 568  
649 for 2 h. Thereafter, incubation with anti-VEGF-A and anti-GFAP antibodies was allowed overnight  
650 in the dark. Finally, anti-mouse IgG labeled with Alexa Fluor 488 and anti-rabbit IgG labeled with  
651 Alexa Fluor 647 were added for 2 h in the dark (Supplementary Table S1).

652 Negative control sections (no exposure to the primary antisera) were processed concurrently with  
653 the other sections for all immunohistochemical studies. Images were acquired using a motorized  
654 Leica DM6000 B microscope equipped with a DFC350FX camera (Leica, Mannheim, Germany).

655 The colocalization area was calculated using the "colocalization" plugin of ImageJ (after evaluating  
656 the threshold value for each channel) and expressed as percentage relative to the value of the  
657 VEGFR-1 or VEGF-A area. The mean fluorescence intensity of VEGF-A in control and oxaliplatin-  
658 treated animals was calculated by subtracting the background (multiplied by the total area) from the  
659 VEGF-A integrated intensity. Analyses were performed on three different images for each animal,  
660 collected through a 20x objective.

661 For confocal analysis, images were acquired with a Leica SP2 AOBS confocal microscope using a  
662 sequential scan setting (exciting lasers 488 nm and 561nm) to avoid channel bleed-through. Images  
663 were acquired through a 63x 1.4NA PL APO objective at voxel size of 232nm (xy) and 121nm (z).

664 Confocal images were processed and analyzed using Fiji (Schindelin *et al*, 2012). Briefly, images  
665 were deconvolved using Deconvolution Lab2 with a synthetic PSF and ICTM algorithm (Sage *et al*,  
666 2017). Colocalization analysis was performed with JACoP (Fiji plugin) (Bolte & Cordelières, 2006)  
667 and manually set thresholds. Colocalization parameters were calculated from 8 confocal z-stacks for  
668 each analysis and are given as mean  $\pm$  SEM.

669

## 670 *Statistics*

671 Results were expressed as means  $\pm$  SEM and the analysis of variance was performed by ANOVA  
672 test. A Bonferroni's significant difference procedure was used as post hoc comparison. P values less  
673 than 0.05 were considered significant. Data were analysed using "Origin® 10" software.



674 Electrophysiological data were analysed through one- way ANOVA followed by Dunnet's multiple  
675 comparison post-hoc test for statistical significance within groups. Two-way ANOVA followed by  
676 Bonferroni post-hoc test for comparison between groups, by using GraphPad Prism 7.0.

677

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682

### 683 **Competing Interests**

684 The authors declare no potential conflicts of interest

685

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691

### 692 **Author contributions**

693 LM performed treatments, behavioral measurements and drafted the manuscript, EL contributed to  
694 behavioral tests and analyzed data, CP, AP, AV and AT performed molecular assay, TM performed  
695 confocal analysis, SB, FR and SM performed electrophysiological measurements, GG, PML, SM,  
696 PF, AP and CG contributed to plan the study, drafted and revised the manuscript, LDCM conceived  
697 the study and drafted the manuscript.

698

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