

Cytoarchitectural analysis of the neuron-to-glia association in the dorsal root ganglia of normal and diabetic mice

*Original*

Cytoarchitectural analysis of the neuron-to-glia association in the dorsal root ganglia of normal and diabetic mice / Ciglieri, E., Vacca, M., Ferrini, F., Atteya, M.A., Aimar, P., Ficarra, E., Di Cataldo, S., Merighi, A., Salio, C.. - In: JOURNAL OF ANATOMY. - ISSN 0021-8782. - 267:5(2020), pp. 988-997. [10.1111/joa.13252]

*Availability:*

This version is available at: 11583/2837479 since: 2020-06-26T15:12:35Z

*Publisher:*

Wiley

*Published*

DOI:10.1111/joa.13252

*Terms of use:*

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

*Publisher copyright*

(Article begins on next page)

1 **Cytoarchitectural analysis of the neuron-to-glia association**  
2 **in the dorsal root ganglia of normal and diabetic mice.**

3

4 **Running title:** Neuron-glia spatial relationship in DRGs

5

6 Elisa Ciglieri<sup>1°</sup>, Maurizia Vacca<sup>2</sup>, Francesco Ferrini<sup>1,#</sup>, Mona A. Atteya<sup>3</sup>, Patrizia  
7 Aimar<sup>1</sup>, Elisa Ficarra<sup>2</sup>, Santa Di Cataldo<sup>2</sup>, Adalberto Merighi<sup>1§</sup>, Chiara Salio<sup>1\*</sup>.

8

9 <sup>1</sup> Department of Veterinary Sciences, University of Turin, Grugliasco, Italy.

10 <sup>°</sup> Max Planck Institute for Metabolism Research, Cologne, Germany.

11 <sup>#</sup> Department of Psychiatry & Neuroscience, Université Laval, Québec, QC, Canada

12 <sup>§</sup> National Institute of Neuroscience, Grugliasco, Italy.

13 <sup>2</sup> Department of Control and Computer Engineering, Politecnico di Torino, Italy.

14 <sup>3</sup> Department of Biochemistry, Faculty of Science, Alexandria University, Egypt.

15

16 **\*Correspondence:**

17 Dr. Chiara Salio

18 Department of Veterinary Sciences

19 University of Turin

20 Largo Braccini, 2

21 10095 Grugliasco, Italy

22 [chiara.salio@unito.it](mailto:chiara.salio@unito.it)

23

24

25

26

27

28

29

30

31

32

33

34

## 35 **Abstract**

36 Dorsal root ganglia (DRGs) host the somata of sensory neurons which convey information from the  
37 periphery to the central nervous system. These neurons have heterogeneous size and  
38 neurochemistry, and those of small-to-medium size, which play an important role in nociception,  
39 form two distinct subpopulations based on the presence (peptidergic) or absence (non-peptidergic)  
40 of transmitter neuropeptides. Few investigations have so far addressed the spatial relationship  
41 between neurochemically different subpopulations of DRG neurons and glia. We used a whole-  
42 mount mouse lumbar DRG preparation, confocal microscopy and computer-aided 3D analysis, to  
43 unveil that IB4<sup>+</sup> non-peptidergic neurons form small clusters of  $4.7 \pm 0.26$  cells, differently from  
44 CGRP<sup>+</sup> peptidergic neurons that are, for the most, isolated ( $1.89 \pm 0.11$  cells). Both subpopulations  
45 of neurons are ensheathed by a thin layer of satellite glial cells (SGCs) that can be observed after  
46 immunolabeling with the specific marker glutamine synthetase (GS). Notably, at the ultrastructural  
47 level we observed that this glial layer was discontinuous, as there were patches of direct contact  
48 between the membranes of two adjacent IB4<sup>+</sup> neurons.

49 To test whether this cytoarchitectonic organization was modified in the diabetic neuropathy, one of  
50 the most devastating sensory pathologies, mice were made diabetic by streptozotocin (STZ). In  
51 diabetic animals, cluster organization of the IB4<sup>+</sup> non-peptidergic neurons was maintained, but the  
52 neuro-glial relationship was altered, as STZ treatment caused a statistically significant increase of  
53 GS staining around CGRP<sup>+</sup> neurons but a reduction around IB4<sup>+</sup> neurons. Ultrastructural analysis  
54 unveiled that SGC coverage was increased at the interface between IB4<sup>+</sup> cluster-forming neurons in  
55 diabetic mice, with a 50% reduction in the points of direct contacts between cells. These  
56 observations demonstrate the existence of a structural plasticity of the DRG cytoarchitecture in  
57 response to STZ.

58

## 59 **Keywords**

60 Dorsal Root Ganglia; 3D computer-aided reconstruction; Satellite Glial Cells; Diabetic Peripheral  
61 Neuropathy; Peptidergic neurons; Non-peptidergic neurons.

62

63

64

65

66

## 67 **Introduction**

68 Except for certain specialized receptors, the cell bodies of the primary sensory neurons are grouped  
69 in a series of ganglia of the peripheral nervous system associated to the brain or the spinal cord. In  
70 the latter they are situated along the dorsal roots of the spinal nerves and commonly referred to as  
71 dorsal root ganglia (DRGs). DRGs are made of pseudounipolar neurons and the surrounding glia.  
72 Neurons may be simply classified into small-to-medium sized cells, mostly specialized in encoding  
73 noxious stimuli, and medium-to-large sized cells, typically encoding innocuous low-threshold  
74 stimuli (Lawson & Waddell, 1991; Lawson, 2002). However, these two populations are highly  
75 heterogeneous, and small-to-medium sized DRG neurons may be further subdivided into  
76 peptidergic or non-peptidergic cells. Peptidergic neurons are about 30-45% of the total number of  
77 DRG neurons, and typically express one or more neuropeptides among which the more common is  
78 the calcitonin gene-related peptide (CGRP) (Gibson et al., 1984; Lawson, 1995), which is now  
79 accepted as the best marker to identify these cells. Non-peptidergic neurons, representing another  
80 third of the total population of DRG neurons, are of smaller size and, at least in mouse, can be  
81 specifically identified after histochemical labeling with the isolectin B4 (IB4) from *Griffonia*  
82 *simplicifolia* (Silverman and Kruger, 1990).

83 Irrespective of their neurochemical heterogeneity, neurons are generally believed to be evenly  
84 distributed within ganglia, and only few studies have probed this view by appropriate anatomical  
85 investigations (Burton & McFarlane, 1973; Wessels et al., 1990; Puigdemívol-Sánchez et al., 1998;  
86 Yan et al., 2002; Ostrowski et al., 2017). Again disregarding their heterogeneity, the cell bodies of  
87 the DRG neurons are individually enwrapped by satellite glial cells (SGCs), so that each neuron  
88 forms a discrete unit, sharply separated anatomically and insulated electrically from the adjacent  
89 nerve cells (Pannese, 2010).

90 Still, the neurochemical diversity of the DRG neurons is very important, because it underscores  
91 their functional diversification, particularly regarding the intervention in the initial processing of  
92 nociceptive stimuli (see Merighi, 2018). Remarkably, whereas many studies have demonstrated that  
93 SGCs go through important functional alterations in pain, specifically in the diabetic neuropathy  
94 (Hanani et al., 2014; Verkhatsky & Fernyhough, 2014), as well as in the presence of sustained  
95 visceral pain (Huang et al., 2010), no data are available as regarding the possibility that the DRG  
96 neurons undergo structural plasticity when pain processing is altered or disturbed.

97 To provide further information about the neuron-to-glia structural association in mouse DRGs, as  
98 well as on its putative plasticity in the diabetic neuropathy, we here used confocal microscopy  
99 coupled with 3D computer-aided analysis in a whole-mount ganglion preparation (Ciglieri et al.,  
100 2016) and transmission electron microscopy (TEM) to study the tridimensional organization of

101 CGRP+ and IB4+ DRG neurons, as well as their SGCs in normal and diabetic mice. Diabetes was  
102 induced by a single high dose injection of streptozotocin (STZ). STZ has a structural similarity with  
103 glucose and is taken up by pancreatic  $\beta$  cells via glucose transporter 2, causing the death of the cells  
104 by DNA fragmentation and impairment of glucose transport (Ventura-Sobrevilla et al., 2011). This  
105 model induces a severe and long lasting neuropathy, characterized by variable alterations of sensory  
106 profiles (Ventura-Sobrevilla et al., 2011).

107 Together, our results showed that the IB4+ non-peptidergic neurons were organized in small  
108 clusters, while the CGRP+ peptidergic neurons were evenly scattered across the DRGs. While this  
109 organization remained unaltered in diabetic mice, the SGCs surrounding the two populations of  
110 DRG neurons were subjected to extensive structural alterations that may be a histological substrate  
111 at the basis of nociceptive alterations in diabetes.

## 112 **Methods**

### 113 **Animals**

114 All experimental procedures were approved by the Italian Ministry of Health and the Committee of  
115 Bioethics and Animal Welfare of the University of Torino (417/2016-PR). Animals were  
116 maintained according to the NIH Guide for the Care and Use of Laboratory Animals and to current  
117 EU and Italian regulations.

118 Male CD1 mice (20-30 g) were housed in a controlled environment and maintained on a 12/12-hour  
119 light/dark cycle with food and water *ad libitum*. All experiments were performed in both control  
120 (normoglycemic) and diabetic (hyperglycemic) mice. To induce diabetes, animals at postnatal day  
121 30 (P30) received one single intraperitoneal injection of streptozotocin (STZ - Sigma, St. Louis,  
122 MO, USA, Cat# S0130). STZ was administered at a dose of 150 mg/kg freshly dissolved in 0.1 M  
123 citrate buffer pH 4.5 to experimental animals, whereas control mice only received the vehicle. Four  
124 weeks later (P60), following tail venipuncture in 5 hour-fasted animals, glycemia was measured  
125 using a glucose oxidase impregnated test strip (Glucocard sensor; Menarini, Firenze, Italy). Only  
126 mice with a blood glucose concentration higher than 300 mg/dL were considered diabetic and used  
127 for the subsequent experiments (see Fig. S1).

128

### 129 **Whole-mount DRG preparation**

130 Immunofluorescence experiments were performed on a whole-mount lumbar DRG preparation, as  
131 previously described (Ciglieri et al., 2016). Briefly, mice (control N=41, diabetic N=31) were  
132 anesthetized with a lethal dose of sodium pentobarbital (30 mg/kg, intraperitoneal). Dissection of  
133 the lumbar DRGs was then performed by constantly maintaining tissues in an ice-cold cutting

134 solution, containing: sucrose 252 mM, KCl 2.5 mM, NaHCO<sub>3</sub> 26 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, D-  
135 glucose 10 mM, kynurenate 1 mM, MgCl<sub>2</sub> 3 mM, CaCl<sub>2</sub> 1.5 mM, saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>.  
136 DRGs were removed after cutting the vertebral column along the midline; then, they were incubated  
137 for 1 hour at 37°C in constantly oxygenated artificial cerebro-spinal fluid (aCSF), containing: NaCl  
138 126 mM, KCl 2.5 mM, D-glucose 10 mM, NaHCO<sub>3</sub> 26 mM, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, CaCl<sub>2</sub> 2mM,  
139 MgCl<sub>2</sub> 1.5 mM and collagenase (7 mg/mL, collagenase type 3; Worthington, NJ, USA, Cat#  
140 LS004180) to digest the outer connective capsule of the ganglia and to allow for better penetration  
141 of the immunoreactants for 3D analysis.

## 142 Immunofluorescence

143 Acutely dissected, collagenase-treated DRGs were fixed for 30 min with 4% paraformaldehyde in  
144 phosphate buffer (PB; 0.1 M, pH 7.4), washed several times in phosphate buffered saline (PBS;  
145 0.02 M, pH 7.4), and then processed for immunofluorescence as follows:

- 146 *i-* They were pre-incubated in PBS containing 6% bovine serum albumin for 1 h, followed by  
147 overnight incubation at 4°C with an IB4 biotin-conjugate (1:250; Sigma, Cat# L2140), washed  
148 in PBS and incubated for 1 h with Extravidin-FITC (1:500; Sigma, Cat# E276);  
149 *ii-* They were pre-incubated in 1% normal goat serum and 0.1% Triton X-100 for 1 h, and then  
150 incubated overnight at 4°C with the following primary antibodies: polyclonal rabbit anti-CGRP  
151 antibody (1:500; Sigma, Cat# C8198; Salio & Ferrini, 2016); monoclonal mouse anti-glutamine  
152 synthetase (GS) antibody, clone GS-6 which specifically stains SGCs (1:50; Merck, Cat#  
153 MAB302; Magni et al., 2015; Rajasekhar et al., 2015). After washing in PBS, DRGs were  
154 incubated for 1 h with appropriate secondary antibodies (1:1000; anti-rabbit Alexa Fluor 633-  
155 Cat# A-21070, anti-rabbit Alexa Fluor 594-Cat# A-11012, anti-mouse Alexa Fluor 546-Cat#  
156 A-11003; Thermo Fisher, Waltham, MA, USA).

157 Negative controls performed by omitting the primary antibodies completely abolished the specific  
158 staining.

159 In a subset of experiments, ganglia were stained with 4',6-diamidino-2-phenylindole  
160 dihydrochloride (DAPI; Sigma, Cat# D9542) by a pre-incubation in PBS containing 0.1% Triton X-  
161 100 for 30 min followed by 15 min in 300 nM DAPI.

162 To obtain Z-series reconstructions, immunostained DRGs were transferred on slides modified *ad*  
163 *hoc* to maintain their 3D volume (Ciglieri et al., 2016) and mounted with Vectashield medium  
164 (Vector Labs, Burlingame, CA, USA, Cat# H-1000). Immunofluorescence was acquired using a  
165 confocal microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany) with a 20x objective  
166 (N.A. 0.17). DAPI was excited with a 405 nm diode laser, FITC with a 488 nm argon laser, Alexa  
167 Fluor 546 and 594 with a 547 nm HeNe laser and Alexa Fluor 633 with a 633 nm HeNe laser.

168 Pinhole was kept at 1 airy unit. Gain and offset were initially set for each fluorophore and  
169 maintained constant in the subsequent acquisitions. Confocal optical sections were taken at 3.5  $\mu\text{m}$   
170 intervals along the Z axis in sequential mode.

171

## 172 **Electron microscopy**

173 Eighteen DRGs (3 DRGs/mouse from three control and three diabetic mice) dissected out from  
174 mice euthanized as previously described, were fixed in 1% paraformaldehyde + 2% glutaraldehyde  
175 in PB (0.1 M, pH7.4) overnight at 4°C. After washing in PB, they were post-fixed in osmium  
176 ferrocyanide (1 volume of 2% aqueous osmium tetroxide : 1 volume of 3% potassium ferrocyanide)  
177 for 1 h at 4°C, dehydrated for 15 min in increasing concentrations of acetone (30%, 60%, 90%,  
178 100%), progressively infiltrated with Spurr resin (Electron Microscopy Sciences, Hatfield, PA,  
179 USA; Cat#14300; data from manufacturer) and embedded in 0.5 mL Eppendorf tubes (24 h at  
180 70°C).

181 Ultrathin sections (80 nm thickness) were cut with an ultramicrotome (EM UC6; Leica), collected  
182 on uncoated nickel grids (200 mesh) and immunostained following a classical post-embedding  
183 protocol. Sections were treated for 1 min with a saturated aqueous solution of sodium  
184 metaperiodate, rinsed in 1% Triton X-100 in Tris-buffered saline (TBS; 0.5 M), and then incubated  
185 for 1 h in 6% bovine serum albumin in TBS. Grids were then transferred overnight on drops of the  
186 IB4 biotin-conjugate (1:20, Sigma, Cat# L2140). After rinsing in TBS, they were incubated in  
187 streptavidin coupled to 20 nm colloidal gold particles (1:15; BBI Solutions, Crumlin, UK, Cat#  
188 EM.STP20), transferred into drops of 2.5% glutaraldehyde in cacodylate buffer 0.05 M and, finally,  
189 washed in distilled water. Sections were counterstained 10 min with lead citrate before observation  
190 with a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan) equipped with a side-  
191 mounted CCD camera (Mega View III, Olympus Soft Imaging System, Munster, Germany).

192 To assess the neuron-glia distribution in the clusters formed by the IB4+ neurons, quantitative  
193 ultrastructural analysis was performed onto sixty randomly selected clusters from control (n=30)  
194 and diabetic (n=30) DRGs. To do so, IB4+ clustered neurons were photographed at 15,000x  
195 magnification by an operator unaware of the experimental group. Individual micrographs were  
196 collated together with Photoshop CS2 9 (Adobe Systems, San Jose, CA, USA) to obtain a single  
197 picture of the cluster and then analyzed with the ImageJ Software (NIH, Bethesda, USA).  
198 Specifically, the length of the plasma membranes' apposition between two IB4+ clustered neurons  
199 was measured, and their distance calculated over 10 equally spaced points. Opposing membranes  
200 were considered in "direct contact" when intermembrane distances were  $\leq 30\text{-}40$  nm. This threshold  
201 was set assuming that the extracellular space is  $\leq 20$  nm and each plasma membrane is about 5 nm

202 thick (Faisal et al., 2005). A contact index was calculated by dividing the number of intermembrane  
203 contact points with distance  $\leq 40$  nm by the length of the neuronal interface. Then, the proportion of  
204 IB4+ profiles sharing at least one point of contact in control and STZ-treated mice was quantified.

205

### 206 **Computerized analysis of neuronal clusterization**

207 The spatial distribution of neurons in DRGs was analyzed by an in-house developed software for  
208 automated 3D analysis (*3DRG*; see Di Cataldo et al., 2016, Supporting Information Data S1 and Fig.  
209 **S2**). Analysis was performed on confocal images of the immunostained DRGs to detect the  
210 peptidergic (CGRP+) and non-peptidergic (IB4+) neuronal populations.

211

### 212 **Analysis of SGCs after immunofluorescence staining**

213 The relationship between SGCs and CGRP+/IB4+ neurons was investigated by 1) counting the  
214 SGCs surrounding each neurochemically identified neuron, 2) measuring the fluorescence intensity  
215 associated with the SGC marker GS around each identified neuron.

216 1) The number of SGCs per sensory neuron was estimated by counting the number of DAPI stained  
217 nuclei surrounding the equatorial optical section of the neuron, i.e. the largest section on the z-axis.  
218 The number of nuclei was normalized to the cross-sectional area to correct for differences in  
219 neuronal size.

220 2) GS fluorescence intensity was measured at the equatorial optical section (see Fig. 3C). To obtain  
221 an unbiased estimate of GS distribution around each neuronal cell body, GS fluorescence intensity  
222 was measured by the ImageJ Software (<https://imagej.nih.gov/ij/>) along four lines passing through  
223 the optical section center and crossing its membrane at 8 equally spaced points. Since GS staining  
224 was concentrated around the neuronal membrane, eight peaks of GS fluorescence were detected. GS  
225 fluorescence intensity was measured at each peak (obtained by averaging three consecutive pixels  
226 around the peak; pixel size = 1.3  $\mu\text{m}$ ) and normalized to the background value (measured at the  
227 center of the neuronal cell body). For each identified neuron, the maximum, minimum and mean GS  
228 fluorescence was obtained in order to estimate both the fluorescence intensity of SGC marker and  
229 its distribution around the sensory neurons (see Fig. 3C).

230

### 231 **Statistics**

232 Statistical analysis was performed with GraphPad Prism 7. Differences were evaluated by using t-  
233 test for independent samples, two-way ANOVA or Mann-Whitney test where appropriate. All data  
234 were reported as mean  $\pm$  SEM, with *n* indicating the number of cells. Values of  $P < 0.05$  were  
235 considered statistically significant.

236

237

## 238 **Results**

### 239 **IB4+ non-peptidergic, but not CGRP+ peptidergic neurons are organized in** 240 **small clusters**

241 After analysis with the *3DRG* software on 147 DRGs obtained from 29 control and 23 diabetic  
242 mice, CGRP+ neurons resulted to be randomly scattered across the entire ganglion volume (Fig.  
243 1A), whereas IB4+ neurons were grouped in clusters (Fig. 1B). Each cluster of IB4+ neurons was  
244 composed of a mean of  $4.7 \pm 0.26$  cells (Fig. 1C). CGRP+ cells were found in clusters that were  
245 made of  $1.89 \pm 0.11$  cells. The difference between the number of neurons/cluster between the two  
246 subpopulations of DRG cells was statistically significant (t-test,  $P < 0.001$ ; Fig. 1C). There were no  
247 numerical alterations in the number of cells/cluster when the DRGs from diabetic mice were  
248 compared to control mice (Fig. 1C).

249

### 250 **The number of SGCs is higher around CGRP+ than IB4+ neurons**

251 After nuclear staining with DAPI (Fig. 2A-D), the number of SGC nuclei surrounding IB4+ and  
252 CGRP+ neurons were calculated and statistically analyzed (Fig. 2E). Artifacts due to the difference  
253 in size of the two subpopulations of sensory neurons were minimized by normalizing the number of  
254 SGC nuclei to the major cross-sectional area of the neurons themselves. Higher numbers of glial  
255 cells nuclei/area were consistently observed around CGRP+ neurons as compared to IB4+ neurons.  
256 In controls, SGCs nuclei were  $12 \pm 0.4 \cdot 10^{-3} / \mu\text{m}^2$  around CGRP+ neurons ( $n=80$ ), while they were  
257  $7 \pm 0.4 \cdot 10^{-3} / \mu\text{m}^2$  around IB4+ neurons ( $n=70$ ; t-test,  $P < 0.001$ ). Similarly, in diabetic mice SGCs  
258 nuclei were  $12 \pm 1 \cdot 10^{-3} / \mu\text{m}^2$  around CGRP+ neurons ( $n=40$ ), while they were  $7 \pm 1 \cdot 10^{-3} / \mu\text{m}^2$  around  
259 IB4+ neurons ( $n=30$ ; t-test,  $P < 0.001$ ). After two-way ANOVA, the differences in the number of  
260 SGCs surrounding the two identified populations of nociceptors resulted to depend on the cell  
261 phenotype but unaffected by the STZ treatment (two-way ANOVA, effect of treatment:  $F(1, 216) =$   
262  $0.02$ ,  $P = 0.89$ ; effect of the cell phenotype:  $F(1, 216) = 84.89$ ,  $P < 0.001$ ; treatment factor-  
263 interaction between factors:  $F(1, 216) = 0.48$ ,  $P = 0.16$  Fig. 2D).

264

### 265 **The SGC marker glutamine synthetase (GS) is differentially affected by** 266 **diabetes according to the cell phenotype.**

267 The distribution of SGCs around CGRP+ and IB4+ sensory neurons was analyzed indirectly, by  
268 measuring GS immunofluorescence (Fig. 3A-C).

269 Under control conditions, mean GS staining was more intense around IB4+ neurons than CGRP+  
270 neurons (Fig. 3D, t-test,  $P = 0.03$ ). Conversely, both minimal and mean fluorescence intensities  
271 were higher around CGRP+ neurons than IB4+ neurons in diabetic mice (Fig. 3E, t-test,  $P = 0.02$   
272 and  $0.03$ ). The two-way ANOVA analysis demonstrated a significant interaction between treatment  
273 and cell phenotype per each level of fluorescence intensity analyzed (Fig 3F, minimal fluorescence,  
274  $F(1, 218) = 9.49$ ,  $P = 0.002$ ; Fig. 3G, mean fluorescence,  $F(1, 218) = 9.26$ ,  $P = 0.003$ ; Fig. 3H,  
275 maximal fluorescence,  $F(1, 218) = 6.13$ ,  $P = 0.01$ ). Specifically, STZ treatment induced a  
276 significant decrease of minimal and mean fluorescence intensity around IB4+ neurons (minimal  
277 fluorescence intensity, t-test,  $P = 0.001$ , Fig. 3F; mean fluorescence intensity, t-test,  $P = 0.005$ , Fig.  
278 3G) and a significant increase of the maximal GS immunofluorescence intensity around CGRP+  
279 neurons (t test  $P=0.048$ , Fig. 3H). Altogether, STZ treatment induced an overall reduction of the  
280 glial layer around IB4 neurons, as detectable by GS staining, while causing a hypertrophic reaction  
281 around CGRP neurons.

282

### 283 **Ultrastructural analysis demonstrates a reduction in the juxtaposition of the** 284 **cell membranes of clustered IB4+ DRG neurons under diabetic conditions**

285 The presence of glia around the DRG neurons can be easily recognized without specific labels as  
286 previously described by Pannese (1981, 2010, Fig. 4A-D). In individual ultrathin sections, IB4+  
287 clusters consisted of two-three cells and the occurrence of clusters was confirmed in both control  
288 (Fig. 5A) and diabetic animals (Fig. 5B). In controls, the SGC sheet became progressively thinner at  
289 the interface between the IB4+ neurons of the same cluster and, in some points, the membrane of  
290 two opposing neurons appeared in direct contact (Fig. 5C). After quantitative analysis, the contact  
291 index between cluster-forming IB4+ neurons was markedly reduced in diabetic animals (Mann-  
292 Whitney test,  $P<0.01$ , Fig. 5D, E). Similarly, the proportion of neuronal interface exhibiting at least  
293 one point of direct contact was reduced of about 50% in these mice (Fisher exact test,  $P<0.05$ , Fig.  
294 5F).

295

296 **Results are graphically summarized in Figure 6.**

297

## 298 **Discussion**

299 In the present study, we found that the non-peptidergic IB4+ neurons in DRGs form small clusters,  
300 differently from the CGRP+ peptidergic neurons. This configuration is unaltered in diabetic mice.  
301 However, hyperglycemic conditions deeply affect the neuron-glia structural relationship between

302 cluster-forming neurons, thus suggesting that the 3D organization of these cells has a functional  
303 impact.

304  
305 **Structural relationship between sensory neurons and SGCs in DRGs**

306 It is widely accepted that there are no chemical synapses between the DRG neurons. Yet several  
307 forms of neuron-to-neuron and neuron-to-glia communication occur in DRGs particularly under  
308 conditions of inflammation and/or pain. Thus, electrical synapses (gap junctions) between DRG  
309 neurons are rare under basal conditions (Ledda et al 2009), but their number may increase together  
310 with neuron-to-neuron dye coupling in experimental inflammation (Ledda et al., 2009; Huang et al.  
311 2010). Noteworthy, it was also demonstrated that coupled activation of DRG neurons was mediated  
312 by an injury-induced upregulation of gap junctions in SGCs and that neuronal coupling contributed  
313 to pain hypersensitivity (Kim et al., 2016). Then, very recently, the gas messenger nitric oxide (NO)  
314 released by the DRG neurons was shown to induce activation of SGCs and to increase gap-  
315 junctional communication *in vitro* (Belzer and Hanani, 2019). Therefore, despite that adult DRG  
316 neurons are insulated by a non-conductive glial layer, which minimizes their direct interactions  
317 (Ohara et al., 2009), SGCs intervene in regulating neuronal excitability in DRGs.  
318 Mono/bidirectional gap junction-mediated neurotransmission between the DRG neurons and the  
319 SGCs may not be the only type of communication between these cells. Namely, other authors have  
320 described the occurrence of “sandwich synapses” between the DRG neurons and glia (Rozanski et  
321 al., 2013). Structurally, the sandwich synapses described by Rozanski and colleagues consist of  
322 neuron–glial cell–neuron trimers, in which membranes are closely apposed in the absence of any  
323 ultrastructural differentiation if not a narrowing of intermembrane clefts. These authors have shown  
324 an unidirectional ionic current following through sandwich synapses whereby a DRG “cis” neuron  
325 forms a first synapse with the SGC that, in turn, forms a second synapse with an adjacent “trans”  
326 neuron (see Figure 6 in Rozanski et al., 2013). According to the original sandwich synapse  
327 hypothesis, stimulation in a given “cis” neuron propagates to neighboring nerve cells following the  
328 activation of the purinergic P2Y<sub>12</sub> receptors expressed by the surrounding SGCs, which have been  
329 hypothesized to release glutamate that acts onto the NMDA receptors expressed at the membrane of  
330 the “trans” neuron (Rozanski et al., 2013). In line with such a possibility, astrocytic glutamate was  
331 shown to evoke NMDA receptor-mediated slow depolarizing inward currents in neurons (Gomez-  
332 Gonzalo et al., 2018). In DRGs, the spread of excitation driven through sandwich synapses is  
333 enhanced in a variety of pathological pain conditions (Ohara et al., 2009; Wu et al., 2012; Kim et  
334 al., 2016). These observations highlight the importance of the spatial contacts among the DRG  
335 neurons and between them and the SGCs to support their electrical coupling. In line with this, we

336 here have demonstrated that the IB4+ non-peptidergic nociceptors were organized in small clusters,  
337 differently from their CGRP+ peptidergic counterpart. Interestingly, these two subpopulations of  
338 DRG neurons also displayed a different association with their surrounding glia. Specifically, IB4+  
339 neurons exhibited a lower number of associated SGCs, and their membranes were directly  
340 juxtaposed within the clusters at TEM observation. That glial coverage was incomplete led us to  
341 speculate that direct neuron-to-neuron communication could occur. Direct neuronal appositions, in  
342 the absence of synaptic specializations, were previously observed between neurochemically  
343 unclassified sensory neurons of several species, including lizards (Pannese, 2010), chicks (Rozanski  
344 et al., 2012), rats (Pannese, 2010) and rabbits (Khan et al., 2009). Such a structural arrangement is  
345 consistent with their functional coupling by mechanisms others than electrical (gap junctions) or  
346 sandwich synapses. In their seminal study, Devor & Wall (1990) were the first to find that about 5%  
347 of DRG neurons induced subthreshold activity in the neighboring nerve cells. Later, slow chemical  
348 transmission between DRG neurons somata was demonstrated to take place with the intervention of  
349 ATP as a neurotransmitter in chick DRG neurons (Rozanski et al., 2012). In line with these  
350 observations, we here identify the contact points between IB4+ neurons as a structural substrate for  
351 electrotonic neuronal coupling.

352

### 353 **STZ-induced alterations in the neuron to glia association**

354 Alterations in SGCs function and in their anatomical relationship with sensory neurons strongly  
355 affect the spread of excitability across DRGs. SGCs were reported to undergo important changes in  
356 their morphology and activity, often described as an activated state, that contributes to pathological  
357 pain and favors pathological pain behavior (Hanani, 2012). In addition, direct coupling among  
358 clusters of 2-5 DRG neurons, particularly the smaller ones (<20  $\mu\text{m}$ ), was elegantly demonstrated  
359 by *in vivo* calcium imaging in mice with pathological pain (Kim et al., 2016). SGCs activation also  
360 occurred in animals where the diabetic neuropathy was experimentally induced (Hanani et al., 2014;  
361 Jia et al., 2018). As early as two weeks after STZ injection, Hanani and coll. (2014) found a  
362 significant increase in the expression of the glial fibrillary acidic protein (GFAP) in mouse and rat  
363 activated SGCs. SGC activation was also characterized by an increased expression of P2Y12  
364 receptors, which participate to the sandwich synapse mechanism, and connexin 43, which promotes  
365 transglial spread of excitation through the gap junctions (Jia et al., 2018).

366 Very recently Jia and coll. (2018) found that, in diabetic animals, SGCs activation was  
367 preferentially detected at the level of the CGRP-expressing neurons. **Our confocal data from STZ-**  
368 **induced diabetic mice** support these observations at CGRP neurons; contrariwise, we found a  
369 decrease of GS staining around IB4+. However, our ultrastructural study unveiled that this

370 reduction in GS staining was not associated to a reduction of glia ensheathment, which increased at  
371 interface between IB4+ cluster-forming neurons.

372 This apparently contradictory finding indicates that the mere immunocytochemical analysis of glia  
373 at the confocal microscopy may not be sensitive enough to detect changes in the SGC morphology  
374 at nanometric scale. Indeed, within neuronal clusters, glial processes separated the neuronal  
375 membranes of few tens of nanometers (Faisal et al., 2005) whose fine alterations may unlikely to be  
376 detected at the light microscopy level. Our data indicate that diabetes induced an overall increase in  
377 glial coverage at both peptidergic and non-peptidergic neurons in DRGs: at the micrometric level in  
378 the former and at the nanometric level in the latter. At neuronal cluster level, diabetes induces a  
379 shift from a condition in which IB4+ neurons share extensive contact areas, to a condition in which  
380 interneuronal responses might be mediated by the interposing SGCs. **Importantly, none of the**  
381 **observed changes in glia distribution around sensory neurons can be explained in terms of change in**  
382 **SGC number, as we were unable to demonstrate any significant change in the number of SGC**  
383 **nuclei in diabetic mice.** An interesting result of our study is that we have also observed the presence  
384 of some gap junctions between the DRG neurons and the SGCs in both normal and diabetic DRGs.  
385 Several papers (quoted above) have in fact demonstrated that neuro-glia gap junctions were the  
386 structural and functional substrate to explain the activation of the SGCs that occurs in diabetes,  
387 inflammation, or pathological pain.

388

389

### 390 **Acknowledgments**

391 This work was funded by Fondazione CRT (grant N. RF= 2015.1690 to FF) and Compagnia San  
392 Paolo (Fondi di Ateneo 2012 to CS).

393

### 394 **Conflict of interest**

395 The authors declare no conflicts of interest.

396

### 397 **Author contributions**

398 E.C., F.F and C.S. conceived the experiments and participated in their design; E.C. performed  
399 immunofluorescence and data acquisition; E.C., M.A.A. and F.F. performed data analysis; M.V;  
400 E.F. and S.D.C developed the Software and 3D-analysis; P.A. and C.S. performed electron

401 microscopy; E.C., F.F., A.M. and C.S contributed to data interpretation; F.F., E.F.; S.D.C.; C.S and  
402 A.M. revised, formulated and finalized the submitted manuscript.

403  
404

## 405 **References**

406 **Belzer V, Hanani M** (2019) Nitric oxide as a messenger between neurons and satellite glial cells in  
407 dorsal root ganglia. *Glia* **67**, 1296-1307.

408 **Burton H, McFarlane JJ** (1973) The organization of the seventh lumbar spinal ganglion of the cat.  
409 *J Comp Neurol* **149**, 215–231.

410 **Ciglieri, E., Ferrini, F., Boggio, E., et al.** (2016) An improved method for in vitro  
411 morphofunctional analysis of mouse dorsal root ganglia. *Annals Anat* **207**, 62-67.

412 **Di Cataldo S, Tonti S, Ciglieri E, et al.** (2016) Automated 3D immunofluorescence analysis of  
413 Dorsal Root Ganglia for the investigation of neural circuit alterations: a preliminary study.  
414 *Annals of Computer Science and Information Systems* **9**, 65–70.

415 **Devor M, Wall PD** (1990) Cross-excitation in dorsal root ganglia of nerve-injured and intact rats. *J*  
416 *Neurophysiol* **64**, 1733–1746.

417 **Faisal AA, White JA, Laughlin SB** (2005) Ion-channel noise places limits on the miniaturization  
418 of the brain's wiring. *Curr Biol* **15**, 1143-1149.

419 **Gibson SJ, Polak JM, Bloom SR, et al.** (1984) Calcitonin gene-related peptide immunoreactivity  
420 in the spinal cord of man and of eight other species. *J Neurosci* **4**, 3101–3111.

421 **Gómez-Gonzalo M, Zehnder T, Reque LM et al.** (2018) Insights Into the Release Mechanism of  
422 Astrocytic Glutamate Evoking in Neurons NMDA Receptor-Mediated Slow Depolarizing  
423 Inward *Curr Glia* **66**, 2188-2199.

424 **Hanani M, Blum E, Liu S, et al.** (2014) Satellite glial cells in dorsal root ganglia are activated in  
425 streptozotocin-treated rodents. *J Cell and Mol Med* **18**, 2367–2371.

426 **Hanani M** (2012) Intercellular communication in sensory ganglia by purinergic receptors and gap  
427 junctions: implications for chronic pain. *Brain Res* **1487**, 183–191.

- 428 **Huang T-Y, Belzer V, Hanani M** (2010) Gap junctions in dorsal root ganglia: possible  
429 contribution to visceral pain. *J Physiol* **14**, 647–660.
- 430 **Jia T, Rao J, Zou L, et al.** (2018) Nanoparticle-Encapsulated Curcumin Inhibits Diabetic  
431 Neuropathic Pain Involving the P2Y12 Receptor in the Dorsal Root Ganglia. *Front*  
432 *Neurosci*, **11**, 755-767.
- 433 **Khan AA, Dilkash MNA, Khan MA, et al.** (2009) Morphologically atypical cervical dorsal root  
434 ganglion neurons in adult rabbit. *Biomed Res* **20**, 45-49.
- 435 **Kim YS, Anderson M, Park K, et al.** (2016) Coupled Activation of Primary Sensory Neurons  
436 Contributes to Chronic Pain. *Neuron* **91**, 1085–1096.
- 437 **Lawson SN** (1995) Neuropeptides in morphologically and functionally identified primary afferent  
438 neurons in dorsal root ganglia: substance P, CGRP and somatostatin. *Prog in Brain Res* **104**,  
439 161–173.
- 440 **Lawson SN** (2002) Phenotype and function of somatic primary afferent nociceptive neurones with  
441 C-, Adelta- or Aalpha/beta-fibres. *Exp Physiol* **87**, 239–244.
- 442 **Lawson SN, Waddell PJ** (1991) Soma neurofilament immunoreactivity is related to cell size and  
443 fibre conduction velocity in rat primary sensory neurons. *J Physiol*, **435**, 41–63.
- 444 **Ledda M, Blum E, De Palo S et al.** (2009) Augmentation in gap junction-mediated cell coupling  
445 in dorsal root ganglia following sciatic nerve neuritis in the mouse. *Neurosci* **164**, 1538-  
446 1545.
- 447 **Magni G, Merli D, Verderio C, et al.** (2015) P2Y2 receptor antagonists as anti-allodynic agents in  
448 acute and sub-chronic trigeminal sensitization: role of satellite glial cells. *Glia*, **63**, 1256–  
449 1269.
- 450 **Merighi A** (2018) Costorage of High Molecular Weight Neurotransmitters in Large Dense Core  
451 Vesicles of Mammalian Neurons. *Front Cell Neurosci* **21**, 12:272.
- 452 **Ohara PT, Vit J-P, Bhargava A, et al.** (2009) Gliopathic pain: when satellite glial cells go bad.  
453 *Neuroscientist* **15**, 450–463.

- 454 **Ostrowski AK, Sperry ZJ, Kulik G, et al.** (2017) Quantitative models of feline lumbosacral  
455 dorsal root ganglia neuronal cell density. *J Neurosci Methods* **290**, 116–124.
- 456 **Pannese E** (1981) The satellite cells of the sensory ganglia. *Adv Anat Embryol Cell Biol* **65**, 1–111.
- 457 **Pannese E** (2010) The structure of the perineuronal sheath of satellite glial cells (SGCs) in sensory  
458 ganglia. *Neuron Glia Biol* **6**, 3–10.
- 459 **Puigdemívol-Sánchez A, Prats-Galino A, Ruano-Gil D, et al.** (1998) Sciatic and femoral nerve  
460 sensory neurones occupy different regions of the L4 dorsal root ganglion in the adult rat.  
461 *Neurosci Lett* **251**, 169–172.
- 462 **Rajasekhar P, Poole DP, Liedtke W, et al.** (2015) P2Y1 Receptor Activation of the TRPV4 Ion  
463 Channel Enhances Purinergic Signaling in Satellite Glial Cells. *J Biol Chem* **290**, 29051–  
464 29062.
- 465 **Rozanski GM, Kim H, Li Q et al.** (2012) Slow chemical transmission between dorsal root  
466 ganglion neuron somata. *Eur J Neurosci* **36**, 3314–3321.
- 467 **Rozanski GM, Li Q, Stanley EF** (2013) Transglial transmission at the dorsal root ganglion  
468 sandwich synapse: glial cell to postsynaptic neuron communication. *Eur J Neurosci* **37**,  
469 1221–1228.
- 470 **Salio C, Ferrini F** (2016) BDNF and GDNF expression in discrete populations of nociceptors. *Ann*  
471 *Anat* **207**, 55–61.
- 472 **Silverman JD, Kruger L** (1990) Selective neuronal glycoconjugate expression in sensory and  
473 autonomic ganglia: relation of lectin reactivity to peptide and enzyme markers. *J Neurocytol*  
474 **19**, 789–801.
- 475 **Ventura-Sobrevilla J, Boone D, Aguilar, C, et al.** (2011). Effect of Varying Dose and  
476 Administration of Streptozotocin on Blood Sugar in Male CD1 Mice. *Proc. West.*  
477 *Pharmacol. Soc.* **54**, 5–9.
- 478 **Verkhatsky A, Fernyhough P** (2014) Calcium signalling in sensory neurones and peripheral glia  
479 in the context of diabetic neuropathies. *Cell Calcium* **56**, 362–371.

480 **Wessels WJ, Feirabend HK, Marani E** (1990) Evidence for a rostrocaudal organization in dorsal  
 481 root ganglia during development as demonstrated by intra-uterine WGA-HRP injections into  
 482 the hindlimb of rat fetuses. *Brain Res Dev Brain Res* **54**, 273–281.

483 **Wu A, Green CR, Rupenthal ID, et al.** (2012) Role of gap junctions in chronic pain. *J Neurosci*  
 484 *Res* **90**, 337–345.

485 **Yan J, Tian R, Horiguchi M** (2002) Distribution of sensory neurons of ventral and dorsal cervical  
 486 cutaneous nerves in dorsal root ganglia of adult rat--a double-label study using DiO and DiI.  
 487 *Okajimas Folia Anat Jpn* **79**, 129–133.

488

### 489 **Figure legends**

490 **Fig. 1** Cluster analysis of CGRP+ and IB4+ neurons in DRGs from control and diabetic mice.  
 491 Representative pictures of CGRP+ (A) and IB4+ (B) neurons in DRGs from CTR. The enlargements  
 492 below illustrate the diameters of representative CGRP+ and IB4+ cells. (C) Histogram showing the  
 493 number of cells per cluster of CGRP+ and IB4+ neurons in vehicle-treated (IB4+ N=48; CGRP,  
 494 N=58; t-test,  $P < 0.001$ ) and STZ-treated mice (IB4+ N=57; CGRP, N=56; t-test,  $P < 0.001$ ).  
 495 Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide; CTR: vehicle-treated mice;  
 496 STZ: streptozotocin-treated mice. \*\*\*\* $P < 0.0001$ .

497 **Fig. 2** Analysis of the changes in the number of SGCs induced by STZ. (A-D) Representative  
 498 pictures of CGRP (blue), IB4 (green) and DAPI (white) staining on whole-mount DRGs. (E)  
 499 Histograms illustrating the number of DAPI+ nuclei surrounding CGRP+ or IB4+ sensory neurons  
 500 normalized to the cross-sectional area in vehicle- and STZ- treated mice. Two-way ANOVA: effect  
 501 of treatment:  $F(1, 216) = 0.02$ ,  $P = 0.89$ ; effect of the cell phenotype:  $F(1, 216) = 84.89$ ,  $P < 0.001$ ;  
 502 interaction between treatment and phenotype:  $F(1, 216) = 0.48$ ,  $P = 0.16$ . T-Test: IB4-CTR vs  
 503 CGRP-CTR, IB4-CTR vs CGRP-STZ, IB4-STZ vs CGRP-CTR, IB4-STZ vs CGRP-STZ,  $P < 0.001$ ;  
 504 IB4-CTR vs IB4-STZ, CGRP-CTR vs CGRP-STZ,  $P > 0.05$ ). Abbreviations: IB4: isolectin B4;  
 505 CGRP: calcitonin gene-related peptide; DAPI, 4',6-diamidino-2-phenylindole; CTR: vehicle-treated  
 506 mice; STZ: streptozotocin-treated mice. \*\*\*\* $P < 0.0001$ .

507 **Fig. 3** GS immunostaining and analysis of SGC coverage of CGRP+ and IB4+ neurons. (A-B)  
 508 Representative images showing double staining for the SGC marker GS (red), the peptidergic DRG  
 509 neuron marker CGRP (blue, A), and the non-peptidergic DRG neuron marker IB4 (green, B) in

510 vehicle-treated and STZ-treated mice. (C) Method for GS staining analysis. On the left,  
 511 representative single optical section of GS staining around a DRG neuron. Fluorescence intensity is  
 512 measured along the four colored lines, which cross the ensheathing SGC in 8-points (*white dots*)  
 513 around the DRG neuron. Quantification of the fluorescence intensity along each colored line is  
 514 illustrated in the graph on the right, using the same color code. GS fluorescence intensity is measured  
 515 at the white dots, *i.e.* the peaks in the graphs, and then normalized to internal background (*yellow*  
 516 *dot*). (D) Minimal, mean and maximal GS fluorescence intensities around CGRP+ or IB4+ neurons  
 517 vehicle-treated mice. T-test: Min,  $P = 0.053$ ; Mean,  $P = 0.028$ ; Max,  $P = 0.079$ . (E) Minimal, mean  
 518 and maximal GS fluorescence intensities around CGRP+ or IB4+ neurons in STZ-treated mice. T-  
 519 test: Min,  $P = 0.016$ ; Mean,  $P = 0.028$ ; Max,  $P = 0.056$ . (F) Dot plot graph of minimal GS  
 520 fluorescence intensity. Two-way ANOVA: interaction between treatment and phenotype:  $F(1, 218)$   
 521  $= 9.49$ ,  $P = 0.002$ ; effect of treatment:  $F(1, 218) = 2.99$ ,  $P = 0.085$ ; effect of the cell phenotype:  $F(1,$   
 522  $218) = 0.02$ ,  $P = 0.88$ . IB4-CTR vs IB4-STZ, t-test,  $P = 0.0014$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P$   
 523  $= 0.32$ . (G) Dot plot graph of mean GS fluorescence intensity. Two-way ANOVA: interaction  
 524 between treatment and phenotype:  $F(1, 218) = 9.26$ ,  $P = 0.003$ ; effect of treatment:  $F(1, 218) = 8.45$ ,  
 525  $P = 0.36$ ; effect of the cell phenotype:  $F(1, 218) = 0.015$ ,  $P = 0.9$ . IB4-CTR vs IB4-STZ, t-test,  $P =$   
 526  $0.005$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P = 0.14$ . (H) Dot plot graph of maximal GS fluorescence  
 527 intensity. Two-way ANOVA: interaction between treatment and phenotype:  $F(1, 218) = 6.12$ ,  $P =$   
 528  $0.014$ ; effect of treatment:  $F(1, 218) = 0.17$ ,  $P = 0.68$ ; effect of the cell phenotype:  $F(1, 218) =$   
 529  $0.017$ ,  $P = 0.69$ . IB4-CTR vs IB4-STZ, t-test,  $P = 0.13$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P = 0.048$ .  
 530 Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide; CTR: vehicle-treated mice;  
 531 STZ: streptozotocin-treated mice; GS: glutamine synthetase. \* $P < 0.05$ , \*\* $P < 0.01$ .

532 **Fig. 4** Ultrastructure of the DRG neurons in control mice. (A) An ensheathing layer made of SGC  
 533 processes (*arrowheads*) separates two adjacent neurons. (B) The neuronal membranes of two  
 534 adjacent neurons are in direct contact (*arrows*), without SGC interposition. (C) High-magnification  
 535 of panel A. Note the presence of an SGC process between the sensory neuron somata (*arrowheads*)  
 536 and gap-junctions between neuron and SGC (*red double arrowheads*). (D) High magnification of  
 537 panel B. Note the lack of SGC interposition between the facing membranes of the two neurons  
 538 (*arrows*). Abbreviation: SGC: Satellite glial cell; N: nucleus; ct: connective tissue.

539 **Fig. 5** Ultrastructural analysis of IB4+ DRG neurons in control and diabetic mice. (A) In CTR, the  
 540 membranes of two adjacent clustered IB4+ sensory neurons are juxtaposed, without the interposition  
 541 of glia (*arrowheads*). (B) In STZ-treated mice, a glial sheath is present between two IB4+ neurons of  
 542 the same cluster (*arrows*). (C) High magnification of panel A. Note the occurrence of 20 nm gold

543 particles indicative of IB4 immunogold staining scattered over the entire length of the juxtaposed  
544 neuronal membranes (*arrowheads*). (D) High-magnification of panel B. Note the glia separating the  
545 membranes of two IB4+ DRG neurons (*arrows*) and a gap-junction between the neuron and SGC  
546 (*red double arrowheads*). (E) Contact index in vehicle-treated mice and STZ-treated mice. The  
547 contact index is markedly reduced in STZ (Mann-Whitney test,  $P < 0.01$ ). (F) Pie charts showing the  
548 proportion of neuronal membranes exhibiting at least one point of contact (Fisher exact test,  $P < 0.05$ )  
549 in CTR and STZ-treated mice. Abbreviation: SGC: Satellite glial cell; N: nucleus; CTR: vehicle-  
550 treated mice; STZ: streptozotocin-treated mice.

551 **Fig. 6** Schematic summary of neuro-glia relationship in DRGs of control and diabetic mice. (A)  
552 Representation of a simplified dorsal root ganglion (DRG) in control (CTR) mice: IB4+ neurons  
553 (green) are grouped in cluster and surrounded by a few satellite glial cells (SGC). SGCs form a  
554 continuous glutamine synthetase (GS)+ sheet around neurons (red thick line) which becomes  
555 thinner at the interface of two IB4+ opposing neurons. At some points, the membranes of cluster-  
556 forming IB4+ neurons are in direct contact (yellow arrowheads). CGRP+ neurons (blue) are  
557 randomly scattered across the DRG and surrounded by numerous SGCs with relatively less bright  
558 GS immunostaining (red thin line). (B) In streptozotocin (STZ)-induced diabetic mice, IB4+  
559 neurons (green) are still grouped in clusters and CGRP+ neurons (blue) randomly scattered across  
560 the DRG. However, GS fluorescence intensity is higher around CGRP+ neurons (red thick line)  
561 than in IB4+ neurons (red thin line). Moreover, the points of the cluster where two IB4+ opposing  
562 neurons are in direct contact are significantly reduced.

563

## 564 **Supporting Information**

565 Additional Supporting Information may be found in the online version of this article:

566 **Data S1** Computerized analysis of neuronal clusterization.

567 **Fig. S1** Blood glucose concentration in control and diabetic mice.

568 **Fig. S2** Schematic flowchart describing the steps in image processing performed using the *3DRG*  
569 software.

570

571

## **SUPPLEMENTARY MATERIALS**

### **Cytoarchitectural analysis of the neuron-to-glia association in the dorsal root ganglia of normal and diabetic mice.**

Elisa Ciglieri<sup>1°</sup>, Maurizia Vacca<sup>2</sup>, Francesco Ferrini<sup>1</sup>, Mona A. Atteya<sup>3</sup>, Patrizia Aimar<sup>1</sup>, Elisa Ficarra<sup>2</sup>, Santa Di Cataldo<sup>2</sup>, Adalberto Merighi<sup>1§</sup>, Chiara Salio<sup>1\*</sup>.

#### **Data S1**

#### **Computerized analysis of neuronal clusterization**

The spatial distribution of neurons in DRGs was analyzed by an in-house developed software for automated 3D analysis (*3DRG*; Di Cataldo et al., 2016). Analysis was performed on confocal images of the immunostained DRGs to detect the peptidergic (CGRP+) and non-peptidergic (IB4+) neuronal populations. To distinguish positively stained neuronal cells from noise and artifacts (e.g. spurious fluorescence, black spots, etc.), a fully-automated 3D segmentation technique was applied, as follows:

- ✓ images were preprocessed by applying contrast enhancement and median image filtering to remove fake signals by preserving significant details of the neuronal borders;
- ✓ a spatial fuzzy c-means clustering (SFCM; Chuang, Tzeng, Chen, Wu, & Chen, 2006) algorithm was applied to distinguish the fluorescent objects from the dark background, thus reducing noise and spurious blobs. SFCM is an improved version of the standard fuzzy c-means algorithm, a widely used technique in pattern recognition that groups similar image pixels by means of so-called membership functions;
- ✓ the cell segmentation provided by SFCM was refined by separating the touching objects in single cells, based on the assumption that individual nuclei are approximately round-shaped;
- ✓ the objects collected from a single 2D image were projected to the neighborhood slices of the z-stack, to perform a 3D reconstruction of the neurons and discard spurious objects. More specifically: if the neighborhood slices contained an object that overlapped by at least 50% with the projected one, this object was interpreted as a part of the projected cell and added to the 3D reconstruction, otherwise it was considered as a sham fluorescence and discarded (see Supplementary Figure 2).

In order to investigate whether neuronal populations were randomly distributed across the DRG volume or organized in clusters (i.e. groups of more than two cells in direct contact), cell-to-cell

contacts were analyzed by measuring the number of cells per cluster ( $\bar{n}_{cell}$ ) and the median clusters volume ( $V_{cls}$ ).

$\bar{n}_{cell}$  was calculated as follows:

$$\bar{n}_{cell} = \sum_{i=1}^{N_{cls}} \frac{V_{cls}^i}{V_{ref}}$$

Where the average number of cells per cluster  $\bar{n}_{cell}$  is the mean of the ratio of all the clusters with a volume higher than the 75<sup>o</sup> percentile to the reference volume  $V_{ref}$ .

$V_{ref}$  was set considering an observed mean diameter size of 20  $\mu\text{m}$  for IB4+ neurons and 25  $\mu\text{m}$  for CGRP+ neurons.

$V_{cls}$  was calculated as follows:

$$V_{cls} = \frac{[\text{med}(V)]_{cls}}{V_{ref}}$$

where the average volume of clusters  $V_{cls}$  is the median value of clusters volume measured normalized by the reference volume  $V_{ref}$ .

## Supplemental figures

### Supplemental Figure 1 (Fig. S1)

**Blood glucose concentration in control and diabetic mice.** Bar chart showing the blood glucose concentration in control (CTR N=16, black bar) and diabetic (STZ N=16, grey bar) mice four weeks after intraperitoneal injection of vehicle or streptozotocin (150 mg/kg), respectively. Control mice are normoglycemic, with a blood glucose concentration < 200 mg/dl, while diabetic mice are hyperglycemic, with a blood glucose concentration > 300 mg/dl (T-test, \*\*\* p < 0.001).

### Supplemental Figure 2 (Fig. S2)

**Schematic flowchart describing the steps in image processing performed using the 3DRG software.**

After an initial preprocessing stage of image denoising and contrast (INPUT), the software proceeded to the segmentation of positive objects and the generation of a 3D cell density map (OUTPUT). Based on the cell density map and custom data, 3D rendering and subsequent cluster analysis were performed. IB4+ staining is *green* and CGRP+ staining is *red*. Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide.

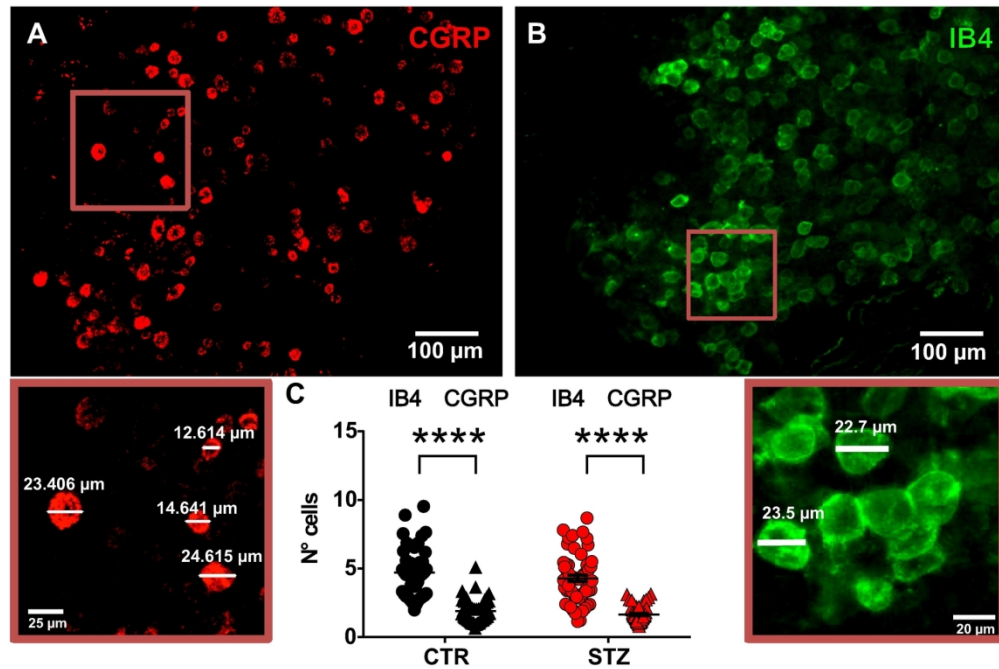


Fig. 1 Cluster analysis of CGRP+ and IB4+ neurons in DRGs from control and diabetic mice. Representative pictures of CGRP+ (A) and IB4+ (B) neurons in DRGs from CTR. The enlargements below illustrate the diameters of representative CGRP+ and IB4+ cells. (C) Histogram showing the number of cells per cluster of CGRP+ and IB4+ neurons in vehicle-treated (IB4+ N=48; CGRP, N=58; t-test,  $P < 0.001$ ) and STZ-treated mice (IB4+ N=57; CGRP, N=56; t-test,  $P < 0.001$ ). Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide; CTR: vehicle-treated mice; STZ: streptozotocin-treated mice. \*\*\*\* $P < 0.0001$ .

172x113mm (300 x 300 DPI)

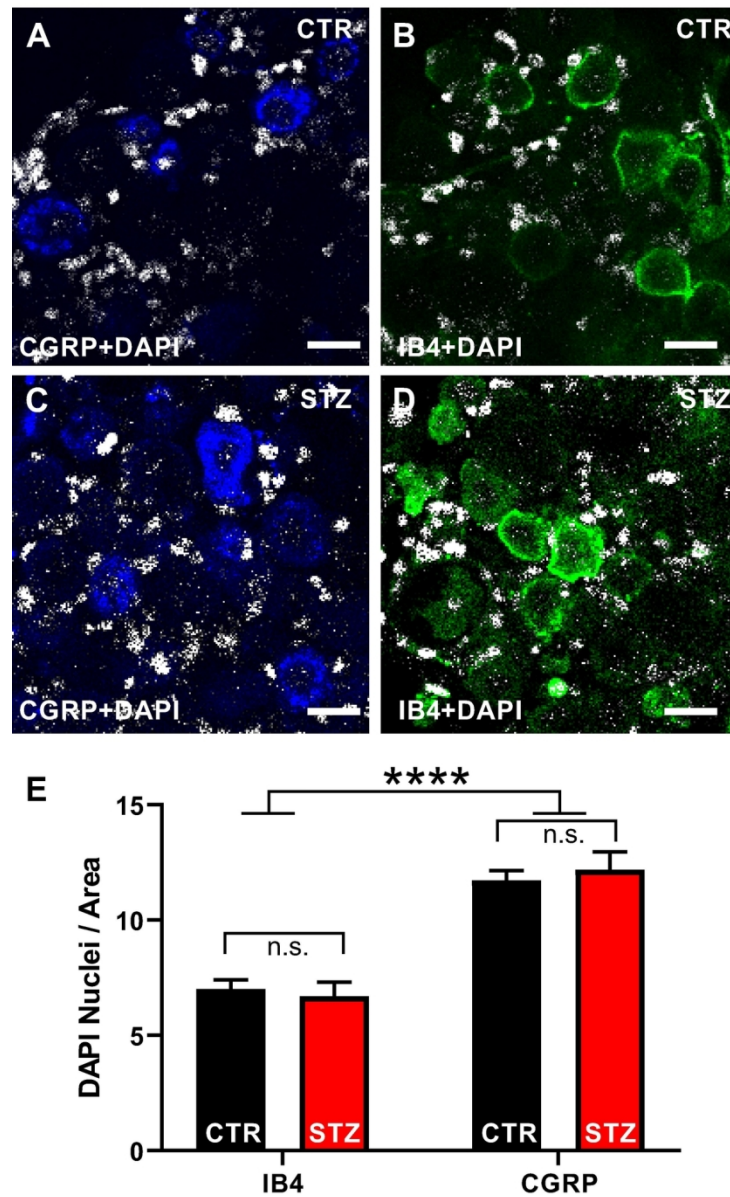


Fig. 2 Analysis of the changes in the number of SGCs induced by STZ. (A-D) Representative pictures of CGRP (blue), IB4 (green) and DAPI (white) staining on whole-mount DRGs. (E) Histograms illustrating the number of DAPI+ nuclei surrounding CGRP+ or IB4+ sensory neurons normalized to the cross-sectional area in vehicle- and STZ- treated mice. Two-way ANOVA: effect of treatment:  $F(1, 216) = 0.02$ ,  $P = 0.89$ ; effect of the cell phenotype:  $F(1, 216) = 84.89$ ,  $P < 0.001$ ; interaction between treatment and phenotype:  $F(1, 216) = 0.48$ ,  $P = 0.16$ . T-Test: IB4-CTR vs CGRP-CTR, IB4-CTR vs CGRP-STZ, IB4-STZ vs CGRP-CTR, IB4-STZ vs CGRP-STZ,  $P < 0.001$ ; IB4-CTR vs IB4-STZ, CGRP-CTR vs CGRP-STZ,  $P > 0.05$ . Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide; DAPI, 4',6-diamidino-2-phenylindole; CTR: vehicle-treated mice; STZ: streptozotocin-treated mice. \*\*\*\* $P < 0.0001$ .

107x176mm (300 x 300 DPI)

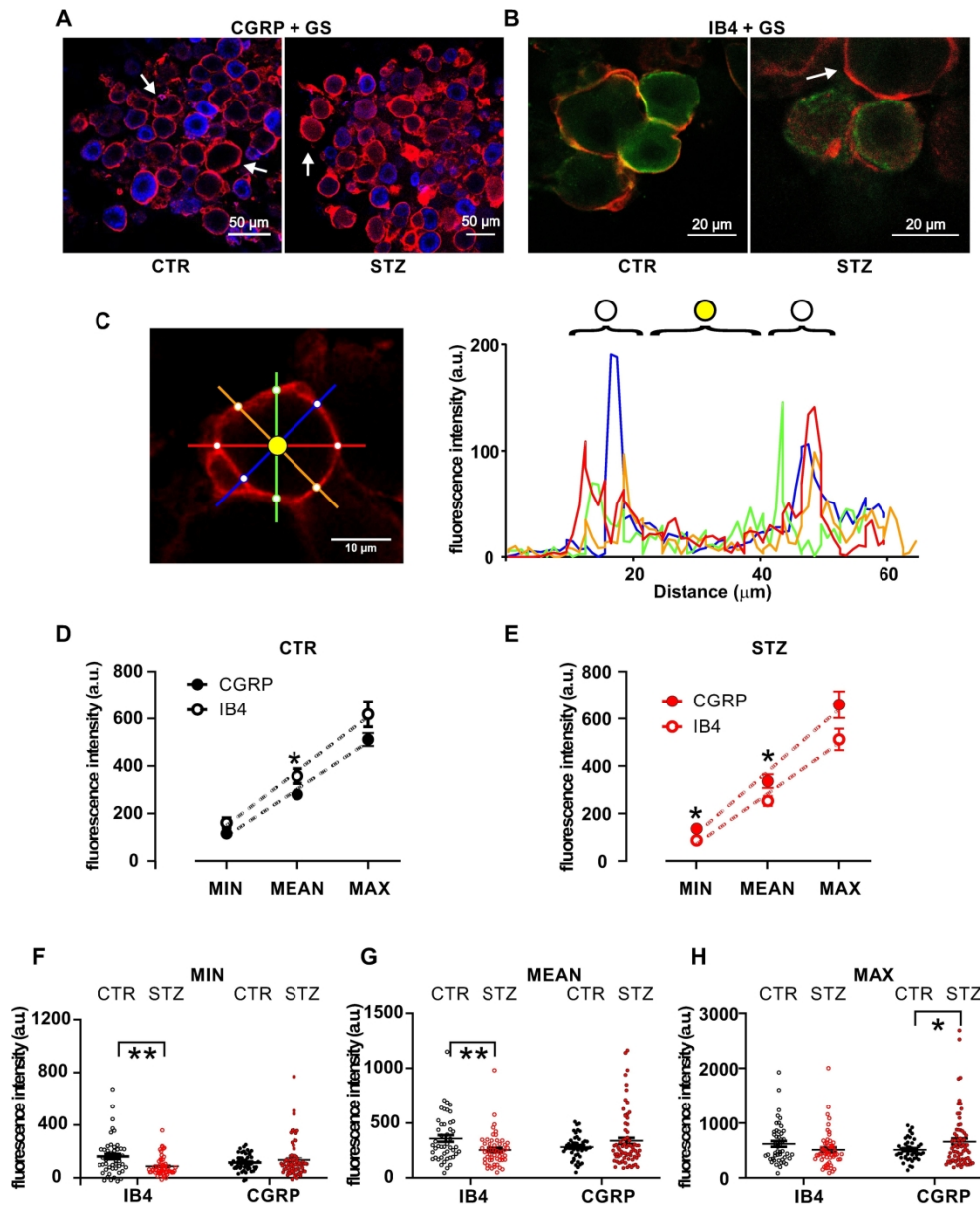


Fig. 3 GS immunostaining and analysis of SGC coverage of CGRP+ and IB4+ neurons. (A-B) Representative images showing double staining for the SGC marker GS (red), the peptidergic DRG neuron marker CGRP (blue, A), and the non-peptidergic DRG neuron marker IB4 (green, B) in vehicle-treated and STZ-treated mice. (C) Method for GS staining analysis. On the left, representative single optical section of GS staining around a DRG neuron. Fluorescence intensity is measured along the four colored lines, which cross the ensheathing SGC in 8-points (white dots) around the DRG neuron. Quantification of the fluorescence intensity along each colored line is illustrated in the graph on the right, using the same color code. GS fluorescence intensity is measured at the white dots, i.e. the peaks in the graphs, and then normalized to internal background (yellow dot). (D) Minimal, mean and maximal GS fluorescence intensities around CGRP+ or IB4+ neurons vehicle-treated mice. T-test: Min,  $P = 0.053$ ; Mean,  $P = 0.028$ ; Max,  $P = 0.079$ . (E) Minimal, mean and maximal GS fluorescence intensities around CGRP+ or IB4+ neurons in STZ-treated mice. T-test: Min,  $P = 0.016$ ; Mean,  $P = 0.028$ ; Max,  $P = 0.056$ . (F) Dot plot graph of minimal GS fluorescence intensity. Two-way ANOVA: interaction between treatment and phenotype:  $F(1, 218) = 9.49$ ,  $P = 0.002$ ; effect of treatment:  $F(1, 218) = 2.99$ ,  $P = 0.085$ ; effect of the cell phenotype:  $F(1, 218) =$

0.02,  $P = 0.88$ . IB4-CTR vs IB4-STZ, t-test,  $P = 0.0014$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P = 0.32$ . (G) Dot plot graph of mean GS fluorescence intensity. Two-way ANOVA: interaction between treatment and phenotype:  $F(1, 218) = 9.26$ ,  $P = 0.003$ ; effect of treatment:  $F(1, 218) = 8.45$ ,  $P = 0.36$ ; effect of the cell phenotype:  $F(1, 218) = 0.015$ ,  $P = 0.9$ . IB4-CTR vs IB4-STZ, t-test,  $P = 0.005$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P = 0.14$ . (H) Dot plot graph of maximal GS fluorescence intensity. Two-way ANOVA: interaction between treatment and phenotype:  $F(1, 218) = 6.12$ ,  $P = 0.014$ ; effect of treatment:  $F(1, 218) = 0.17$ ,  $P = 0.68$ ; effect of the cell phenotype:  $F(1, 218) = 0.017$ ,  $P = 0.69$ . IB4-CTR vs IB4-STZ, t-test,  $P = 0.13$ ; CGRP-CTR vs CGRP-STZ, t-test,  $P = 0.048$ . Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide; CTR: vehicle-treated mice; STZ: streptozotocin-treated mice; GS: glutamine synthetase. \* $P < 0.05$ , \*\* $P < 0.01$ .

211x256mm (300 x 300 DPI)

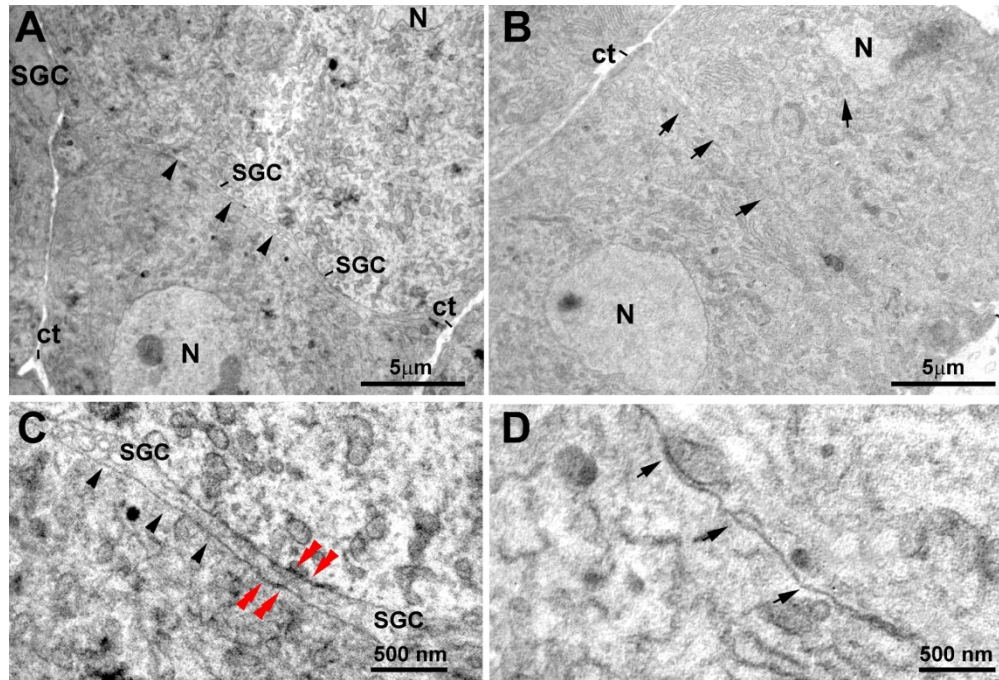


Fig. 4 Ultrastructure of the DRG neurons in control mice. (A) An ensheathing layer made of SGC processes (arrowheads) separates two adjacent neurons. (B) The neuronal membranes of two adjacent neurons are in direct contact (arrows), without SGC interposition. (C) High-magnification of panel A. Note the presence of an SGC process between the sensory neuron somata (arrowheads) and gap-junctions between neuron and SGC (red double arrowheads). (D) High magnification of panel B. Note the lack of SGC interposition between the facing membranes of the two neurons (arrows). Abbreviation: SGC: Satellite glial cell; N: nucleus; ct: connective tissue.

180x122mm (300 x 300 DPI)

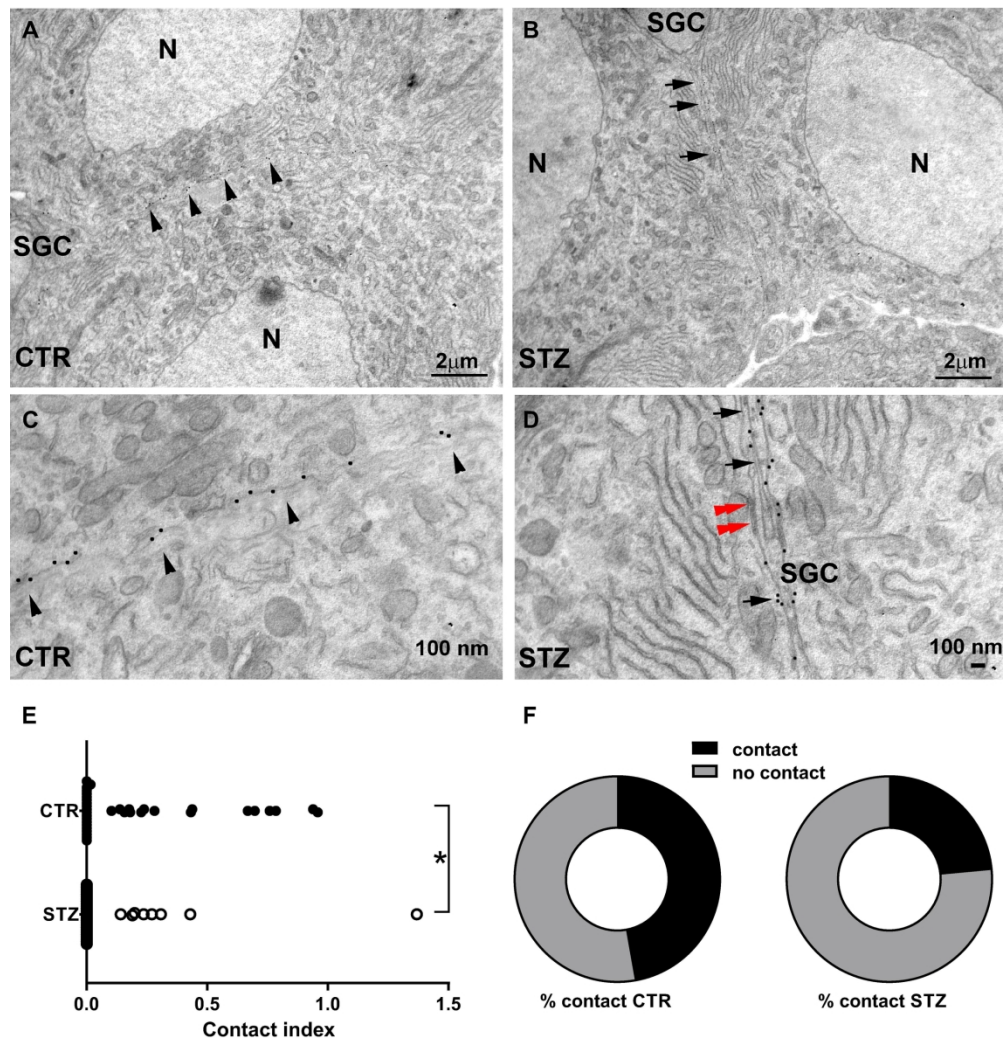


Fig. 5 Ultrastructural analysis of IB4+ DRG neurons in control and diabetic mice. (A) In CTR, the membranes of two adjacent clustered IB4+ sensory neurons are juxtaposed, without the interposition of glia (arrowheads). (B) In STZ-treated mice, a glial sheath is present between two IB4+ neurons of the same cluster (arrows). (C) High magnification of panel A. Note the occurrence of 20 nm gold particles indicative of IB4 immunogold staining scattered over the entire length of the juxtaposed neuronal membranes (arrowheads). (D) High-magnification of panel B. Note the glia separating the membranes of two IB4+ DRG neurons (arrows) and a gap-junction between the neuron and SGC (red double arrowheads). (E) Contact index in vehicle-treated mice and STZ-treated mice. The contact index is markedly reduced in STZ (Mann-Whitney test,  $P < 0.01$ ). (F) Pie charts showing the proportion of neuronal membranes exhibiting at least one point of contact (Fisher exact test,  $P < 0.05$ ) in CTR and STZ-treated mice. Abbreviation: SGC: Satellite glial cell; N: nucleus; CTR: vehicle-treated mice; STZ: streptozotocin-treated mice.

210x216mm (300 x 300 DPI)

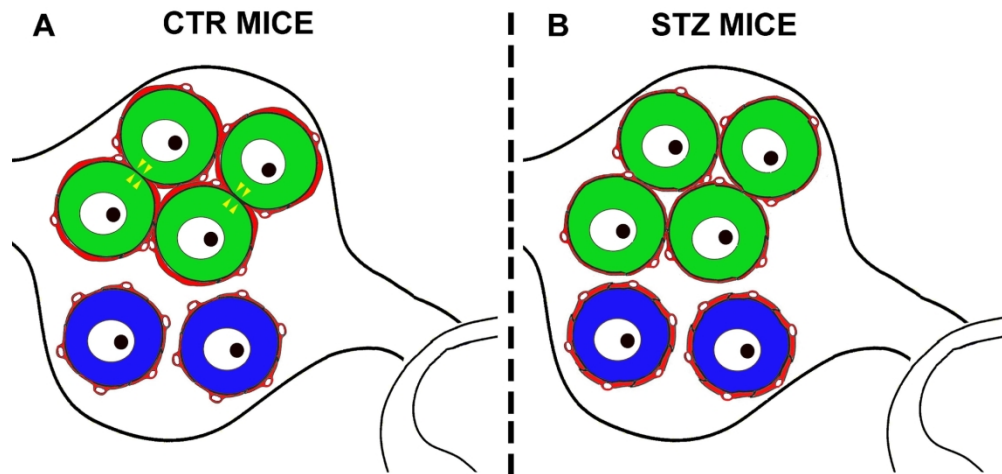
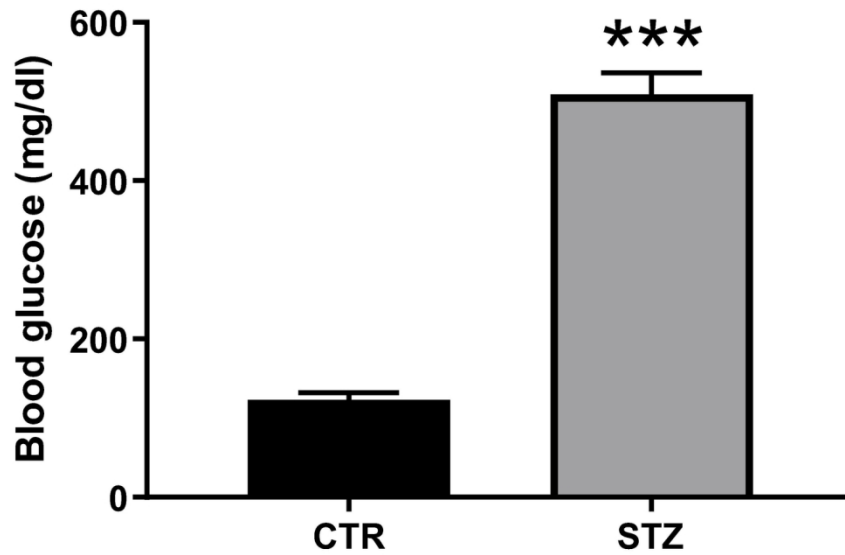


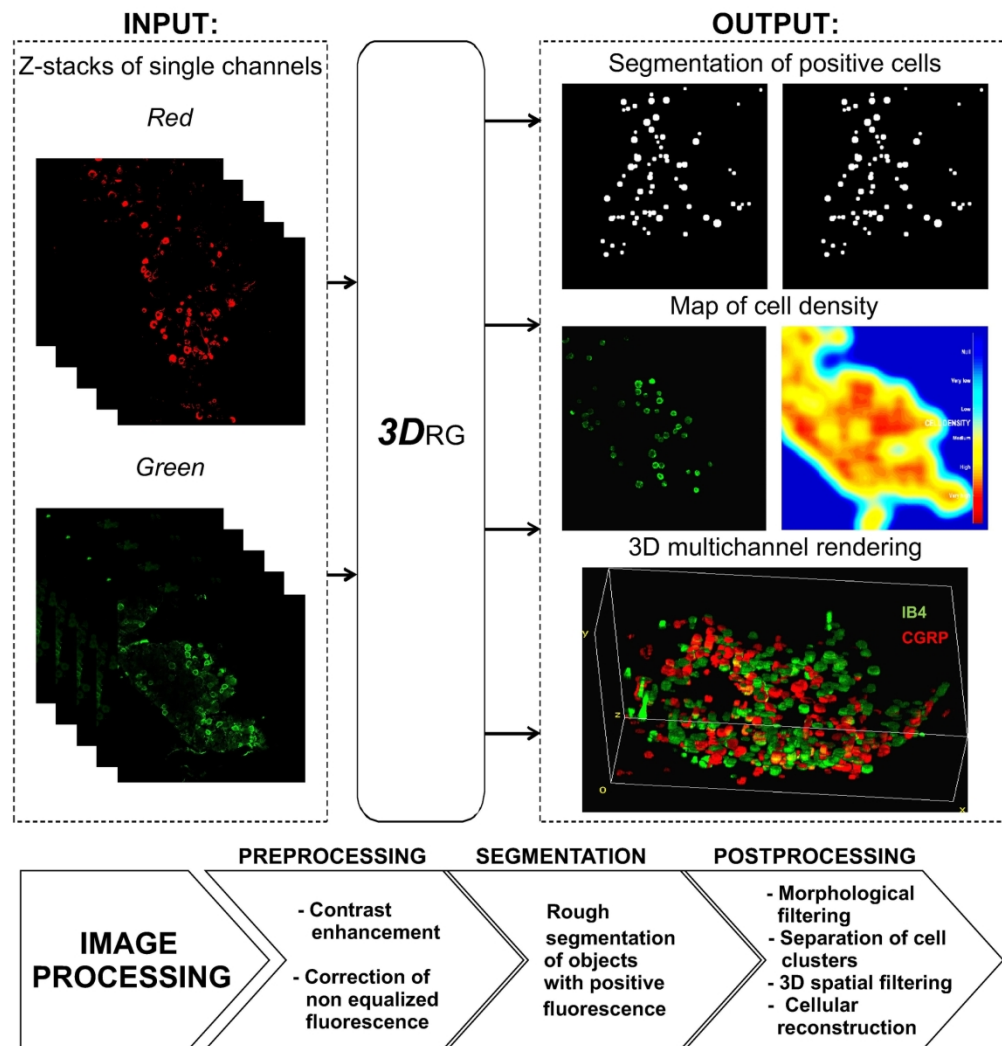
Fig. 6 Schematic summary of neuro-glia relationship in DRGs of control and diabetic mice. (A) Representation of a simplified dorsal root ganglion (DRG) in control (CTR) mice: IB4+ neurons (green) are grouped in cluster and surrounded by a few satellite glial cells (SGC). SGCs form a continuous glutamine synthetase (GS)+ sheet around neurons (red thick line) which becomes thinner at the interface of two IB4+ opposing neurons. At some points, the membranes of cluster-forming IB4+ neurons are in direct contact (yellow arrowheads). CGRP+ neurons (blue) are randomly scattered across the DRG and surrounded by numerous SGCs with relatively less bright GS immunostaining (red thin line). (B) In streptozotocin (STZ)-induced diabetic mice, IB4+ neurons (green) are still grouped in clusters and CGRP+ neurons (blue) randomly scattered across the DRG. However, GS fluorescence intensity is higher around CGRP+ neurons (red thick line) than in IB4+ neurons (red thin line). Moreover, the points of the cluster where two IB4+ opposing neurons are in direct contact are significantly reduced.

179x116mm (300 x 300 DPI)



Supplemental Figure 1 (Fig. S1) Blood glucose concentration in control and diabetic mice. Bar chart showing the blood glucose concentration in control (CTR N=16, black bar) and diabetic (STZ N=16, grey bar) mice four weeks after intraperitoneal injection of vehicle or streptozotocin (150 mg/kg), respectively. Control mice are normoglycemic, with a blood glucose concentration < 200 mg/dl, while diabetic mice are hyperglycemic, with a blood glucose concentration > 300 mg/dl (T-test, \*\*\*  $p < 0.001$ ).

141x89mm (300 x 300 DPI)



Supplemental Figure 2 (Fig. S2) Schematic flowchart describing the steps in image processing performed using the 3DRG software. After an initial preprocessing stage of image denoising and contrast (INPUT), the software proceeded to the segmentation of positive objects and the generation of a 3D cell density map (OUTPUT). Based on the cell density map and custom data, 3D rendering and subsequent cluster analysis were performed. IB4+ staining is green and CGRP+ staining is red. Abbreviations: IB4: isolectin B4; CGRP: calcitonin gene-related peptide.

176x187mm (300 x 300 DPI)