TITLE: **The greater occipital nerve and its spinal and brainstem afferent projections: A stereological and tract-tracing study in the rat**

RUNNING TITLE: The greater occipital nerve in the rat

AUTHORS: Nuria García-Magro[1], Yasmina B. Martin[2], Pilar Negredo[1]* and Carlos Avendaño[1]*

AFFILIATION: [1] Autonoma University of Madrid, Medical School, Department of Anatomy, Histology & Neuroscience, c/ Arzobispo Morcillo 2, 28029 Madrid, Spain.
[2] Francisco de Vitoria University (UFV), Department of Anatomy, Faculty of Health Sciences, Ctra. M-115 Pozuelo-Majadahonda Km 1,800, Pozuelo de Alarcón, 28223 Madrid, Spain.

Nº of text pages: 40

Nº of Figures: 15

Nº of Tables: 3

CORRESPONDENCE TO: Dr. C. Avendaño, Departamento de Anatomía, Histología y Neurociencia, Facultad de Medicina, Universidad Autónoma, c/ Arzobispo Morcillo 2, 28029 Madrid, Spain.
E-mail: carlos.avendano@uam.es
Phone: +34 91 497 5335

Dr. P. Negredo, Departamento de Anatomía, Histología y Neurociencia, Facultad de Medicina, Universidad Autónoma, c/ Arzobispo Morcillo 2, 28029 Madrid, Spain.
E-mail: pilar.negredo@uam.es
Phone: +34 91 497 5354

ACKNOWLEDGMENTS: The authors would like to thank Begoña Rodríguez Menéndez for her skilled technical assistance. This work was supported by Grant BFU2015-66941R from Spain's Ministerio de Economía Industria y Competitividad/Fondo Europeo de Desarrollo Regional (MINECO/FEDER).

CONFLICT OF INTEREST: The authors declare no competing financial interests.

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37 FUNDING INFORMATION

38 Grant BFU2015-66941R from Spain's Ministerio de Economía Industria y
39 Competitividad/Fondo Europeo de Desarrollo Regional (MINECO/FEDER).

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ABSTRACT

The neuromodulation of the greater occipital nerve (GON) has proved effective to treat chronic refractory neurovascular headaches, in particular migraine and cluster headache. Moreover, animal studies have shown convergence of cervical and trigeminal afferents on the same territories of the upper cervical and lower medullary dorsal horn (DH), the so-called trigeminocervical complex (TCC), and recent studies in rat models of migraine and craniofacial neuropathy have shown that GON block or stimulation alter nociceptive processing in TCC. The present study examines in detail the anatomy of GON and its central projections in the rat applying different tracers to the nerve and quantifying its ultrastructure, the ganglion neurons subserving GON, and their innervation territories in the spinal cord and brainstem. With considerable intersubject variability in size, GON contains on average 900 myelinated and 3300 unmyelinated axons, more than 90% of which emerge from C_2 ganglion neurons. Unmyelinated afferents from GON innervates exclusively laminae I-II of the lateral DH, mostly extending along segments C_{2-3}. Myelinated fibers distribute mainly in laminae I and III-V of the lateral DH between C_1 and C_6 and, with different terminal patterns, in medial parts of the DH at upper cervical segments, and ventrolateral rostral cuneate, paratrigeminal, and marginal part of the spinal caudal and interpolar nuclei. Sparse projections also appear in other locations nearby. These findings will help to better understand the bases of sensory convergence on spinomedullary systems, a critical pathophysiological factor for pain referral and spread in severe painful craniofacial disorders.

KEY WORDS: Spinal cord; Dorsal horn; Cervical afferents; Dorsal root ganglia; Trigeminocervical

ABBREVIATIONS: 3dON, third occipital nerve; AS, m. auricularis superior; AT, m. acromiotrapezius; Bi, Bischoff nucleus; C_2, second spinal nerve; ChAT, choline acetyltransferase; CT, m. clavotrapezius; CTB, cholera toxin B; Cu, cuneate nucleus; CuE, external cuneate nucleus; CuM, cuneate nucleus medial division; CuR, cuneate nucleus rostral division; DC, dorsal column; DCN, dorsal column nuclei; DH, dorsal horn; DRG, dorsal root ganglion; FA, m. frontalis, pars auricularis; FB, Fast blue; GON, greater occipital nerve; Gr,

71 gracile nucleus; IB4, isolectin IB4 from Griffonia simplicifolia; LAL, levator auris longus;
72 LC, lateral column; MAx, myelinated axons; NBi, internal basilar nucleus; Pa5,
73 paratrigeminal nucleus; SC, m. splenius capitis; Sol, solitary nucleus; Sp5c, caudal trigeminal
74 nucleus; Sp5cc, caudal trigeminal nucleus (caudal level); Sp5cr, caudal trigeminal nucleus
75 (rostral level); Sp5i, interpolar trigeminal nucleus; T, m. temporalis; TCC, trigeminocervical
76 complex; TH, tyrosine hydroxylase; UAx, unmyelinated axons; x, nucleus x; XII, hypoglossal
77 nucleus; z, nucleus z.

78

79 ## 1. INTRODUCTION

80 The Greater Occipital Nerve (GON) is the major component of the second spinal
81 nerve (C_2). It forms the medial division of the posterior branch of C_2, which emerges after this
82 branch sends communicating branches to C_1 and C_3 and a few twigs to deep nuchal muscles.
83 In humans, the GON pierces the muscles supraspinalis capitis (often) and trapezius
84 (sometimes), as well as the deep fascia, to supply sensory innervation to a wide occipital part
85 of the scalp (reviewed in Kemp, Tubbs & Cohen-Gadol, 2011). The anesthetic block of the
86 GON has been used for decades to alleviate some types of primary headaches, and the interest
87 in its anatomy has not ceased to grow in parallel to the development of more refined
88 diagnostic and therapeutic manipulations of the GON to deal with a variety of craniofacial
89 pain disorders (reviewed in Chen et al., 2015; Dodick et al., 2015; Inan et al., 2015; Tang,
90 Kang, Zhang & Zhang, 2017; Young, 2010).

91 An intriguing feature of some primary headaches is their extensive location from the
92 face to occipital regions, a distribution that spans innervation territories of the first and second
93 trigeminal branches and the upper cervical spinal nerves. Likewise, artificial or pathological
94 stimulation of occipital or nuchal structures, or the infratentorial dura, often evoke referred
95 pain in frontal regions (Goadsby & Bartsch, 2010; Piovesan et al., 2001). Among the various
96 mechanisms proposed to explain referred cephalic pain, a convergence of afferents from
97 different territories and submodalities on central neurons has been favored, following early
98 reports of electrophysiological and anatomical convergence on upper cervical or medullary
99 dorsal horn (DH) in cats (Abrahams, Anstee, Richmond & Rose, 1979; Kaube, Keay, Hoskin,
100 Bandler & Goadsby, 1993; Kerr, 1972; Kerr & Olafson, 1961; Sessle, Hu, Amano & Zhong,

1986). More recent reports in the rat examined the topographical convergence of primary afferents on the first cervical segments and caudal spinal trigeminal nucleus (Pfaller & Arvidsson, 1988; Xiong & Matsushita, 2000) and proved the existence of direct functional coupling, particularly of meningeal and cervical afferents, on DH neurons (Bartsch & Goadsby, 2002; Goadsby & Bartsch, 2010).

The GON has recently become a useful target for testing how its block or stimulation in a rat model of migraine affects mechanical and nociceptive thresholds in the head and elsewhere (De La Cruz et al., 2015; Walling et al., 2017), and alters trigeminovascular nociceptive processing in the spinal trigeminal nucleus (Lyubashina, Panteleev & Sokolov, 2017). However, available data on the GON anatomy and connections in the rat is scanty, being limited to two early studies using HRP transport (Neuhuber & Zenker, 1989; Scheurer, Gottschall & Groh, 1983). Therefore, we decided to examine in further qualitative and quantitative detail the fiber content of the GON, the primary sensory neurons from which they emerge, and their central axonal projections in the spinal cord and the brainstem of the rat.

2. MATERIAL AND METHODS

2.1. Subjects

Twenty-eight 3-month-old male Sprague-Dawley rats born from different litters from Harlan (Harlan Iberia, Barcelona, Spain) and Charles River (Charles River Laboratories, Barcelona, Spain) were used in this study. All procedures followed the regulations issued by the Ethical Committee of the Autonoma University of Madrid and the European Community's Council Directive 2010/63/UE.

2.2. Surgery and tracer delivery to the GON

Tracer deposits were made in 16 rats anesthetized by intramuscular injection of a 0.2 ml/100 g of a solution containing a mixture of Ketamine (Ketolar, 55 mg/kg), Xylazine (Rompun, 15 mg/kg) and Atropine (0.2 g/kg). A midline incision was made from the interparietal suture rostral to lambda to the 3d cervical vertebra. The skin was retracted laterally and the GON and the 3d occipital nerve were identified through the thin layers of fascia and superficial muscles (Fig. 1). About 2-3 mm of the GON just before the nerve gives its first superficial twigs were isolated and exposed by carefully separating it from the

131 surrounding muscular and connective tissue.

132 One group of animals (Group 1, n=13) received an intraneural injection in the GON
133 (Figs. 2, 3) of a mixture of 1% cholera toxin B (CTB, Sigma-Aldrich), 2% isolectin IB4 from
134 Griffonia simplicifolia (IB4, Vector Laboratories) and traces of the supravital dye light green
135 (Sigma-Aldrich), using a glass micropipette (A-M Systems, Sequim, WA; 20–30 µm tip outer
136 diameter) coupled to a 10 µl Hamilton syringe. As previously described (Fernández-Montoya,
137 Martin, Negredo & Avendano, 2017) a narrow pliable metal plate was carefully placed under
138 the exposed nerve and 1-1.5 µl of the tracer mixture were injected in 3-4 pulses over 30
139 minutes. Finally, the region was rinsed with saline and the skin was sutured with a 2/0 silk
140 suture.

141 In order to examine the possibility of local contamination by the tracers injected, in a
142 second group of cases (Group 2, n=2) the GON was exposed in the same manner but left
143 undamaged, and the same amount of CTB-IB4 was instilled and left in place for 30 min
144 before rinsing and closing the wound. Another group of animals (Group 3, n=3) received an
145 intraneural deposit in the GON of a 5% suspension of Fast Blue (FB, Sigma-Aldrich) in
146 DH_2O. The nerve was exposed and transected, and the proximal stump was introduced in a
147 small glass or plastic vial containing the tracer. After 1 hour, the vial was removed, the region
148 was rinsed and the skin sutured.

149 2.3. Histological processing of tracer-injected cases

150 Animals from Groups 1 and 2 (CTB/IB4 applications) survived 4 days. They were
151 euthanized by an intraperitoneal injection of sodium pentobarbital (Dolethal, 50 mg/kg) and
152 perfused through the heart with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4).
153 Ganglia C_2 and C_3 and the cervical spinal cord and caudal two-thirds of the brainstem were
154 dissected out, postfixed in the same fixative overnight at 4ºC and cryoprotected with a 30%
155 sucrose solution in 0.1M PB for 2 days. The ganglia were frozen and serially cut at 15 µm in a
156 Leica CM1950 cryostat (Leica Biosystems, Nussloch, GmbH). Every second section was
157 incubated overnight at 4ºC with a combination of two primary antibodies (Table 1), goat anti-
158 IB4 and rabbit anti-CTB. After several washes with saline PB (PBS), the sections were
159 incubated for 2 h in the dark in a mixture of secondary antibodies: donkey anti-goat
160 AlexaFluor 488 and donkey anti-rabbit AlexaFluor 647. Nuclei were labeled with

161 Bisbenzimide. The blocks including cervical spinal cord and brainstem were frozen cut in
162 horizontal (n=13) or coronal (n=2) sections of 40 µm in a sliding microtome (Leica SM2400,
163 Leica Biosystems, Nussloch). In two cases of Group 1, alternate sections were processed free-
164 floating using the same immunofluorescence protocol. The remaining cases were processed
165 free-floating using the avidin–biotin–peroxidase method (ABC), with diaminobenzidine
166 (DAB) as a chromogen as previously described (Fernández-Montoya et al., 2017). Briefly,
167 after endogenous peroxidase inactivation and preincubation in a blocking solution containing
168 2% Triton X-100, alternate series were incubated with either goat anti-IB4 (1:1000; Vector
169 Laboratories) or rabbit anti-CTB (1:500; Sigma Aldrich) overnight at 4ºC under agitation.
170 After several washes, sections were then incubated for two hours at room temperature, with
171 the rabbit anti-goat (1:250; Vector Laboratories) or goat anti-rabbit (1:500; Sigma Aldrich)
172 biotinylated secondary antibodies corresponding to each series. Finally, both series were
173 incubated in an avidin-biotin (Kit ABC Elite®, Vector laboratories) solution in PBS 0.02M
174 with 2% Triton X-100, and revealed in 0.05% DAB in 0.1M PB with 0.008% cobalt chloride
175 and 0.0064% nickel sulfamate, adding 0.001% H_2O_2. In the two animals cut on the coronal
176 plane, a series of alternate sections were stained with 0.1% cresyl violet. All sections were
177 mounted on subbed glass slides, dehydrated, defatted and coverslipped with DePeX.

178 Animals from Group 2 (FB deposits in GON) survived 6 days and then were deeply
179 anesthetized, perfused and had their C_2 and C_3 ganglia and a spinal cord-brainstem block
180 extracted as in Group 1. The ganglia were cryoprotected, frozen and serially cut at 20 µm in a
181 cryostat; the spinal cord-brainstem block was frozen cut in horizontal sections of 40 µm in a
182 sliding microtome. After mounting and drying in the dark, sections were quickly dehydrated
183 in ethanol, defatted in xylene and coverslipped with DePeX.

184 2.4. Antibody characterization

185 All antibodies used in this study are commercially available and their supplier, host
186 species and dilutions are indicated in Table 1.

187 The rabbit polyclonal anti-Cholera Toxin antibody consists of two subunits, A (27
188 kDa) and B (12 kDa). This antiserum showed no binding to Staphylococcus enterotoxins A or
189 B, or Pseudomonas exotoxin, but it bound specifically to cholera toxin, a widely used axonal
190 tracer (manufacturer´s datasheet). In several studies, the specificity was demonstrated by the

191 presence of immunolabeling in specific locations on the ipsilateral side of the spinal cord and
192 lack of immunolabeling on the contralateral side (reviewed in Shehab et al., 2015). This
193 antibody was used with Alexa Fluor 647 donkey anti-rabbit secondary antibody.

194 The goat monoclonal Anti- Griffonia Simplicifolia Lectin I antibody has been well
195 characterized previously. For example, it was shown that in spinal cord sections from a
196 control rat which were did not receive IB4, this antibody did not show specific staining (Kim
197 et al., 2008; Shehab, 2009). This antibody was used with Alexa Fluor 488 donkey anti-goat
198 secondary antibody.

199 The same antibodies against CTB and IB4 were used in a previous study from our
200 laboratory (Fernández-Montoya et al., 2017), and the results were comparable to those
201 presented here.

202 The goat polyclonal anti-choline acetyltransferase antibody specifically recognizes a
203 single band of 68-70 kDa on Western blots of rat peripheral nerves (Brunelli et al, 2005) and
204 mouse brain lysates (manufacturer´s technical information). ChAT has been widely used for
205 detecting motoneurons and their axons (Castro, Negredo & Avendaño, 2008; Kaneyama &
206 Shirasaki, 2018).

207 The rabbit polyclonal anti-Tyrosine Hydroxylase recognizes a single band of 62 kDa
208 on Western blots (according to manufacturer´s datasheet). This antibody has proven useful to
209 label sympathetic postganglionic axons and terminals (Castro et al., 2008; Gautron et al.
210 2010).

211 Negative controls, omitting the primary antibodies, yielded no staining in the GON.
212 Positive controls were simultaneously done on spinal cord sections for ChAT, and cerebral
213 cortex sections for TH, resulting in well-characterized labeling of motoneurons and
214 intracortical axons, respectively.

215 2.5. Methacrylate embedding of C_2 and C_3 dorsal root ganglia

216 Animals in Group 4 (n=10) were deeply anesthetized and perfused with 4%
217 paraformaldehyde-0.1% glutaraldehyde in 0.1M PB and. In eight of these rats, ganglia C_2 and
218 C_3 were collected bilaterally and postfixed in fresh-buffered 4% paraformaldehyde for 2-3
219 days. They were then dehydrated in graded ethanol solutions and infiltrated in

220 glycolmethacrylate (Technovit 7100 Kulzer, Wehrheim, Germany) for 3 days. Both ganglia
221 from each side and case were rolled free and embedded in one block, which was exhaustively
222 sectioned in a rotary microtome (Microm HM 350 S, Thermo-Fisher, Walldorf, Germany) at
223 40 µm. All sections were serially mounted on glass slides and Nissl-stained with 0.1% cresyl
224 violet.

225 2.6. Treatment of GONs for electron microscopy and immunostaining

226 In eight animals of Group 4, a 2 mm piece of the GON was excised, uni- or bilaterally,
227 and postfixed for 2 days in a buffered 2% paraformaldehyde and 2% glutaraldehyde mixture.
228 The nerves were osmicated (1% OsO_4 in 0.1M PB), dehydrated and embedded in Durcupan
229 resin (Fluka AG, Buchs, Switzerland). Consecutive semithin and ultrathin sections were
230 obtained using a Leica Ultracut UCT ultramicrotome (Leica Microsystems AG, Wetzlar,
231 Germany) and a diamond knife (Drukker International B.V., Cuijk, The Netherlands).
232 Semithin sections (0.75-1 µm) were stained with 0.5% toluidine blue (Merck). Ultrathin
233 sections (60-70 nm) were collected on 200-mesh copper grids and stained with uranyl acetate
234 and lead citrate.

235 In semithin sections of three GONs randomly chosen, immunohistochemistry was
236 performed for tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) to examine
237 whether GON contains sympathetic or motor fibers, respectively, as reported previously
238 (Castro et al., 2008). In short, mounted sections were etched with a mixture of sodium
239 ethanolate and propylene oxide and de-osmicated with 2% sodium metaperiodate. Before
240 carrying out the immunostaining endogenous peroxidase was inactivated with 1% hydrogen
241 peroxide, and sections were incubated in citrate buffer for 10 min at 90°C for antigen
242 retrieval. Semithin sections were incubated with polyclonal primary anti-TH (1:100) or anti-
243 ChAT (1:50) antibodies (Table 1). Secondary antibodies were biotinylated rabbit-anti-goat
244 (1:50, Chemicon) and goat-anti-rabbit (1:50, Vector Laboratories). After washes, sections
245 were incubated in avidin-biotin (1:250, Kit ABC Elite®, Vector Laboratories) in PBS for 2
246 hours and reacted with diaminobenzidine (DAB, 0.05%, Sigma-Aldrich). The reaction was
247 stopped by washing with PB 0.1 M and intensified with 0.02% buffered osmium tetroxide for
248 2 min. After thorough washing, the sections were dehydrated, defatted and coverslipped with
249 DePeX. Negative controls, omitting the primary antibodies, yielded no staining. Positive

250 controls were simultaneously done on thin cryostat sections of the spinal cord and produced
251 well characterized labeling.

252 2.7. Microscopy

253 Semithin GON sections, methacrylate-embedded ganglia, DAB-reacted tissues and in
254 general control material was examined and photographed under transmitted light microscopy
255 in an Olympus BX61 microscope using dry (2x planapochromatic, 4x, 10x and 20x
256 superplanapochromatic, Olympus) and oil-immersion (100x, 1.40 NA, UPlanSApo, Olympus)
257 objectives, and a DP71 video-camera (Olympus-Europa, Hamburg, Germany).

258 Confocal microscopy images of fluorescent material were obtained with a TCS SP5
259 Spectral Leica confocal microscope using a 40x oil immersion objective for the ganglia; the
260 spinal cord-brainstem sections were studied with dry 10x and oil-immersion 40x lenses. The
261 images were acquired at 1024×1024 pixels. Image stacks and merged channel panels were
262 obtained with the Leica LAS AF software.

263 The ultrastructural study of the GON was carried out in a Jeol 1010 electron
264 microscope (Tokyo, Japan) equipped with a CCD camera (Gatan BioScan, Pleasanton, CA,
265 USA). Low power (120x) images of the nerves were obtained for reference and sampling
266 design (see Stereology, below); higher power (8000x) images were captured to show in
267 sufficient detail both myelinated and unmyelinated axons.

268 Corel Photopaint and CorelDraw software (v. X3, Corel, Ottawa, Canada) was used
269 for figure composition and digital processing, which was limited to adjustments in gray scale,
270 brightness and/or contrast, to enhance information.

271 2.8. Stereology

272 *Immunofluorescent neuron numbers in DRG*

273 The total number of labeled neurons with CTB and IB4 in the C_2 and C_3 ganglia was
274 estimated using the physical fractionator method (Gundersen, 1986; Howard & Reed, 1998).
275 One in four sections was examined and photographed in the confocal microscope using
276 separately the red and green channels and with both channels merged. A series of pairs of 0.5
277 µm-thick pictures were taken with a 5 µm intrapair separation in the z-axis, covering
278 completely the sampled section. Each section was systematically sampled off-line with

279 unbiased frames overlaid on the images with the help of CorelDraw software. Somatic
280 profiles of immunofluorescent neuronal perikarya were used as counting units. The labeled
281 profiles fulfilling the counting criteria were quantified in both directions of the disector
282 separately for CTB- and IB4-labeled, and cells co-localizing both tracers. Final sampling
283 fraction (F_T) was 1:24, and 120 neurons were counted on average (ΣQ^-) on about 70 disector
284 fields in C_2 and C_3 per side. Total estimates were computed as $\widehat{N} = \Sigma Q^- \cdot F_T^{-1}$. A similar
285 procedure was performed for cases receiving the tracer FB.

286 The precision of the individual estimates was approximated by estimating the error
287 variance due to the stereological procedure, which is represented by the coefficient of error
288 (CE), as previously reported (Avendaño, Machin, Bermejo & Lagares, 2005; Cruz-Orive,
289 2004). The mean CEs of the estimates for the total number of labeled neurons ranged between
290 8.9–10.4%. As expected, estimates of much sparser subpopulations (e.g., double labeled
291 neurons, or labeled neurons in C_3, if counted separately from those in C_2) resulted in higher
292 CEs.

293 *Number and somatic volume of neurons in C_2 and C_3 ganglia*

294 The total number of neurons in C_2 and C_3 was estimated using the optical fractionator
295 (West, Slomianka & Gundersen, 1991) on the methacrylate embedded ganglia. In all
296 appropriately sampled neurons the somatic volume was estimated with the planar rotator
297 (Tandrup, Gundersen & Jensen, 1997). All stereological analyses were carried out in an
298 Olympus BX51 microscope equipped with a high-precision motorized microscope stage
299 (Prior Proscan II, Prior Scientific Inc., Rockland, MA), an internal 0.3 µm-resolution
300 microcator, and an Olympus DP71 digital camera. The control of the stage movements and
301 the interactive test grids were provided by the NewCast stereological software (ver. 4.6.1.630,
302 Visiopharm, Hørsholm, Denmark) running in a Dell Precision 390 computer.

303 Average final sampling fraction was either 1:70 or 1:140 –both strategies yielding
304 essentially the same results. Number estimation was computed as for the physical fractionator
305 (see above). Section thickness measurements, cell typing and perikaryal volume estimations
306 were performed with an oil-immersion 100x lens (1.40 NA, UPlanSApo, Olympus) at a
307 3900x final magnification on screen. Neuron types were identified according to well
308 established morphological criteria (Lawson, 2005; Lieberman, 1976; Rambourg, Clermont &

309 Beaudet, 1983). The few (less than 5%) cells that could not be clearly identified as A- or B-
310 type were split in half and each half was ascribed to either A3 or B1 subtypes. The average
311 precision of the stereological procedure among individual cases was 10.9% (range 7.3-
312 14.2%).

313 *Volume of CTB- and IB4-innervated territories*

314 The volume occupied by labeled axons and their varicosities was estimated by point
315 counting, applying the Cavalieri principle (Gundersen et al., 1988) on 40 μm-thick IB4- or
316 CTB-immunolabeled sections at fixed intervals (d = 160 μm), as previously reported
317 (Fernández-Montoya et al., 2017). With the integrated stereological equipment described in
318 the previous section, and using a planopochromatic 20x dry objective (0.75 NA, Olympus
319 UPLSAPO), two quadratic lattices of points of different density were superimposed on the
320 immunolabeled regions. Dorsal horn laminae III-IV, displaying the densest and most
321 extensive innervation, was sampled with low intensity (a(p) = 4127 μm^2); for the remaining
322 territories a denser grid (a(p) = 1337 μm^2) was used. All points displaying at least one axonal
323 swelling within a radius of 4 μm, at any depth of the section, were included in the count.
324 Labeling with IB4 turned out to be more diffuse, characterized by thinner axons and
325 varicosities of small size, and its delineation was less precise in peripheral areas and areas
326 with little labeling. To help identifying the innervated structures, separate cases with Nissl-
327 and cytochrome oxidase-stained horizontal sections were prepared.

328 Volume estimates were obtained as $\hat{V} = \Sigma P \cdot a(p) \cdot \bar{d}$ for a number of pre-defined
329 territories: Lamina I, lamina II, laminae III-IV (lateral parts), and deep laminae in the DH of
330 the spinal cord and caudal medulla; patches in the medial parts of the spinal DH; projections
331 in and rostral to the dorsal column nuclei (DCN); and the interpolar nucleus of the trigeminal
332 complex (Sp5i). Sparse projections in other regions were not measured. Values were not
333 corrected for shrinkage. Average precision in the measuring procedure varied much,
334 depending on the size of the target region: The CEs obtained ranged between 6-7% for
335 laminae III-IV or all territories combined, to 29% for the deep DH laminae, with the weakest
336 innervation.

337 *Morphometry of the GONs*

Measurements were performed at basically the same level of the GON in all cases (Fig. 2). The myelinated (MAx) and unmyelinated axons (UAx), as well as the interstitium of the GON were morphometrically assessed on ultrastructural images captured at 8000x and handled off-line with CorelDraw X3 software. A 2D fractionator design was used for estimating axon numbers, and the 2D-nucleator was applied on sampled profiles for estimating cross-sectional areas of axons and myelin sheaths (Larsen, 1998). From the areal measurements of MAx, the axonal and fiber diameters were derived to compute the g-ratio (the ratio of the inner axonal diameter to the total fiber diameter), widely held as a reliable indicator of myelination (Chomiak & Hu, 2009). The predicted CE for number estimation in each case was estimated as in Larsen (1998; Eq. A2). The mean CE was 6.3% for MAx and 3.9% for UAx. Areal fractions of the GON occupied by MAx, myelin, UAx and interstitium (including endo- and perineurium, Schwann cell nuclei and cytoplasm, and other cells; excluding epineurium and large blood vessels) were estimated by point counting.

2.9. Statistics

Descriptive statistics (means and SEM or SD) were obtained from the Excel program (Microsoft Office Professional Plus 2010 for Windows 10). Statistical comparisons between sides were made for neuronal numbers and perikaryal volumes of methacrylate-embedded ganglia with the help of the SPSS program (v. 15.0 for Windows). For this purpose, the Wilcoxon signed rank test for two correlated groups was applied. Statistical significance was set at a P-value < 0.05.

3. RESULTS

3.1. Macroscopic anatomy of the GON

As its homonymous nerve in humans and other mammalian species, the GON in the rat is the main component of the posterior branch of the second cervical spinal nerve. The earliest and nearly the only anatomical description of this nerve was provided by Scheurer et al. (1983), and present findings are consistent with that description. The posterior branch of the spinal nerve C_2 is joined by communicating branches with C_3 and, often, C_1, and then divides into a thinner lateral branch, and a larger medial branch, which makes the GON. This nerve penetrates first the muscle semispinalis capitis, and then the clavotrapezius and/or the

368 splenius capitis before piercing the superficial fascia and the anterior belly of the levator auris
369 longus (LAL, Fig. 1). Along its course, the nerve gives off a variable number of small
370 branches that apparently penetrate local muscles. In its most superficial course, the GON
371 curves rostrolaterally and divides into a variable number of branches that distribute on the
372 occipital region and near the medial base of the pinna. Along this course it is possible that the
373 GON or some of its branches also pierce thin muscles that are not readily seen during surgery,
374 such as the abductor auricularis longus or the interscutularis (Murray, Gillingwater & Parson,
375 2010). After piercing the LAL, and before turning rostralwards and dividing into terminal
376 branches, the GON is easily accessible for at least 2 mm, and this was the locus chosen to
377 carry out the tracer injections or deposits and the ultrastructural study of the nerve (Fig. 2).

378 3.2. Tracer delivery to the GON and neuronal labeling in sensory ganglia

379 The success of the intraneural injections of CTB-IB4 was ascertained by several lines
380 of evidence, which pointed to a consistent and substantial uptake of the tracers by GON
381 axons: 1, all cases showed topographically similar patterns of distribution of primary afferents
382 in the spinal cord and brainstem. 2, the number of ganglion cell bodies that were
383 immunofluorescent for CTB, IB4 or both tracers did not differ much across cases (coefficient
384 of variation below 10%; Figs. 4, 5). 3, however, those numbers were significantly lower than
385 the number of FB-labeled neurons in ganglia C_2 and C_3 after exposing the proximal stump of
386 the transected GON to the tracer (Fig. 6), for reasons that will be explained in the Discussion.
387 And 4, axon counts in the GON backed these results, as will also be discussed below.

388 Labeled neurons were much more abundant in C_2 (2016±209, CTB+IB4; 2453±133,
389 FB) than in C_3 (184±52, CTB+IB4; 256±32, FB). About 20% of neurons in C_2, and 6% in C_3
390 co-expressed CTB and IB4. As expected, CTB labeled predominantly large or medium-sized
391 neurons, while IB4 mainly appeared in small and some medium-sized neurons. However,
392 there were occasional instances of large IB4-labeled neurons, and, more frequently, small
393 CTB-labeled neurons. Most double-labeled neurons were small or medium-sized (Fig. 5).

394 3.3. Central distribution of primary afferents from the GON

395 *IB4-labeled afferents: restricted regional and laminar distribution in the spinal cord*

396 Both in tissue reacted with DAB, and in immunofluorescent material, IB4-labeled
397 axons enter the spinal cord through the dorsal roots of C_2 (very few were found in C_3). In the

398 posterolateral (Lissauer's) tract the axons divide into short ascending and longer descending
399 fibers that give collateral or terminal branches that enter the lateral part of the DH from the
400 rostralmost levels of spinal segment C_4 to the caudalmost levels of segment C_1. No IB4-
401 labeling appears elsewhere in the spinal cord or the caudal brainstem. The innervated territory
402 is consistently expressed as a dense band of labeling restricted to the outer two-thirds of
403 lamina II, with more scattered immunostaining in lamina I (Fig. 7). Labeling remains in
404 lateral parts of the DH, and mainly consists of thin axons and small varicosities.

405 *CTB-labeled afferents: widespread innervation in the spinal cord and caudal brainstem*

406 Numerous CTB-immunolabeled axons enter through all rootlets of C_2 and, to a lesser
407 extent, C_3, and divide in T in a relatively wide medio-lateral stretch of the spinal cord above
408 the DH, from Lissauer's tract to the lateral part of the dorsal column (DC). Fibers ascending
409 and descending in the tract give collaterals into the DH all along their course, more
410 prominently at segmental levels C_2 and C_3, but which nevertheless extend abundantly and
411 uninterruptedly between spinal segments C_4 and C_1. This lateral component of CTB-labeled
412 afferents generates a dense meshwork of preterminal and terminal axons and varicosities of
413 different sizes, with a predominance of small and medium-sized ones, in laminae III and IV of
414 the spinal DH. Also, a narrow band of immunoreactivity labels lamina I that extends further
415 caudally to segment C_6, and sparse fibers and varicosities appear scattered in laminae V and
416 VI (Fig. 8). At segmental levels of the DH where CTB- and IB4-immunolabeling coexist, the
417 inner one-half of lamina II displays a mixture of axons and terminals expressing either of the
418 two tracers, and others expressing both (Fig. 7).

419 CTB-labeled fibers coursing caudalwards in the lateral parts of the DC from C_2 soon
420 penetrate the DH to end as a few small terminal patches in laminae III-IV. No similar
421 afferents are seen originating from C_3 dorsal roots. In contrast, rostrally directed axons in the
422 DC are more abundant and distribute in a variety of terminal territories (Figs. 8, 9). These
423 axons can be followed from their origin in the division of primary afferents, advancing near
424 the medial surface of the upper cervical and caudal medullary DH with a slight dorsal shift
425 until reaching the lateral surface of the cuneate nucleus. They then proceed into the wedge-
426 shaped space that exists between the rostral and external cuneate nuclei and the medial edge
427 of the trigeminal tract, where nucleus x, the dorsolateral reticular nucleus, and the ventral part

428 of the inferior (spinal) vestibular nucleus have been defined previously (Bermejo, Jiménez,
429 Torres & Avendaño, 2003; Paxinos & Watson, 1986), and vanish in this region about 1.5 mm
430 rostral to the obex. Along their whole course adjacent to the DH small bundles of axons
431 descend and terminate at regular intervals in 10-12 restricted patches that apparently end in
432 the most medial part of lamina III. None of them seemingly invades the internal basilar
433 nucleus. At the level of the most rostral of these patches, about 1 mm caudal to the obex, a
434 few scattered varicose fibers descend further towards the central canal, and some cross the
435 midline dorsal to the canal within the commissural division of the nucleus of the solitary tract
436 to end soon at the base of the contralateral DH. Other scattered fibers appear in deeper
437 laminae (deep IV and V) from the caudal part of Sp5c through spinal segment C_3 probably
438 reaching, very sparsely, the central cervical nucleus and the dorsomedial part of the ventral
439 horn. It could not be decided which of these deep afferents derive from the fibers coursing
440 medially or laterally along the DH.

441 The fiber bundle that advances rostrally abutting the DCN is relatively compact and
442 rich in varicosities. These could innervate adjoining parts of the medial, rostral or external
443 divisions of the cuneate nucleus complex (Bermejo et al., 2003), but without sending obvious
444 offshoots into these nuclei. Near the rostral end of this bundle, a moderate number of fibers
445 turn laterally into the dorsal part of the trigeminal tract above the trigeminal nucleus Sp5i.
446 Some remain there distributing within the paratrigeminal nucleus (Pa5), but others collect into
447 a few small bundles that descend dropwise along the innermost surface of the trigeminal tract.
448 Fibers originating from these bundles enter the trigeminal nuclei to form patches of terminal
449 fields irregularly distributed along the dorsoventral extent of Sp5c and the caudal one-half of
450 Sp5i (Figs. 8, 9). No labeled fibers appear further rostrally, into the oral or principal divisions
451 of the trigeminal nuclei.

452 *Quantitative data on the central innervation of GON fibers*

453 On a superficial observation, it was noticed that thin axons and small varicosities were
454 dominant in lamina II and in the lateral portions of laminae III-IV in the DH and in Sp5i,
455 while they were less prominent among relatively more abundant thicker axons and larger
456 varicosities in the DCN, in the medial patches and at deeper levels of the DH, and in lamina I.
457 Also, both terminal and *en passant* varicosities were found in all territories (Fig. 10).

458 Although no attempt was made to quantitate the different types of labeled axons and
459 varicosities in this study, the overall volume of the innervated territories was estimated by
460 stereological means. It was found that the volume of IB4-labeled tissue, nearly all of it in
461 lamina II, covered 0.203±0.012 mm^3, which represents more than one-half of the overall
462 volume of the territories innervated by CTB-labeled fibers (0.393±0.036 mm^3). And within
463 the latter, more than three-quarters (0.303±0.022 mm^3) corresponded to laminae III-IV in the
464 DH (Fig. 11).

465 *Retrograde labeling*

466 In all cases, motoneurons appeared retrogradely labeled by CTB or FB (but not IB4) in
467 the medial division of the facial nucleus and in the ventral horn of spinal segments C_1-C_3.
468 Their cell bodies and dendrites were strongly labeled, and their axons were readily followed
469 along the central course of the facial nerve and the ventral roots. None or very few of the
470 labeled primary afferents were traced to the vicinity of these neurons, and their axons were
471 smooth and gave no collaterals that could be mistaken for primary afferents. This
472 motoneuronal labeling also occurred in the sham-injected animals and was due presumably to
473 contamination of local muscles or nerve twigs, not to the presence of skeletomotor fibers in
474 the GON (see Discussion).

475 3.4. The primary sensory neurons in C_2 and C_3

476 All morphological types of sensory neurons described in DRG of various mammalian
477 species were identified in the present material. A-type neurons are large, and contain small or
478 medium-sized clumps of Nissl substance, homogeneously distributed across the cytoplasm, or
479 more abundant in the central portions of the cytoplasm and sparser at the periphery. Their
480 nucleus is large and lightly stained, with a prominent and heavily stained nucleolus centrally
481 located. The B-type neurons are smaller and rather heterogeneous in shape and Nissl-based
482 cytological features. In general, they contain coarser clumps of Nissl substance that are
483 scantier or even missing altogether, in perinuclear regions of the cytoplasm. The aggregation
484 of Nissl clumps can adopt various forms, from a single peripheral rim to a relatively uniform
485 cytoplasmic distribution. In a small fraction of B-cells, the nuclei display more than one
486 nucleolus, and smaller intranuclear accessory bodies are frequently observed. C-type neurons

487 are very small, display little amount of Nissl substance sometimes forming local aggregates,
488 and often have more than one relatively indistinct nucleolus.

489 Applying these criteria, A-cells only represented about 15% of all neurons in C_2 and
490 C_3, B-cells stood out at 52%, and C-cells made up 33%. Although C_3 contained about 16%
491 fewer neurons (mostly involving small cells) than C_2 this difference did not reach statistical
492 significance, nor were there significant differences in neuron numbers between the right and
493 left ganglia (Table 2). Significant lateral differences, however, were detected in the somatic
494 volume of large neurons. A-cells were on average 16% larger in the right compared to the left
495 ganglia, a difference that increased to 22% when only C_2 was considered. Lateral differences
496 were not found for other cell types, nor for all neurons pooled (Fig. 12).

497 3.5. Light- and electron-microscopy of the GON

498 At the level of the superficial fascia, the GON exhibits an apparent width around 0.5-
499 1.0 mm. A large fraction of the nerve, however, consists of a thick and loose epineurium,
500 which hides a notably thinner nerve trunk, often divided into up to 4 separate fascicles, each
501 surrounded by a thin but dense layer of perineurium (Fig. 13). The absolute values obtained
502 from cross-sectional areal measurements of the GON are of little informative value, since its
503 fascicles, and fiber groups therein, often follow a somewhat winding course inside the
504 epineurium. In relative terms, however, it was found that myelinated fibers occupied a fairly
505 stable 61% of the nerve, while the interstitium (including as such the endo- and perineurium,
506 plus Schwann cell nuclei and cytoplasm excluding myelin, and other cell types) took a sizable
507 34% (Table 3).

508 Stereological estimates of axon numbers (Table 3) showed a notable variability among
509 the 11 GONs (5 on the left, 6 on the right) examined, with coefficients of variation ranging
510 between 18% for MAx on the right to 53% for MAx on the left, and around 30% for UAx on
511 either side. On average, MAx represented only 21% of all axons in the nerve. In several cases,
512 an additional 1-2% of myelinated profiles apparently corresponded to degenerating MAx. Up
513 to 20 UAx appeared in some cases ensheathed by a single non-myelinating Schwann cell;
514 more commonly, these Remak bundles contained 8-10 UAx, while glial profiles ensheathing
515 1-2 UAx were rarely found. The degree of myelination of MAx assessed by the g-ratio was
516 essentially identical on both sides, and correlated significantly with the size of MAx, the

517 thickest fibers exhibiting values twice as high as the thinnest (Fig. 14). Not a single MAx
518 displayed ChAT-immunolabeling, indicating that the GON does not have motor fibers at least
519 along its subcutaneous course. In contrast, about 20% UAx expressed TH-immunolabeling;
520 although not formally quantified this value represents a guesstimate based on the examination
521 of randomly sampled spots in adjacent ultrathin sections and immunoreacted semithin
522 sections of two GONs (Fig. 15).

523

524 4. DISCUSSION

525 The main goal of this study was to provide a comprehensive anatomical assessment of
526 the GON in the rat. The motivation for choosing this particular nerve was twofold: First, the
527 sensory innervation of the occipital region and posterior aspect of the neck was for a long time
528 an active area of research to understand the role of its extero- and proprioceptive contribution
529 to postural control and coordination of eye movements. Not surprisingly, the organization of
530 central projections from upper cervical spinal nerves was profusely investigated with every
531 anatomical or functional method that came to life along the last century (Bolton & Ray, 2000;
532 Escolar, 1948; Kerr, 1972; Kerr & Olafson, 1961; Neuhuber & Zenker, 1989; Pfaller &
533 Arvidsson, 1988; Yee & Corbin, 1939) . Yet, only in two studies, using transganglionic
534 transport of HRP, were the GON and/or its central projections specifically examined
535 (Neuhuber & Zenker, 1989; Scheurer et al., 1983). And second, as the interest in the upper
536 cervical afferents in that context seemed to wane, the GON got renewed attention after
537 evidence accumulated on its involvement in the pathophysiology of referred pain in some
538 chronic headache disorders (Magis & Schoenen, 2012; Rodrigo, Acin & Bermejo, 2017; Tang
539 et al., 2017). Clinical findings were supported by the demonstration in different species of
540 overlapping projections of trigeminal and cervical afferents in the upper cervical and caudal
541 spinal trigeminal nucleus (Bartsch & Goadsby, 2002; Goadsby & Bartsch, 2010; Pfaller &
542 Arvidsson, 1988; Xiong & Matsushita, 2000). However, given the paucity of new anatomical
543 information on the GON in the rat, the time seemed ripe for a reappraisal of its structure and
544 connections using more sensitive and precise tracing and quantitative studies. The present
545 study provides a new and detailed qualitative and quantitative description of ganglia C_2 and
546 C_3, as well as of the ultrastructure of the GON and its central projection pattern and

547 innervation territories in the spinal cord and brainstem. The use of two neuron-specific
548 tracers, CTB and IB4, has also made it possible to define subpopulations of the primary
549 sensory neurons that subserve the GON, and recognize regional and laminar particularities in
550 the central projections of putative myelinated and unmyelinated primary afferents coursing
551 through the GON.

552 4.1. Comments on the tracing and labeling methods

553 *The cervical ganglia contributing to GON*

554 It was consistently found that ganglion C_3 displayed only a small fraction of labeled
555 neurons after any type of tracer delivery to GON. Lacking a direct proof, it may be suggested
556 that those neurons sent their axons (or axon collaterals) to GON through the communicating
557 branch between the dorsal branches of the spinal nerves C_2 and C_3 (Fig. 2). In two cases
558 ganglion C_4 was extracted but showed no labeled neurons. Ganglion C_1 was not identified
559 during surgery, however, in two cases the thin dorsal root of the first cervical nerve was
560 examined histologically. Since no ganglion neurons were found, no further attempt was made
561 to dissect C_1. Ganglion C_1 is often absent in humans (reviewed in Campos et al, 2012), and
562 rats (Neuhuber & Zenker, 1989), although in this species it may be represented by a group of
563 scattered neurons in only a small percentage of cases (Pfaller & Arvidsson, 1988).

564 *Spurious labeling following delivery of tracers to GON*

565 In all cases that received intraneural injections of CTB labeled motoneurons were
566 found in the facial nucleus and the spinal ventral horn at C_1-C_3 levels. Such labeling also
567 occurred when, in the first cases injected, the area of injection was thoroughly rinsed with
568 saline, and the nerve was carefully wrapped in Parafilm® to prevent leakage of the tracer.
569 Moreover, similar retrograde labeling resulted when the GON was exposed in the same
570 manner but left undamaged, and the same amount of tracer was instilled and left in place for
571 the same time as during actual injections before rinsing and closing the wound. Since ChAT
572 immunostaining failed to label myelinated axons in the GON, it could be ruled out that the
573 GON contained motoneuron axons. It seemed, therefore, that some tracer was taken up by
574 local muscles or muscle nerve twigs that resulted damaged during surgery. The topographical
575 distribution of labeled motoneurons was consistent: in the facial nucleus they remained
576 confined within its medial division, which innervates dorsal and posterodorsal superficial

577 auricular muscles (Friauf & Herbert, 1985; Watson, Sakai & Armstrong, 1982); in the ventral
578 horn they were restricted to medial and central parts of this horn at caudal medullary and
579 upper cervical levels, presumably corresponding to motoneurons of the spinal accessory
580 nucleus innervating the clavotrapezius and sternocleidomastoid muscles (Kitamura & Sakai,
581 1982; Ullah, Mansor, Ismail, Kapitonova & Sirajudeen, 2007).

582 *On the completeness of GON fiber labeling*

583 The procedure used here to inject a CTB-IB4 mixture into a sensory nerve was
584 successfully tried before (Fernández-Montoya et al., 2017). However, in that study the target
585 nerve was smaller and consisted of a single fascicle, whereas the GON often appeared divided
586 into two or more fascicles (Fig. 12), which could raise a concern regarding the barrier
587 imposed by the perineurium, if intact, to tracer uptake. Although the trauma caused by the
588 injection is likely to induce a transitory general increase in perineurial permeability (Mizisin
589 & Weerasuriya, 2011), this issue was directly addressed by two procedures: First, by applying
590 FB to the transected GON, a method that is supposed to label all motoneurons and primary
591 sensory neurons that send their fibers through the target nerve (Negredo, Castro, Lago,
592 Navarro & Avendaño, 2004; Puigdellívol-Sánchez, Prats-Galino, Ruano-Gil & Molander,
593 2000). This procedure yielded 18% more labeled neurons in C2 and C3, suggesting that the
594 tracers CTB and IB4 administered by intraneural injection had access to most, but not all
595 fibers in the nerve. An additional factor that may play a role in this mismatch is that a small
596 subpopulation of peptidergic neurons fail to take up CTB or IB4 (Shehab and Hughes 2011).
597 The second method relied on counting the number of MAx in the GON, to assess whether it
598 matched the number of neurons labeled by CTB in the ganglia. Although axon numbers in the
599 GON varied greatly, and they were obtained from cases that lacked tracer injections, it was
600 comforting that about 1000 MAx were found on average in the right GON, a value slightly
601 below the number of neurons labeled by CTB in the ganglia, suggesting a successful uptake of
602 the tracers by all MAx.

603 It is more difficult to explain the notable mismatch between the number of UAx in the
604 GON (about 3700 on the right side) and the number of IB4-labeled neurons in the (right) C_2
605 and C_3 ganglia (about 1300). Although, as mentioned above, it is not possible to rule out that
606 some axons were not sufficiently exposed to the injected IB4, and that a subpopulation of

607 axons in the GON may not be able to take up the tracers, other explanations should be
608 considered. For example, it was proposed that central or peripheral axons originating from
609 ganglion cells bifurcate near the ganglion (Chung & Coggeshall, 1984; Langford &
610 Coggeshall, 1981). More recent studies disproved this suggestion for the spinal dorsal roots
611 (Tandrup, 1995) and the trigeminal root (L. Vazquez and C. Avendaño, Master Thesis, 2017,
612 unpublished). It remains possible, however, that such bifurcations may take place in
613 peripheral nerves farther away from the ganglion, at least for UAx. A second source of the
614 high number of UAx is the population of visceromotor autonomic axons that is known to exist
615 in peripheral nerves, and which after being removed by an extensive sympathectomy was
616 estimated to account for 1/3 of all UAx in the sciatic nerve and its main branches
617 (Schmalbruch, 1986). The proportion of putative autonomic axons found in the sciatic nerve
618 was only 19% using the approach of counting TH-immunoreactive profiles on semithin axons
619 (Castro et al., 2008). Although not quantified, the same procedure applied to the GON in the
620 present study revealed frequent TH-immunoreactive profiles coincident with UAx profiles.
621 These findings suggest that the numerical mismatch between the UAx in the GON and IB4-
622 labeled ganglion neurons could be partly explained by the presence of autonomic motor axons
623 in the nerve, and partly by an incomplete uptake of the tracer by UAx following intraneural
624 tracer injection.

625 ### 4.2. The central projections of GON as part of the spinal ganglia afferents

626 Our results show that GON afferents, including IB4- and CTB-labeled fibers,
627 distribute in central territories similar to, but more extensive than those described in previous
628 studies using more 'classical' techniques (reviewed in Neuhuber & Zenker, 1989; see also
629 Scheurer et al., 1983). These territories coincide to a large extent with many of the structures
630 innervated by fibers labeled by HRP, WGA-HRP or biotinylated dextran injected directly into
631 spinal ganglia C_2 and C_3 (Neuhuber & Zenker, 1989; Pfaller & Arvidsson, 1988; Zhan,
632 Pongstaporn & Ryugo, 2006), except for a lack of GON projections to vestibular, cochlear,
633 perihypoglossal, Sp5c, the central cervical, and Clarke's dorsal nuclei in thoracic segments,
634 which were however innervated to various degrees following C_2 and/or C_3 injections. It was
635 unsettled in previous reports whether the GON projected to the external cuneate nucleus, and
636 whether GON afferents in the spinal DH remained within the first four cervical segments, or
637 extended as far caudal as segments T_3-T_4. Our findings showed no more than a few terminal

638 fibers in the caudoventral region of the external cuneate, and failed to identify labeled fibers
639 in the spinal cord caudal to segment C_6.

640 4.3. The trigeminocervical complex (TCC) and the central projections of the GON

641 Primary afferents from neurons in the trigeminal ganglion project extensively to nuclei
642 outside the central targets of GON afferents, but trigeminal and GON projection domains are
643 not entirely segregated. Some of the trigeminal afferent territories in the spinal and medullary
644 DH (Abrahams et al., 1979; Kerr, 1972; Kerr & Olafson, 1961; Marfurt & Rajchert, 1991;
645 Sessle et al., 1986) match well with upper cervical terminal fields. This topographical
646 convergence of exteroceptive, proprioceptive and visceroceptive afferents that originate from
647 diverse head and neck regions prompted to examine the involvement of this region in
648 craniocervical pain disorders whose pathophysiology had escaped explanation for a long time.
649 The term 'trigeminocervical complex' (TCC, Hoskin, Zagami & Goadsby, 1999) was coined
650 to encompass the caudal Sp5c and the upper 2 cervical spinal segments, particularly their
651 laminae I-II, and the commissural division of the nucleus of the solitary tract and its caudal
652 extension into spinal lamina X, where the electrical stimulation of the middle meningeal
653 artery or the superior sagittal sinus induced up-regulation of c-Fos expression in cats and
654 monkeys (Goadsby & Hoskin, 1997; Kaube et al., 1993). Supratentorial dural stimuli in the
655 clinical setting are often accompanied by pain referred to the ophthalmic trigeminal territory
656 in the face, which could be explained by the proximity, although not identity, of sensory
657 neurons in the trigeminal ganglion that innervate the forehead and supratentorial dura
658 (O'Connor & Van der Kooy, 1986). However, putatively painful stimuli in the infratentorial
659 cranial fossa, upper cervical canal, or occipital or upper dorsal cervical structures may also
660 elicit referred pain in the forehead (reviewed in Bartsch & Goadsby, 2002; Bogduk, 2001),
661 suggesting that the TCC receives not only dural and neurovascular afferents from trigeminal-
662 innervated supratentorial region, but also those originating from upper cervical sensory
663 domains including vertebrae, joints, muscles, and internal carotid and vertebrobasilar arteries.

664 When our findings are compared with reports of trigeminal projections in the caudal
665 brainstem and cervical spinal cord, several conclusions emerge:

666 1, Overall, most GON projection territories also had variable amounts of trigeminal
667 afferents, except for the lateral DH, whose laminae I-III were heavily innervated by the GON

668 between segments C_1 and C_6, but were essentially free from trigeminal afferents below C_2
669 (Jacquin, Semba, Egger & Rhoades, 1983; Marfurt & Rajchert, 1991; Pfaller & Arvidsson,
670 1988).

671 2, Trigeminal afferents to the lateral part of C_1 and or C_2, where a heavy GON
672 projection distributes, originate from some, but not all, components of the ophthalmic
673 division. By transganglionic tracing, afferents have been shown mainly in laminae III-IV from
674 the frontal, supraorbital and anterior ethmoidal nerve (Panneton, Gan & Juric, 2006;
675 Panneton, Hsu & Gan, 2010; Shigenaga et al., 1986; Takemura, Sugimoto & Shigenaga,
676 1991), and c-Fos expression was induced in laminae I-II at the same spot by painful thermal
677 stimuli applied to the supraorbital region (Strassman & Vos, 1993). Afferents from the middle
678 meningeal artery also had terminals in laminae I-IV in a similar region of the DH (Liu,
679 Broman & Edvinsson, 2008). In addition, conjunctival, but not corneal, afferents distributed
680 in a slightly more medial position in laminae I-III of segment C1 (Panneton et al., 2010).

681 3, Afferents from the maxillary territory, including the whiskerpad, reached the rostral
682 portions of C_1, but their location in the DH was at an intermediate position (Fernández-
683 Montoya et al., 2017; Panneton et al., 2010; Shigenaga et al., 1986; Strassman & Vos, 1993),
684 dorsal and lateral to the main focus of CTB- and IB4-labeled terminals from the GON.

685 4, The discontinuous patches of GON afferents in the medial DH are coincident with
686 the central projections reported for the inferior alveolar and mylohyoid branches of the
687 mandibular nerve (Jacquin et al., 1983; Strassman & Vos, 1993). The auriculotemporal nerve,
688 another branch of the mandibular nerve whose cutaneous distribution is placed laterally and
689 rostrally to that of the GON, innervates in C_1 a patch (Jacquin et al., 1983) that lies
690 immediately lateral to the GON patches.

691 4.4. GON projections outside the TCC: overlap with other trigeminal and craniocervical
692 afferents.

693 GON afferents consistently distribute in three regions outside the TCC, which also
694 receive afferents from the trigeminal, intermediate, vagus and/or upper cervical nerves other
695 than GON. First, the ventrolateral zone of the rostral cuneate nucleus, which receives dense
696 CTB-labeled afferents from GON, also receives trigeminal innervation from the mandibular
697 nerve (Jacquin et al., 1983). This region is also known to receive neck muscle and cutaneous

698 afferents (Abrahams et al., 1979; Bolton & Tracey, 1992; Edney & Porter, 1986), which
699 likely arise from neurons in upper cervical ganglia (Imamura, Saunders & Keller, 1986;
700 Pfaller & Arvidsson, 1988), as well as fibers from periauricular structures innervated by the
701 intermediate nerve (Arvidsson & Thomander, 1984), the auricular branch of the vagus nerve
702 in cats (Nomura & Mizuno, 1984), and extraocular muscles in monkeys (Porter, 1986).
703 Second, a moderate amount of CTB-labeled fibers from GON innervates a slender marginal
704 band at the lateral surface of Sp5i, where trigeminal fibers end respecting a somatotopy
705 whereby caudal parts of the face are represented more laterally (Jacquin, Stennett, Renehan &
706 Rhoades, 1988; Jacquin et al., 1986), and where most of the nociceptive afferents in Sp5i
707 preferentially distribute (Hayashi, Sumino & Sessle, 1984). Moreover, afferents from the
708 palpebral and bulbar conjunctiva, but not the cornea, are known to terminate ventrolaterally in
709 Sp5i in rabbits (Van Ham & Yeo, 1996) and rats (Panneton et al., 2010). It is noteworthy that
710 extraocular muscle afferents (Buisseret-Delmas & Buisseret, 1990; Porter & Donaldson,
711 1991) and afferents from the territory of innervation of the auricular branch of the vagus
712 nerve (Arvidsson & Thomander, 1984; Ichiyama et al., 1997; Nomura & Mizuno, 1984),
713 similar to those projecting to the rostral cuneate, also innervate the marginal part of Sp5i.

714 The third structure outside TCC where GON and trigeminal afferents converge is Pa5.
715 This is the major interstitial component within the spinal trigeminal tract just above the caudal
716 half of Sp5i (Phelan & Falls, 1989), which is heavily innervated by trigeminal afferents,
717 particularly from its mandibular division (Jacquin et al., 1983; Jacquin et al., 1988; Marfurt &
718 Rajchert, 1991; Shigenaga et al., 1986). In Pa5 a multitude of nociceptive and visceroceptive
719 afferents converge that are involved in the modulation of cardiovascular and nocifensive
720 responses to widely diverse stimuli: being a target for glossopharyngeal afferents, probably
721 arising from its jugular ganglion, Pa5 plays a role in the baroreceptor reflex arch (Yu &
722 Lindsey, 2003), and relays to the diencephalon sensory signals from the upper respiratory
723 tract (Driessen, Farrell, Mazzone & McGovern, 2015; McGovern et al., 2015). In addition,
724 Pa5 responds to nociceptive stimuli from other disparate somatic and visceral territories,
725 including the sciatic and orofacial regions, esophagus and urinary bladder, putatively
726 mediating not only sensations but also cardiovascular responses that accompany
727 somatosensory-evoked reflexes (Alioto, Lindsey, Koepp & Caous, 2008; Arvidsson &
728 Thomander, 1984; Bon, Lanteri-Minet & Menetrey, 1997; Caous, Koepp, Couture, Balan &

729 Lindsey, 2008; Imbe & Ren, 1999; McGovern et al., 2015; Suwanprathes, Ngu, Ing, Hunt &
730 Seow, 2003; Yu & Lindsey, 2003).

731 4.5. The GON as a target for therapeutic interventions: the value of a rat model

732 Clinical phenomena of spread and referral of pain are common in primary painful
733 craniofacial disorders. Although the pathophysiology of these phenomena is not entirely clear
734 yet, it is widely accepted that nociceptive input-induced sensitization has to occur in second-
735 order neurons on which primary afferents from separate territories converge (Arendt-Nielsen,
736 2015; Basbaum, Bautista, Scherrer & Julius, 2009; Woolf, 1991). This mechanism has been
737 proposed to underlie the origin of pain in neurovascular headaches, in particular migraine and
738 trigeminal autonomic cephalalgias such as cluster headache (Bartsch & Goadsby, 2003;
739 Goadsby, 2006; Goadsby et al., 2017; Jacobs & Dussor, 2016). Once the sensitization is
740 established, sensory stimuli on specific targets (trigger points) would spark pain in the
741 territories whose innervation converges upon the sensitized neurons. As described above,
742 some neural regions in the TCC and neighboring structures receive convergent afferents from
743 the GON and various cutaneous, muscular, dural, and visceral trigeminal sources. Using the
744 rat as a model Peter Goadsby and his colleagues were the first to demonstrate direct functional
745 coupling of nociceptive supratentorial dural afferents and cervical afferents conveyed through
746 the GON on upper cervical DH neurons. These neurons, located in laminae IV-VI and to a
747 lesser extent in laminae I-II, mainly corresponded to the wide dynamic range type and showed
748 sensitization in their responses to dural stimulation following GON electrical stimulation
749 (Bartsch & Goadsby, 2002). Conversely, dural stimulation increased the responses of these
750 neurons to GON electrical stimulation, and lowered their response thresholds and expanded
751 their receptive fields upon innocuous mechanical testing in the occipito-cervical region and
752 the ophthalmic and caudal parts of the maxillary and mandibular territories (Bartsch &
753 Goadsby, 2003).

754 These findings gave experimental support to the clinical practice of pharmacologically
755 blocking the GON, which for many years was used to treat various headaches (reviewed in
756 Young, 2010), and which has received renewed attention more recently as a proven therapy
757 for refractory migraine and cluster headache (Cuadrado et al., 2017; Gul, Ozon, Karadas, Koc
758 & Inan, 2017; Tang et al., 2017). Moreover, after a number of open-label clinical trials

759 reported beneficial effects of GON subcutaneous stimulation for various painful craniofacial
760 conditions, a randomized, double-blind and controlled study (Saper et al., 2010) showed that
761 GON stimulation provided significant improvement to medically intractable chronic migraine
762 cases, including some who did not respond to GON block. Unsurprisingly, GON stimulation
763 has been increasingly used along the last decade to treat refractory migraine and trigeminal
764 autonomic cephalalgias (Dodick et al., 2015; Lambru et al., 2014; Lambru, Shanahan,
765 Watkins & Matharu, 2014; Miller, Watkins & Matharu, 2016; Miller, Watkins & Matharu,
766 2017; Rodrigo et al., 2017).

767 The surge in clinical interest in GON neuromodulation propelled a search for the
768 mechanisms involved, an endeavor in which the use of rat craniofacial pain models is
769 invaluable. So far, only a few acute and short-term studies are available in rats, that indicate
770 that GON stimulation induces lasting elevations of mechanical (but not thermal) thresholds in
771 the forepaw and periorbital region (De La Cruz et al., 2015), and decreases spontaneous
772 activity and responsivity to receptive field stimulation of neurons in laminae II-IV neurons of
773 the medullary DH that receive converging input from the supra- and/or infraorbital skin, the
774 supratentorial dura, and the GON (Lyubashina et al., 2017). A neuromodulatory effect of
775 GON stimulation has also been reported on thalamic neurons upstream the trigeminal
776 somatosensory pathways, which become sensitized in a migraine model, but reduce their
777 spontaneous and facial and forepaw mechanical stimulation-evoked activity during GON
778 stimulation (Walling et al., 2017).

779 In sum, the GON in the rat is a relatively easily accessible sensory nerve that may be a
780 key target for examining a variety of mechanisms of the physiology of craniocervical
781 nociception, as well as the pathophysiology of severe chronic painful disorders strongly
782 linked to human clinical conditions. Present findings may be valuable for a better
783 understanding of the neural bases of sensory convergence on spinomedullary systems, which
784 could help refine and advance available experimental approaches in this field.

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786 **REFERENCES**

787

788 Abrahams V. C., Anstee G., Richmond F. J. & Rose P. K. (1979). Neck muscle and trigeminal input to
789 the upper cervical cord and lower medulla of the cat. *Canadian Journal of Physiology and*
790 *Pharmacology 57(6)*, currently 642-651.

791 Alioto O. E., Lindsey C. J., Koepp J. & Caous C. A. (2008). Sensory sciatic nerve afferent inputs to
792 the dorsal lateral medulla in the rat. *Autonomic Neuroscience Basic and Clinical 140 (1-2)*, 80-87.
793 Arendt-Nielsen L. (2015). Central sensitization in humans: assessment and pharmacology. *Handb Exp*
794 *Pharmacol 227:79-102. doi: 10.1007/978-3-662-46450-2_5*.
795 Arvidsson J., Thomander L. (1984). An HRP study of the central course of sensory intermediate and
796 vagal fibers in peripheral facial nerve branches in the cat. *Journal of Comparative Neurology 223*
797 *(1)*, 35-45.
798 Avendaño C., Machin R., Bermejo P. E. & Lagares A. (2005). Neuron numbers in the sensory
799 trigeminal nuclei of the rat: A GABA- and glycine-immunocytochemical and stereological
800 analysis. *Journal of Comparative Neurology 493 (4)*, 538-553.
801 Bartsch T., Goadsby P. J. (2002). Stimulation of the greater occipital nerve induces increased central
802 excitability of dural afferent input. *Brain 125 (7)*, 1496-1509.
803 Bartsch T., Goadsby P. J. (2003). Increased responses in trigeminocervical nociceptive neurons to
804 cervical input after stimulation of the dura mater. *Brain 126 (8)*, 1801-1813.
805 Basbaum A. I., Bautista D. M., Scherrer G. & Julius D. (2009). Cellular and molecular mechanisms of
806 pain. *Cell 139 (2)*, 267-284.
807 Bermejo P. E., Jiménez C. E., Torres C. V. & Avendaño C. (2003). Quantitative stereological
808 evaluation of the gracile and cuneate nuclei and their projection neurons in the rat. *Journal of*
809 *Comparative Neurology 463 (4)*, 419-433.
810 Bogduk N. (2001). Cervicogenic headache: anatomic basis and pathophysiologic mechanisms.
811 *Current Pain and Headache Reports 5 (4)*, 382-386.
812 Bolton P. S., Ray C. A. (2000). Neck afferent involvement in cardiovascular control during
813 movement. *Brain Research Bulletin 53 (1)*, 45-49.
814 Bolton P. S., Tracey D. J. (1992). Neurons in the dorsal column nuclei of the rat respond to stimulation
815 of neck mechanoreceptors and project to the thalamus. *Brain Research 595 (1)*, 175-179.
816 Bon K., Lanteri-Minet M. & Menetrey D. (1997). Involvement of the dorsal paratrigeminal nucleus in
817 visceral pain-related phenomena. *Comptes Rendus de l'Académie des Sciences - Series III 320 (7)*,
818 533-539.
819 Brunelli, G., Spano, P., Barlati, S., Guarneri, B., Barbon, A., Bresciani, R., & Pizzi, M. (2005).
820 Glutamatergic reinnervation through peripheral nerve graft dictates assembly of glutamatergic
821 synapses at rat skeletal muscle. *Proceedings of the National Academy of Sciences 102(24)*, 8752-
822 8757.
823 Buisseret-Delmas C., Buisseret P. (1990). Central projections of extraocular muscle afferents in cat.
824 *Neuroscience Letters 109 (1-2)*, 48-53.
825 Campos D., Goerck M. L., Ellwanger J. H., Corbellini R. O., Hoelscher R. H., Souza M. S. & Rieger
826 A. (2012). Anatomy of the first spinal nerve - a review. *Journal of Morphological Sciences 29*
827 *(2)*, 65-68.
828 Caous C. A., Koepp J., Couture R., Balan A. C. & Lindsey C. J. (2008). The role of the paratrigeminal
829 nucleus in the pressor response to sciatic nerve stimulation in the rat. *Autonomic Neuroscience*
830 *Basic and Clinical 140 (1-2)*, 72-79.
831 Castro J., Negredo P. & Avendaño C. (2008). Fiber composition of the rat sciatic nerve and its
832 modification during regeneration through a sieve electrode. *Brain Research 1190*, 65-77.

833 Chen Y. F., Bramley G., Unwin G., Hanu-Cernat D., Dretzke J., Moore D., … & Lilford R. (2015).
834 Occipital nerve stimulation for chronic migraine-a systematic review and meta-analysis. *PLoS*
835 *ONE 10 (3)*, e0116786.
836 Chomiak T., Hu B. (2009). What is the optimal value of the g-ratio for myelinated fibers in the rat
837 CNS? A theoretical approach. *PLoS ONE 4 (11)*, e7754.
838 Chung K., Coggeshall R. E. (1984). The ratio of dorsal root ganglion cells to dorsal root axons in
839 sacral segments of the cat. *Journal of Comparative Neurology 225 (1)*, 24-30.
840 Cruz-Orive L. M. (2004). Precision of the fractionator from Cavalieri designs. *Journal of Microscopy*
841 *213 (2)*, 205-211.
842 Cuadrado M. L., Aledo-Serrano A., Navarro P., Lopez-Ruiz P., Fernandez-de-Las-Penas C.,
843 Gonzalez-Suarez I., … & Fernandez-Perez C. (2017). Short-term effects of greater occipital nerve
844 blocks in chronic migraine: A double-blind, randomised, placebo-controlled clinical trial.
845 *Cephalalgia 37 (9)*, 864-872.
846 De La Cruz P., Gee L., Walling I., Morris B., Chen N., Kumar V., … & Pilitsis J. G. (2015).
847 Treatment of Allodynia by Occipital Nerve Stimulation in Chronic Migraine Rodent.
848 *Neurosurgery 77 (3)*, 479-485.
849 Dodick D. W., Silberstein S. D., Reed K. L., Deer T. R., Slavin K. V., Huh B., … & Mekhail N.
850 (2015). Safety and efficacy of peripheral nerve stimulation of the occipital nerves for the
851 management of chronic migraine: long-term results from a randomized, multicenter, double-
852 blinded, controlled study. *Cephalalgia 35 (4)*, 344-358.
853 Driessen A. K., Farrell M. J., Mazzone S. B. & McGovern A. E. (2015). The Role of the
854 Paratrigeminal Nucleus in Vagal Afferent Evoked Respiratory Reflexes: A Neuroanatomical and
855 Functional Study in Guinea Pigs. *Front Physiol 6:378. doi: 10.3389/fphys.2015.00378.*
856 Edney D. P., Porter J. D. (1986). Neck muscle afferent projections to the brainstem of the monkey:
857 implications for the neural control of gaze. *Journal of Comparative Neurology 250 (3)*, 389-398.
858 Escolar J. (1948). The afferent connections of the 1st, 2nd, and 3rd cervical nerves in the cat; an
859 analysis by Marchi and Rasdolsky methods. *Journal of Comparative Neurology 89 (2)*, 79-92.
860 Fernández-Montoya J., Martin Y. B., Negredo P. & Avendano C. (2017). Changes in the axon
861 terminals of primary afferents from a single vibrissa in the rat trigeminal nuclei after active touch
862 deprivation or exposure to an enriched environment. *Brain Structure and Function 223 (1), 47-61*
863 *doi: 10.1007/s00429-017-1472-5*, 1-15.
864 Friauf E., Herbert H. (1985). Topographic organization of facial motoneurons to individual pinna
865 muscles in rat (Rattus rattus) and bat (Rousettus aegyptiacus). *Journal of Comparative Neurology*
866 *240 (2)*, 161-170.
867 Gautron, L., Lee, C., Funahashi, H., Friedman, J., Lee, S., & Elmquist, J. (2010). Melanocortin-4
868 receptor expression in a vago-vagal circuitry involved in postprandial functions. *Journal of*
869 *Comparative Neurology 518(1)*, 6-24.
870 Goadsby P. J. (2006). In: S. B. McMahon & M. Koltzenburg (Eds.), *Wall and Melzack's Textbook of*
871 *Pain*. (5 ed., pp. 851-874). Elsevier / Churchill Livingstone.
872 Goadsby P. J., Bartsch T. (2010). In: C. Fernández de las Peñas, L. Arendt-Nielsen & R. D. Gerwin
873 (Eds.), *Tension-type and Cervicogenic Headache: Pathophysiology, Diagnosis and Treatment*.
874 pp. 109-116). Sudbury, MA: Jones and Barlett.

875 Goadsby P. J., Holland P. R., Martins-Oliveira M., Hoffmann J., Schankin C. & Akerman S. (2017).
876 Pathophysiology of Migraine: A Disorder of Sensory Processing. *Physiological Reviews 97 (2)*,
877 553-622.
878 Goadsby P. J., Hoskin K. L. (1997). The distribution of trigeminovascular afferents in the nonhuman
879 primate brain Macaca nemestrina: a c-fos immunocytochemical study. *Journal of Anatomy 190*
880 *(3)*, 367-375.
881 Gul H. L., Ozon A. O., Karadas O., Koc G. & Inan L. E. (2017). The efficacy of greater occipital
882 nerve blockade in chronic migraine: A placebo-controlled study. *Acta Neurologica Scandinavica*
883 *136 (2)*, 138-144.
884 Gundersen H. J. (1986). Stereology of arbitrary particles. A review of unbiased number and size
885 estimators and the presentation of some new ones, in memory of William R. Thompson. *Journal*
886 *of Microscopy 143 (1)*, 3-45.
887 Gundersen H. J. G., Bendtsen T. F., Korbo L., Marcussen N., Møller A., Nielsen K., … & West M. J.
888 (1988). Some new, simple and efficient stereological methods and their use in pathological
889 research and diagnosis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica 96 (1-*
890 *6)*, 379-394.
891 Hayashi H., Sumino R. & Sessle B. J. (1984). Functional organization of trigeminal subnucleus
892 interpolaris: nociceptive and innocuous afferent inputs, projections to thalamus, cerebellum, and
893 spinal cord, and descending modulation from periaqueductal gray. *Journal of Neurophysiology 51*
894 *(5)*, 890-905.
895 Hoskin K. L., Zagami A. S. & Goadsby P. J. (1999). Stimulation of the middle meningeal artery leads
896 to Fos expression in the trigeminocervical nucleus: a comparative study of monkey and cat.
897 *Journal of Anatomy 194 (4)*, 579-588.
898 Howard C. V., Reed M. G. (1998). *Unbiased Stereology. Three-dimensional Measurement in*
899 *Microscopy*. Oxford: BIOS Scientific Publishers.
900 Ichiyama M., Itoh M., Miki T., Xie Q., Kaneto T. & Takeuchi Y. (1997). Central distribution of
901 sensory fibers in the facial nerve: an anatomical and immunohistochemical study. *Okajimas Folia*
902 *Anat Jpn 74 (1)*, 53-63.
903 Imamura J., Saunders M. C. & Keller J. T. (1986). Projections of cervical nerves to the rat medulla.
904 *Neuroscience Letters 70 (1)*, 46-51.
905 Imbe H., Ren K. (1999). Orofacial deep and cutaneous tissue inflammation differentially upregulates
906 preprodynorphin mRNA in the trigeminal and paratrigeminal nuclei of the rat. *Molecular Brain*
907 *Research 67 (1)*, 87-97.
908 Inan L. E., Inan N., Karadas O., Gul H. L., Erdemoglu A. K., Turkel Y. & Akyol A. (2015). Greater
909 occipital nerve blockade for the treatment of chronic migraine: a randomized, multicenter,
910 double-blind, and placebo-controlled study. *Acta Neurologica Scandinavica 132 (4)*, 270-277.
911 Jacobs B., Dussor G. (2016). Neurovascular contributions to migraine: Moving beyond vasodilation.
912 *Neuroscience 338:130-144. doi: 10.1016/j.neuroscience.2016.06.012.*
913 Jacquin M. F., Semba K., Egger M. D. & Rhoades R. W. (1983). Organization of HRP-labeled
914 trigeminal mandibular primary afferent neurons in the rat. *Journal of Comparative Neurology 215*
915 *(4)*, 397-420.
916 Jacquin M. F., Stennett R. A., Renehan W. E. & Rhoades R. W. (1988). Structure-function
917 relationships in the rat brainstem subnucleus interpolaris: II. Low and high threshold trigeminal
918 primary afferents. *Journal of Comparative Neurology 267 (1)*, 107-130.

919 Jacquin M. F., Woerner D., Szczepanik A. M., Riecker V., Mooney R. D. & Rhoades R. W. (1986).
920 Structure-function relationships in rat brainstem subnucleus interpolaris. I. Vibrissa primary
921 afferents. *Journal of Comparative Neurology 243 (2)*, 266-279.
922 Kaneyama, T., & Shirasaki, R. (2018). Post-crossing segment of dI1 commissural axons forms
923 collateral branches to motor neurons in the developing spinal cord. *Journal of Comparative*
924 *Neurology 526(12)*, 1943-1961.
925 Kaube H., Keay K. A., Hoskin K. L., Bandler R. & Goadsby P. J. (1993). Expression of c-Fos-like
926 immunoreactivity in the caudal medulla and upper cervical spinal cord following stimulation of
927 the superior sagittal sinus in the cat. *Brain Research 629 (1)*, 95-102.
928 Kemp W. J., III, Tubbs R. S. & Cohen-Gadol A. A. (2011). The innervation of the scalp: A
929 comprehensive review including anatomy, pathology, and neurosurgical correlates. *Surgical*
930 *Neurology International 2:178. doi: 10.4103/2152-7806.90699.*
931 Kerr F. W. (1972). Central relationships of trigeminal and cervical primary afferents in the spinal cord
932 and medulla. *Brain Research 43 (2)*, 561-572.
933 Kerr F. W. L., Olafson R. A. (1961). Trigeminal and cervical volleys. Convergence on single units in
934 the spinal gray at C-1 and C-2. *Archives of Neurology 5 (2)*, 171-178.
935 Kim, Y. S., Paik, S. K., Cho, Y. S., Shin, H. S., Bae, J. Y., Moritani, M., ... & Moon, C. (2008).
936 Expression of P2X3 receptor in the trigeminal sensory nuclei of the rat. *Journal of Comparative*
937 *Neurology 506(4)*, 627-639
938 Kitamura S., Sakai A. (1982). A study on the localization of the sternocleidomastoid and trapezius
939 motoneurons in the rat by means of the HRP method. *Anatomical Record 202 (4)*, 527-536.
940 Lambru G., Abu B. N., Stahlhut L., McCulloch S., Miller S., Shanahan P. & Matharu M. S. (2014).
941 Greater occipital nerve blocks in chronic cluster headache: a prospective open-label study.
942 *European Journal of Neurology 21 (2)*, 338-343.
943 Lambru G., Shanahan P., Watkins L. & Matharu M. S. (2014). Occipital nerve stimulation in the
944 treatment of medically intractable SUNCT and SUNA. *Pain Physician 17*, 29-41.
945 Langford L. A., Coggeshall R. E. (1981). Branching of sensory axons in the peripheral nerve of the
946 rat. *Journal of Comparative Neurology 203 (4)*, 745-750.
947 Larsen J. O. (1998). Stereology of nerve cross sections. *Journal of Neuroscience Methods 85 (1)*, 107-
948 118.
949 Lawson S. N. (2005). In: P. J. Dyck & P. K. Thomas (Eds.), *Peripheral Neuropathy*. pp. 163-202).
950 New York: W.B. Saunders; Elsevier Inc..
951 Lieberman A. R. (1976). In: D. N. Landon (Ed.), *The Peripheral Nerve*. pp. 188-278). London:
952 Chapman & Hall.
953 Liu, Y., Broman, J. & Edvinsson, L. (2008). Central projections of the sensory innervation of the rat
954 middle meningeal artery. *Brain Research 1208*:103-110. *doi: 10.1016/j.brainres.2008.02.078*.
955 Lyubashina O. A., Panteleev S. S. & Sokolov A. Y. (2017). Inhibitory effect of high-frequency greater
956 occipital nerve electrical stimulation on trigeminovascular nociceptive processing in rats. *Journal*
957 *of Neural Transmission 124 (2)*, 171-183.
958 Magis D., Schoenen J. (2012). Advances and challenges in neurostimulation for headaches. *Lancet*
959 *Neurology 11 (8)*, 708-719.
960 Marfurt C. F., Rajchert D. M. (1991). Trigeminal primary afferent projections to "non-trigeminal"
961 areas of the rat central nervous system. *Journal of Comparative Neurology 303 (3)*, 489-511.

962 McGovern A. E., Driessen A. K., Simmons D. G., Powell J., Davis-Poynter N., Farrell M. J. &
963 Mazzone S. B. (2015). Distinct brainstem and forebrain circuits receiving tracheal sensory neuron
964 inputs revealed using a novel conditional anterograde transsynaptic viral tracing system. *Journal*
965 *of Neuroscience 35 (18)*, 7041-7055.
966 Miller S., Watkins L. & Matharu M. (2016). Long-term outcomes of occipital nerve stimulation for
967 chronic migraine: a cohort of 53 patients. *J Headache Pain 17 (1)*, 68 *doi: 10.1186/s10194-016-*
968 *0659-0 -0659.*
969 Miller S., Watkins L. & Matharu M. (2017). Treatment of intractable chronic cluster headache by
970 occipital nerve stimulation: a cohort of 51 patients. *European Journal of Neurology 24 (2)*, 381-
971 390.
972 Mizisin A. P., Weerasuriya A. (2011). Homeostatic regulation of the endoneurial microenvironment
973 during development, aging and in response to trauma, disease and toxic insult. *Acta*
974 *Neuropathologica 121 (3)*, 291-312.
975 Murray L. M., Gillingwater T. H. & Parson S. H. (2010). Using mouse cranial muscles to investigate
976 neuromuscular pathology in vivo. *Neuromuscular Disorders 20 (11)*, 740-743.
977 Negredo P., Castro J., Lago N., Navarro X. & Avendaño C. (2004). Differential growth of axons from
978 sensory and motor neurons through a regenerative electrode: a stereological, retrograde tracer,
979 and functional study in the rat. *Neuroscience 128 (3)*, 605-615.
980 Neuhuber W. L., Zenker W. (1989). Central distribution of cervical primary afferents in the rat, with
981 emphasis on proprioceptive projections to vestibular, perihypoglossal, and upper thoracic spinal
982 nuclei. *Journal of Comparative Neurology 280 (2)*, 231-253.
983 Nomura S., Mizuno N. (1984). Central distribution of primary afferent fibers in the Arnold's nerve (the
984 auricular branch of the vagus nerve): a transganglionic HRP study in the cat. *Brain Research 292*
985 *(2)*, 199-205.
986 O'Connor T. P., Van der Kooy D. (1986). Pattern of intracranial and extracranial projections of
987 trigeminal ganglion cells. *Journal of Neuroscience 6 (8)*, 2200-2207.
988 Panneton W. M., Gan Q. & Juric R. (2006). Brainstem projections from recipient zones of the anterior
989 ethmoidal nerve in the medullary dorsal horn. *Neuroscience 141 (2)*, 889-906.
990 Panneton W. M., Hsu H. & Gan Q. (2010). Distinct central representations for sensory fibers
991 innervating either the conjunctiva or cornea of the rat. *Experimental Eye Research 90 (3)*, 388-
992 396.
993 Paxinos G., Watson C. (1986). *The Rat Brain in Stereotaxic Coordinates*. 2 ed. Centrecourt: Academic
994 Press Australia.
995 Pfaller K., Arvidsson J. (1988). Central distribution of trigeminal and upper cervical primary afferents
996 in the rat studied by anterograde transport of horseradish peroxidase conjugated to wheat germ
997 agglutinin. *Journal of Comparative Neurology 268 (1)*, 91-108.
998 Phelan K. D., Falls W. M. (1989). The interstitial system of the spinal trigeminal tract in the rat:
999 anatomical evidence for morphological and functional heterogeneity. *Somatosensory and Motor*
1000 *Research 6 (4)*, 367-399.
1001 Piovesan E. J., Kowacs P. A., Tatsui C. E., Lange M. C., Ribas L. C. & Werneck L. C. (2001).
1002 Referred pain after painful stimulation of the greater occipital nerve in humans: evidence of
1003 convergence of cervical afferences on trigeminal nuclei. *Cephalalgia 21 (2)*, 107-109.
1004 Porter J. D. (1986). Brainstem terminations of extraocular muscle primary afferent neurons in the
1005 monkey. *Journal of Comparative Neurology 247 (2)*, 133-143.

1006 Porter J. D., Donaldson I. M. (1991). The anatomical substrate for cat extraocular muscle
1007 proprioception. *Neuroscience 43 (2-3)*, 473-481.
1008 Puigdellívol-Sánchez A., Prats-Galino A., Ruano-Gil D. & Molander C. (2000). Fast Blue and
1009 Diamidino Yellow as retrograde tracers in peripheral nerves: efficacy of combined nerve injection
1010 and capsule application to transected nerves in the adult rat. *Journal of Neuroscience Methods 95*
1011 *(2)*, 103-110.
1012 Rambourg A., Clermont Y. & Beaudet A. (1983). Ultrastructural features of six types of neurons in rat
1013 dorsal root ganglia. *Journal of Neurocytology 12 (1)*, 47-66.
1014 Rodrigo D., Acin P. & Bermejo P. (2017). Occipital Nerve Stimulation for Refractory Chronic
1015 Migraine: Results of a Long-Term Prospective Study. *Pain Physician 20 (1)*, 151-159.
1016 Saper J. R., Dodick D. W., Silberstein S. D., McCarville S., Sun M. & Goadsby P. J. (2010). Occipital
1017 nerve stimulation for the treatment of intractable chronic migraine headache: ONSTIM feasibility
1018 study. *Cephalalgia 31 (3)*, 271-285.
1019 Scheurer S., Gottschall J. & Groh V. (1983). Afferent projections of the rat major occipital nerve
1020 studied by transganglionic transport of HRP. *Anatomy and Embryology (Berlin) 167 (3)*, 425-438.
1021 Schmalbruch H. (1986). Fiber composition of the rat sciatic nerve. *Anatomical Record 215 (1)*, 71-81.
1022 Sessle B. J., Hu J. W., Amano N. & Zhong G. (1986). Convergence of cutaneous, tooth pulp, visceral,
1023 neck and muscle afferents onto nociceptive and non-nociceptive neurones in trigeminal
1024 subnucleus caudalis (medullary dorsal horn) and its implications for referred pain. *Pain 27 (2)*,
1025 219-235.
1026 Shehab, S. A. S. (2009). Acute and chronic sectioning of fifth lumbar spinal nerve has equivalent
1027 effects on the primary afferents of sciatic nerve in rat spinal cord. *Journal of Comparative*
1028 *Neurology 517(4)*, 481-492
1029 Shehab, S. A., & Hughes, D. I. (2011). Simultaneous identification of unmyelinated and myelinated
1030 primary somatic afferents by co-injection of isolectin B4 and Cholera toxin subunit B into the
1031 sciatic nerve of the rat. Journal of neuroscience methods, 198(2), 213-221.
1032 Shehab, S., Anwer, M., Galani, D., Abdulkarim, A., Al-Nuaimi, K., Al-Baloushi, A., ... &
1033 Ljubisavljevic, M. (2015). Anatomical evidence that the uninjured adjacent L4 nerve plays a
1034 significant role in the development of peripheral neuropathic pain after L5 spinal nerve ligation in
1035 rats. *Journal of Comparative Neurology 523(12)*, 1731-1747.
1036 Shigenaga Y., Chen I. C., Suemune S., Nishimori T., Nasution I. D., Yoshida A., Sato H., Okamoto
1037 T., Sera M. & Hosoi M. (1986). Oral and facial representation within the medullary and upper
1038 cervical dorsal horns in the cat. *Journal of Comparative Neurology 243 (3)*, 388-408.
1039 Strassman A. M. & Vos B. P. (1993). Somatotopic and laminar organization of fos-like
1040 immunoreactivity in the medullary and upper cervical dorsal horn induced by noxious facial
1041 stimulation in the rat. *Journal of Comparative Neurology 331 (4),* 495-516.
1042 Suwanprathes P., Ngu M., Ing A., Hunt G. & Seow F. (2003). c-Fos immunoreactivity in the brain
1043 after esophageal acid stimulation. *American Journal of Medicine 115 (3), Suppl 3A:31S-38S.*,
1044 31S-38S.
1045 Takemura M., Sugimoto T. & Shigenaga Y. (1991). Difference in central projection of primary
1046 afferents innervating facial and intraoral structures in the rat. *Experimental Neurology 111 (3)*,
1047 324-331.

Tandrup T. (1995). Are the neurons in the dorsal root ganglion pseudounipolar? A comparison of the number of neurons and number of myelinated and unmyelinated fibres in the dorsal root. *Journal of Comparative Neurology 357 (3)*, 341-347.

Tandrup T., Gundersen H. J. G. & Jensen E. B. V. (1997). The optical rotator. *Journal of Microscopy 186 (2)*, 108-120.

Tang Y., Kang J., Zhang Y. & Zhang X. (2017). Influence of greater occipital nerve block on pain severity in migraine patients: A systematic review and meta-analysis. *American Journal of Emergency Medicine 35 (11)*, 1750-1754.

Ullah M., Mansor O., Ismail Z. I., Kapitonova M. Y. & Sirajudeen K. N. (2007). Localization of the spinal nucleus of accessory nerve in rat: a horseradish peroxidase study. *Journal of Anatomy 210 (4)*, 428-438.

Van Ham J. J., Yeo C. H. (1996). The central distribution of primary afferents from the external eyelids, conjunctiva, and cornea in the rabbit, studied using WGA-HRP and B-HRP as transganglionic tracers. *Experimental Neurology 142 (2)*, 217-225.

Walling I., Smith H., Gee L. E., Kaszuba B., Chockalingam A., Barborica A., … & Pilitsis J. G. (2017). Occipital Nerve Stimulation Attenuates Neuronal Firing Response to Mechanical Stimuli in the Ventral Posteromedial Thalamus of a Rodent Model of Chronic Migraine. *Neurosurgery 81 (4)*, 696-701.

Watson C. R., Sakai S. & Armstrong W. (1982). Organization of the facial nucleus in the rat. *Brain, Behavior and Evolution 20 (1-2)*, 19-28.

West, M. J., Slomianka, L., and Gundersen, H. J. G. (1991). Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anatomical Record 231 (4)*, 482-497.

Woolf C. J. (1991). Generation of acute pain: central mechanisms. *British Medical Bulletin 47 (3)*, 523-533.

Xiong G., Matsushita M. (2000). Upper cervical afferents to the motor trigeminal nucleus and the subnucleus oralis of the spinal trigeminal nucleus in the rat: an anterograde and retrograde tracing study. *Neuroscience Letters 286 (2)*, 127-130.

Yee J., Corbin K. B. (1939). The intramedullary course of the upper five, cervical, dorsal root fibers in the rabbit. *The Journal of Comparative Neurology 70 (2)*, 297-304.

Young W. B. (2010). Blocking the greater occipital nerve: utility in headache management. *Current Pain and Headache Reports 14 (5)*, 404-408.

Yu Y. G., Lindsey C. J. (2003). Baroreceptor-sensitive neurons in the rat paratrigeminal nucleus. *Autonomic Neuroscience Basic and Clinical 105 (1)*, 25-34.

Zhan X., Pongstaporn T. & Ryugo D. K. (2006). Projections of the second cervical dorsal root ganglion to the cochlear nucleus in rats. *Journal of Comparative Neurology 496 (3)*, 335-348.

1086 **TABLE 1**

1087 List of antibodies used in this study

Antibodies	Immunogen	Manufacturer	Dilution
Anti- Griffonia Simplicifolia Lectin I	Lectin I purified from Griffonia (Bandeiraea) Simplicifolia	Vector Laboratories goat polyclonal, Cat no. AS2104 RRID: AB_2314660	1µg/ml
Anti-Cholera Toxin	Nondenatured CTB isolated from Vibria cholerae	Sigma-Aldrich, rabbit polyclonal, Cat no. C3062, RRID: AB_258833	1:500
Rabbit Anti-Tyrosine Hydroxylase	Denatured TH from rat pheochromocytoma	Merk, rabbit polyclonal, Cat no. AB152, RRID: AB_390204	1:100
Goat Anti-Choline Acetyltransferase	Human placental enzyme.	Merck, goat polyclonal, Cat no. AB144P RRID:AB_2079751	1:50
AlexaFluor488 Donkey Anti-Goat	Gamma Immunoglobins Heavy and Light chains	ThermoFisher, goat polyclonal, Cat no. A11055, RRID:AB_2534102	1:200
AlexaFluor647 Donkey Anti-Rabbit	Gamma Immunoglobin	ThermoFisher, rabbit polyclonal, Cat no. A10040, RRID:AB_2534016	1:200
Biotinylated Rabbit Anti-Goat IgG	Purified goat IgG	Vector Laboratories, rabbit polyclonal, Cat no. BA5000, RRID:AB_2336126	1:250
Anti-Rabbit IgG Biotin Conjugate	Purified rabbit IgG	Sigma Aldrich, goat polyclonal, Cat no. B7389, RRID:AB_258613	1:500

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1090 **TABLE 2**

1091 Neuron number (**N**; mean±sd) and cell body volume (**V**; mean±sem) in the left (**L**, n=6) and right (**R**,
1092 n=8) DRG C_2 and C_3. †p=0.030 and *p=0.028 (R vs. L; Wilcoxon paired test)

		N ($x10^{-3}$)				V ($\mu m^3 x10^{-3}$)			
Ganglion	Side	A	B	C	All	A	B	C	All
C_2	L	1.49±0.6	6.16±3.1	3.08±0.9	10.72±4.3	25.58±1.4	7.82±0.3	2.57±0.1	8.99±0.4
	R	1.25±0.6	4.16±0.8	3.55±1.4	8.97±2.4	31.14±1.8†	7.43±0.2	2.59±0.1	9.00±0.6
	L+R	2.87±0.7	10.67±3.2	7.30±1.8	20.83±5.4				
C_3	L	1.59±0.4	4.44±0.9	3.12±0.9	9.16±2.1	25.09±1.9	7.23±0.3	2.45±0.1	8.94±0.4
	R	1.35±0.3	4.28±1.2	3.40±1.1	9.03±1.9	27.30±0.8	7.62±0.3	2.61±0.1	9.24±0.6
	L+R	2.87±0.3	8.40±2.1	6.25±1.6	17.51±3.7				
C_2+C_3	L	3.04±0.7	10.96±2.8	6.29±1.1	20.29±3.8	24.94±0.6	7.41±0.1	2.55±0.03	8.94±0.1
	R	2.69±0.7	8.65±1.4	6.91±1.4	18.25±2.6	28.91±0.3*	7.61±0.01	2.54±0.02	8.90±0.2

1093

1094 **TABLE 3**

1095 Estimates of morphometric parameters in the rat left (L,
1096 n=5) and right (R, n=6) GONs: **N**, number of myelinated
1097 (MAx) and unmyelinated axons (UAx); **g-ratio**, the ratio
1098 between the diameters of the axon and the nerve fiber
1099 obtained from the area measurements of each myelinated
1100 fiber**;** $\mathbf{A_{Int}}$, percentage of the nerve occupied by interstitium
1101 (including endo- and perineurium, Schwann and other cells;
1102 excluding epineurium and large blood vessels). $\mathbf{A_{Mf}}$,
1103 percentage of the nerve occupied by myelinated fibers. CV,
1104 coefficient of variation.

	L			R		
	Mean	sd	CV	Mean	sd	CV
N(Max)	825	441	0.53	975	173	0.18
N(UAx)	2913	958	0.33	3688	998	0.27
g-ratio (%)	47.2	2.4	0.05	47.5	4.0	0.08
A_{Mf} (%)	61.9	4.0	0.06	60.2	5.2	0.09
A_{Int} (%)	34.2	3.1	0.09	34.9	5.2	0.15

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1107 **Figure 1.** Illustration of a dorsal view of occipital and neck regions of the rat, with the
1108 skin removed to show the superficial muscles and the course of the GON and 3d occipital
1109 nerve. Only the muscles readily identifiable are depicted. On the left, the superficial fascia is
1110 removed; on the right the most superficial muscles are also partially removed to show more
1111 clearly the course of the nerves at deeper levels. Exposed nerves are drawn in solid white;
1112 their muscle-covered courses are marked as gray traces. 3dON, third occipital nerve; AS, m.
1113 auricularis superior; AT, m. acromiotrapezius; CT, m. clavotrapezius; FA, m. frontalis, pars
1114 auricularis; GON, greater occipital nerve; LAL, m. levator auris longus; SC, m. splenius
1115 capitis; T, m. temporalis.

1116 **Figure 2.** Sketch of the dorsal roots, ganglia and spinal nerves C_2 and C_3, the
1117 communicating branches between adjacent spinal nerves and the distribution of the GON and
1118 3dON. The loaded pipette points to the location of the tracer deposits in the GON before it
1119 divides into its two main terminal branches.

1120 **Figure 3.** Example of a tracer injection in the GON. **A:** Exposure of the nerve during
1121 surgery. **B:** Intraneural injection of a mixture of CTB-IB4, with traces of the supravital dye
1122 Light Green to facilitate the visualization of the injection; the photo was taken immediately
1123 after the injection. *Scale bar* 1 mm.

1124 **Figure 4.** Examples of immunofluorescent neurons in sections of the right DRG C_2 (**A**)
1125 and C_3 (**B**). Magenta, green and blue channels are used to identify CTB-, IB4- and
1126 Bisbenzimide-labeled cell bodies and nuclei, respectively. Double-labeled neuronal perikarya
1127 appear in C_2 in the merged image (panel on the right; see also Fig. 5). *Scale bars* 100 µm.
1128 Histograms show the estimations of the total number (mean+sd) of single- and double-labeled
1129 neurons in each ganglion.

1130 **Figure 5.** High-power view of CTB- (magenta) and/or IB4-labeled (green) neurons in C_2.
1131 While most CTB-labeled neurons are large or medium-sized, some are definitely small (**B** and
1132 **C**, thin arrows). Likewise, most IB4-labeled cells are small-to-medium sized, but a few
1133 examples occur of strongly labeled fairly large neurons (**A**, thick arrow). Most double labeled
1134 neurons belong within the small or medium-size range (**C**, arrowheads). *Scale bar* 50 µm.

1135 **Figure 6.** Low-power confocal images of C_2 (**A**) and C_3 (**B**) ganglia sections displaying
1136 maximum retrograde labeling after a FB deposit in GON. *Scale bar* 200 μm. The inset shows
1137 the stereological estimate of total number (mean+sd) of FB-labeled neurons in these ganglia.

1138 **Figure 7.** **A:** Low-power view of IB4-immunofluorescent terminal field in lamina II
1139 under confocal microscopy on a horizontal section of the upper cervical spinal cord. *Arrows*
1140 point to needle holes that were made midway between the insertion on the spinal cord of
1141 dorsal roots C_2-C_3 and C_3-C_4 before extracting the tissue after perfusion to serve as fiducial
1142 marks. **B**: Higher-power detail of the nearly complementary laminar terminal pattern of CTB-
1143 (magenta) and IB4-immunofluorescent (green) central projections of the GON in the DH of
1144 the spinal cord. While IB4-labeled fibers and varicosities are largely restricted to the outer
1145 two-thirds of lamina II, CTB-labeled profiles are present in lamina I and lamina III-IV, and
1146 also appears in the deeper part of lamina II, intermingling to some extent with IB4 labeling.
1147 Where there is topographical overlap, also a fraction of the terminals show co-localization of
1148 both tracers (not shown). Short vertical lines (top right corner) mark laminar boundaries, from
1149 the pial surface (right) to laminae II-III border (left). *Scale bars* 500 μm (A) and 100 μm (B).

1150 **Figure 8.** Distribution of CTB-immunolabeled afferent projections from the GON in the
1151 cervical spinal cord and caudal brainstem. **A:** Series of eight DAB-reacted horizontal sections
1152 arranged dorso-ventrally ('a' through 'h', from left to right), with a 240 μm separation
1153 between adjacent sections. Sections are aligned rostro-caudally to preserve relative locations
1154 of labeling at different depths. *Arrowhead* in section 'a' marks the approximate location of the
1155 obex, and thick arrows in section 'd' point to the center of C_2 and C_3 dorsal roots, as indicated
1156 by the nearby reference needle holes (see Fig. 7). Contrast and brightness were adjusted to
1157 enhance visualization. **B:** Close up pictures of the labeled territories numbered in A (*long*
1158 *arrows*): 1 and 6, uneven projections at dorsal and ventral parts of the outermost region of
1159 Sp5i; 2 and 3, dense labeling in laminae III and IV of the DH, that extends from segmental
1160 level C_1, where spinal and medullary DH merge, to segment C_6; 4, separate patches of dense
1161 terminal fields that rostrally directed GON afferents leave medially in upper cervical and
1162 medullary DH; 5, in contrast, caudal divisions of GON afferents entering through C_2 dorsal
1163 root (top) leave a few small and discrete dense terminal patches more laterally in the DH.
1164 *Short arrows* in A indicate the dense bundle of labeled fibers placed at more dorsal and

1165 rostral levels ('a'), strongly labeled C_2 rootlets ('b'), and a few labeled fibers in the solitary
1166 complex crossing to the contralateral side ('d'). Sp5c, caudal trigeminal nucleus; Sp5i,
1167 interpolar trigeminal nucleus; Cu, cuneate nucleus; DC, dorsal column; Gr, gracile nucleus;
1168 LC, lateral column. *Scale bars* 1 mm (A) and 50 µm (B).

1169 **Figure 9:** Distribution of CTB- (magenta) and IB4-labeled (blue) GON afferents in
1170 spinal cord and brainstem territories in one case cut on the coronal plane. Labeled fibers and
1171 boutons in selected sections at the indicated levels were digitally enhanced and superimposed
1172 on attenuated greyscale images of adjacent Nissl-stained sections. Relevant nuclear or laminar
1173 boundaries are outlined. Anatomical terms follow Paxinos & Watson (1986) and Bermejo et
1174 al (2003): Bi, nucleus Bischoff; C_{1-4}, spinal segmental levels; CuE, external cuneate nucleus;
1175 CuM, cuneate nucleus, medial division; CuR, cuneate nucleus, rostral division; Gr, gracile
1176 nucleus; NBi, internal basilar nucleus; Pa5, paratrigeminal nucleus; Sol, solitary nucleus;
1177 Sp5cc, caudal trigeminal nucleus (caudal level); Sp5cr, caudal trigeminal nucleus (rostral
1178 level); Sp5i, interpolar trigeminal nucleus; x, nucleus x; z, nucleus z; I-V, dorsal horn or Sp5c
1179 laminae; XII, hypoglossal nucleus. *Scale bar* 500 µm.

1180 **Figure 10:** Examples of different types of CTB-labeled axons and varicosities in six
1181 central territories innervated from the GON. **A:** Large and medium-sized varicosities
1182 predominate in the terminal patches in medial lamina III of the spinal DH (Fig. 8c). **B:** In
1183 contrast, small- and medium-sized varicosities dominate in the rostral Cu (Fig. 8a). **C:** Small,
1184 mainly *en passant* varicosities with scattered very large ones appear in the paratrigeminal
1185 nucleus. **D:** Isolated varicose axons that aggregate in occasional islands in lamina V of the
1186 spinal cord. **E:** Dense terminal field with heterogeneous varicosities of a wide range of sizes
1187 in lamina III of the spinal DH (Fig. 8d). **F:** Long axons thickly populated by *en passant*
1188 varicosities, most of them small, in lamina I of the spinal cord (Fig. 8d). Not uncommonly,
1189 varicosities accumulate in dense shapes that appear to delineate the typical flattened and
1190 elongated profiles of neurons in this lamina (arrowhead). *Scale bar* 10 µm.

1191 **Figure 11:** Volume of central structures innervated by GON primary afferents in millions
1192 of cubic micra (mean+sem). In "Sp5i" it is included the territory of innervation in the
1193 paratrigeminal nucleus. "DCN" includes terminals in the rostral and external cuneate nuclei
1194 and less well defined adjacent cell groups, such as nucleus x or the dorsolateral reticular

1195 nucleus. And “DH-deep” refers to all labeled fibers and varicosities in or deeper to inner part
1196 of lamina IV.

1197 **Figure 12:** **A:** Distribution of perikaryal volumes of all neuron types in ganglia C_2 and C_3
1198 pooled, plotted as frequencies over a log-transformed size scale (n = 10 rats). No statistical
1199 differences exist between right and left sides. **B:** Differences in cell body size exist, however,
1200 between the large, A-type cells in the left and right ganglia. As shown in the inset, the volume
1201 of A-type cells is significantly higher on the right side with respect to the left (p=0.028,
1202 Wilcoxon signed-rank test).

1203 **Figure 13:** **A:** Toluidine-stained semithin cross-sections of the GON in three cases that
1204 illustrate the variety of sizes and fascicle composition of this nerve at the level of the tracer
1205 injection. The extensive epineurium has been trimmed on the edges for better visualization of
1206 the fascicles. **B:** Typical ultrastructure of the GON, showing large and small myelinated
1207 fibers, and two Remak bundles (asterisks) with 7 and 13 unmyelinated axons, respectively.
1208 *Scale bars* 100 µm (A) and 1 µm (B).

1209 **Figure 14:** As an index of myelination, the g-ratio correlates highly significantly with the
1210 size of the myelinated axons (MAx) in the GON, fitting an exponential regression, with the
1211 finely MAx tending to have lower values of g-ratio. Values represent mean±sem for each
1212 axon cross-sectional size (on a log scale).

1213 **Figure 15:** Two examples of electron micrographs of selected spots in transverse ultrathin
1214 sections of the GON (**A**, **B**) followed by adjacent corresponding semithin sections
1215 immunoreacted for TH or ChAT. Arrows point to small groups of TH-positive inmunolabeled
1216 UAx. Not a single MAx profile displayed ChAT inmunolabeling. *Scale bar* 2 µm.