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

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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 C2 ganglion neurons. Unmyelinated afferents from GON innervates exclusively laminae I-II of the lateral DH, mostly extending along segments C2-3. Myelinated fibers distribute mainly in laminae I and III-V of the lateral DH between C1 and C6 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; C2, 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,
gracile nucleus; IB4, isolectin IB4 from Griffonia simplicifolia; LAL, levator auris longus;
LC, lateral column; MAx, myelinated axons; NBi, internal basilar nucleus; Pa5,
paratrigeminal nucleus; SC, m. splenius capitis; Sol, solitary nucleus; Sp5c, caudal trigeminal
nucleus; Sp5cc, caudal trigeminal nucleus (caudal level); Sp5cr, caudal trigeminal nucleus
(rostral level); Sp5i, interpolar trigeminal nucleus; T, m. temporalis; TCC, trigeminocervical
complex; TH, tyrosine hydroxylase; UAx, unmyelinated axons; x, nucleus x; XII, hypoglossal
nucleus; z, nucleus z.

1. INTRODUCTION

The Greater Occipital Nerve (GON) is the major component of the second spinal
nerve (C2). It forms the medial division of the posterior branch of C2, which emerges after this
branch sends communicating branches to C1 and C3 and a few twigs to deep nuchal muscles.
In humans, the GON pierces the muscles supraspinalis capitis (often) and trapezius
(sometimes), as well as the deep fascia, to supply sensory innervation to a wide occipital part
of the scalp (reviewed in Kemp, Tubbs & Cohen-Gadol, 2011). The anesthetic block of the
GON has been used for decades to alleviate some types of primary headaches, and the interest
in its anatomy has not ceased to grow in parallel to the development of more refined
diagnostic and therapeutic manipulations of the GON to deal with a variety of craniofacial
pain disorders (reviewed in Chen et al., 2015; Dodick et al., 2015; Inan et al., 2015; Tang,
Kang, Zhang & Zhang, 2017; Young, 2010).

An intriguing feature of some primary headaches is their extensive location from the
face to occipital regions, a distribution that spans innervation territories of the first and second
trigeminal branches and the upper cervical spinal nerves. Likewise, artificial or pathological
stimulation of occipital or nuchal structures, or the infratentorial dura, often evoke referred
pain in frontal regions (Goadsby & Bartsch, 2010; Piovesan et al., 2001). Among the various
mechanisms proposed to explain referred cephalic pain, a convergence of afferents from
different territories and submodalities on central neurons has been favored, following early
reports of electrophysiological and anatomical convergence on upper cervical or medullary
dorsal horn (DH) in cats (Abrahams, Anstee, Richmond & Rose, 1979; Kaube, Keay, Hoskin,
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
surrounding muscular and connective tissue.

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

In order to examine the possibility of local contamination by the tracers injected, in a second group of cases (Group 2, n=2) the GON was exposed in the same manner but left undamaged, and the same amount of CTB-IB4 was instilled and left in place for 30 min before rinsing and closing the wound. Another group of animals (Group 3, n=3) received an intraneural deposit in the GON of a 5% suspension of Fast Blue (FB, Sigma-Aldrich) in DH2O. The nerve was exposed and transected, and the proximal stump was introduced in a small glass or plastic vial containing the tracer. After 1 hour, the vial was removed, the region was rinsed and the skin sutured.

2.3. Histological processing of tracer-injected cases

Animals from Groups 1 and 2 (CTB/IB4 applications) survived 4 days. They were euthanized by an intraperitoneal injection of sodium pentobarbital (Dolethal, 50 mg/kg) and perfused through the heart with 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). Ganglia C2 and C3 and the cervical spinal cord and caudal two-thirds of the brainstem were dissected out, postfixed in the same fixative overnight at 4°C and cryoprotected with a 30% sucrose solution in 0.1M PB for 2 days. The ganglia were frozen and serially cut at 15 µm in a Leica CM1950 cryostat (Leica Biosystems, Nussloch, GmbH). Every second section was incubated overnight at 4°C with a combination of two primary antibodies (Table 1), goat anti-IB4 and rabbit anti-CTB. After several washes with saline PB (PBS), the sections were incubated for 2 h in the dark in a mixture of secondary antibodies: donkey anti-goat AlexaFluor 488 and donkey anti-rabbit AlexaFluor 647. Nuclei were labeled with
Bisbenzimide. The blocks including cervical spinal cord and brainstem were frozen cut in horizontal (n=13) or coronal (n=2) sections of 40 μm in a sliding microtome (Leica SM2400, Leica Biosystems, Nussloch). In two cases of Group 1, alternate sections were processed free-floating using the same immunofluorescence protocol. The remaining cases were processed free-floating using the avidin–biotin–peroxidase method (ABC), with diaminobenzidine (DAB) as a chromogen as previously described (Fernández-Montoya et al., 2017). Briefly, after endogenous peroxidase inactivation and preincubation in a blocking solution containing 2% Triton X-100, alternate series were incubated with either goat anti-IB4 (1:1000; Vector Laboratories) or rabbit anti-CTB (1:500; Sigma Aldrich) overnight at 4ºC under agitation. After several washes, sections were then incubated for two hours at room temperature, with the rabbit anti-goat (1:250; Vector Laboratories) or goat anti-rabbit (1:500; Sigma Aldrich) biotinylated secondary antibodies corresponding to each series. Finally, both series were incubated in an avidin-biotin (Kit ABC Elite®, Vector laboratories) solution in PBS 0.02M with 2% Triton X-100, and revealed in 0.05% DAB in 0.1M PB with 0.008% cobalt chloride and 0.0064% nickel sulfamate, adding 0.001% H2O2. In the two animals cut on the coronal plane, a series of alternate sections were stained with 0.1% cresyl violet. All sections were mounted on subbed glass slides, dehydrated, defatted and coverslipped with DePeX.

Animals from Group 2 (FB deposits in GON) survived 6 days and then were deeply anesthetized, perfused and had their C2 and C3 ganglia and a spinal cord-brainstem block extracted as in Group 1. The ganglia were cryoprotected, frozen and serially cut at 20 μm in a cryostat; the spinal cord-brainstem block was frozen cut in horizontal sections of 40 μm in a sliding microtome. After mounting and drying in the dark, sections were quickly dehydrated in ethanol, defatted in xylene and coverslipped with DePeX.

2.4. Antibody characterization

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

The rabbit polyclonal anti-Cholera Toxin antibody consists of two subunits, A (27 kDa) and B (12 kDa). This antiserum showed no binding to Staphylococcus enterotoxins A or B, or Pseudomonas exotoxin, but it bound specifically to cholera toxin, a widely used axonal tracer (manufacturer’s datasheet). In several studies, the specificity was demonstrated by the
presence of immunolabeling in specific locations on the ipsilateral side of the spinal cord and lack of immunolabeling on the contralateral side (reviewed in Shehab et al., 2015). This antibody was used with Alexa Fluor 647 donkey anti-rabbit secondary antibody.

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

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

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

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

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

2.5. Methacrylate embedding of C2 and C3 dorsal root ganglia

Animals in Group 4 (n=10) were deeply anesthetized and perfused with 4% paraformaldehyde-0.1% glutaraldehyde in 0.1M PB and. In eight of these rats, ganglia C2 and C3 were collected bilaterally and postfixed in fresh-buffered 4% paraformaldehyde for 2-3 days. They were then dehydrated in graded ethanol solutions and infiltrated in
glycolmethacrylate (Technovit 7100 Kulzer, Wehrheim, Germany) for 3 days. Both ganglia
from each side and case were rolled free and embedded in one block, which was exhaustively
sectioned in a rotary microtome (Microm HM 350 S, Thermo-Fisher, Walldorf, Germany) at
40 μm. All sections were serially mounted on glass slides and Nissl-stained with 0.1% cresyl
violet.

2.6. Treatment of GONs for electron microscopy and immunostaining

In eight animals of Group 4, a 2 mm piece of the GON was excised, uni- or bilaterally,
and postfixed for 2 days in a buffered 2% paraformaldehyde and 2% glutaraldehyde mixture.
The nerves were osmicated (1% OsO4 in 0.1M PB), dehydrated and embedded in Durcupan
resin (Fluka AG, Buchs, Switzerland). Consecutive semithin and ultrathin sections were
obtained using a Leica Ultracut UCT ultramicrotome (Leica Microsystems AG, Wetzlar,
Germany) and a diamond knife (Drukker International B.V., Cuijk, The Netherlands).
Semithin sections (0.75-1 μm) were stained with 0.5% toluidine blue (Merck). Ultrathin
sections (60-70 nm) were collected on 200-mesh copper grids and stained with uranyl acetate
and lead citrate.

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

2.7. Microscopy

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

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

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

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

2.8. Stereology

*Immunofluorescent neuron numbers in DRG*

The total number of labeled neurons with CTB and IB4 in the C2 and C3 ganglia was estimated using the physical fractionator method (Gundersen, 1986; Howard & Reed, 1998). One in four sections was examined and photographed in the confocal microscope using separately the red and green channels and with both channels merged. A series of pairs of 0.5 µm-thick pictures were taken with a 5 µm intrapair separation in the z-axis, covering completely the sampled section. Each section was systematically sampled off-line with
unbiased frames overlaid on the images with the help of CorelDraw software. Somatic profiles of immunofluorescent neuronal perikarya were used as counting units. The labeled profiles fulfilling the counting criteria were quantified in both directions of the disector separately for CTB- and IB4-labeled, and cells co-localizing both tracers. Final sampling fraction \( F_T \) was 1:24, and 120 neurons were counted on average \( \Sigma Q^- \) on about 70 disector fields in \( C_2 \) and \( C_3 \) per side. Total estimates were computed as \( \hat{N} = \Sigma Q^- \cdot F_T^{-1} \). A similar procedure was performed for cases receiving the tracer FB.

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

**Number and somatic volume of neurons in \( C_2 \) and \( C_3 \) ganglia**

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

Average final sampling fraction was either 1:70 or 1:140 –both strategies yielding essentially the same results. Number estimation was computed as for the physical fractionator (see above). Section thickness measurements, cell typing and perikaryal volume estimations were performed with an oil-immersion 100x lens (1.40 NA, UPlanSApo, Olympus) at a 3900x final magnification on screen. Neuron types were identified according to well established morphological criteria (Lawson, 2005; Lieberman, 1976; Rambourg, Clermont &
Beaudet, 1983). The few (less than 5%) cells that could not be clearly identified as A- or B-type were split in half and each half was ascribed to either A3 or B1 subtypes. The average precision of the stereological procedure among individual cases was 10.9% (range 7.3-14.2%).

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

The volume occupied by labeled axons and their varicosities was estimated by point counting, applying the Cavalieri principle (Gundersen et al., 1988) on 40 μm-thick IB4- or CTB-immunolabeled sections at fixed intervals (d = 160 μm), as previously reported (Fernández-Montoya et al., 2017). With the integrated stereological equipment described in the previous section, and using a planopochromatic 20x dry objective (0.75 NA, Olympus UPLSAPO), two quadratic lattices of points of different density were superimposed on the immunolabeled regions. Dorsal horn laminae III-IV, displaying the densest and most extensive innervation, was sampled with low intensity (a(p) = 4127 μm²); for the remaining territories a denser grid (a(p) = 1337 μm²) was used. All points displaying at least one axonal swelling within a radius of 4 μm, at any depth of the section, were included in the count. Labeling with IB4 turned out to be more diffuse, characterized by thinner axons and varicosities of small size, and its delineation was less precise in peripheral areas and areas with little labeling. To help identifying the innervated structures, separate cases with Nissl- and cytochrome oxidase-stained horizontal sections were prepared.

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

**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 C2 is joined by communicating branches with C3 and, often, C1, 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
splenius capitis before piercing the superficial fascia and the anterior belly of the levator auris longus (LAL, Fig. 1). Along its course, the nerve gives off a variable number of small branches that apparently penetrate local muscles. In its most superficial course, the GON curves rostrolaterally and divides into a variable number of branches that distribute on the occipital region and near the medial base of the pinna. Along this course it is possible that the GON or some of its branches also pierce thin muscles that are not readily seen during surgery, such as the abductor auricularis longus or the interscutularis (Murray, Gillingwater & Parson, 2010). After piercing the LAL, and before turning rostralwards and dividing into terminal branches, the GON is easily accessible for at least 2 mm, and this was the locus chosen to carry out the tracer injections or deposits and the ultrastructural study of the nerve (Fig. 2).

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

The success of the intraneural injections of CTB-IB4 was ascertained by several lines of evidence, which pointed to a consistent and substantial uptake of the tracers by GON axons: 1, all cases showed topographically similar patterns of distribution of primary afferents in the spinal cord and brainstem. 2, the number of ganglion cell bodies that were immunofluorescent for CTB, IB4 or both tracers did not differ much across cases (coefficient of variation below 10%; Figs. 4, 5). 3, however, those numbers were significantly lower than the number of FB-labeled neurons in ganglia C2 and C3 after exposing the proximal stump of the transected GON to the tracer (Fig. 6), for reasons that will be explained in the Discussion. And 4, axon counts in the GON backed these results, as will also be discussed below.

Labeled neurons were much more abundant in C2 (2016±209, CTB+IB4; 2453±133, FB) than in C3 (184±52, CTB+IB4; 256±32, FB). About 20% of neurons in C2, and 6% in C3 co-expressed CTB and IB4. As expected, CTB labeled predominantly large or medium-sized neurons, while IB4 mainly appeared in small and some medium-sized neurons. However, there were occasional instances of large IB4-labeled neurons, and, more frequently, small CTB-labeled neurons. Most double-labeled neurons were small or medium-sized (Fig. 5).

3.3. Central distribution of primary afferents from the GON

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

Both in tissue reacted with DAB, and in immunofluorescent material, IB4-labeled axons enter the spinal cord through the dorsal roots of C2 (very few were found in C3). In the
posterolateral (Lissauer’s) tract the axons divide into short ascending and longer descending fibers that give collateral or terminal branches that enter the lateral part of the DH from the rostralmost levels of spinal segment C₄ to the caudalmost levels of segment C₁. No IB4-labeling appears elsewhere in the spinal cord or the caudal brainstem. The innervated territory is consistently expressed as a dense band of labeling restricted to the outer two-thirds of lamina II, with more scattered immunostaining in lamina I (Fig. 7). Labeling remains in lateral parts of the DH, and mainly consists of thin axons and small varicosities.

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

Numerous CTB-immunolabeled axons enter through all rootlets of C₂ and, to a lesser extent, C₃, and divide in T in a relatively wide medio-lateral stretch of the spinal cord above the DH, from Lissauer’s tract to the lateral part of the dorsal column (DC). Fibers ascending and descending in the tract give collaterals into the DH all along their course, more prominently at segmental levels C₂ and C₃, but which nevertheless extend abundantly and uninterruptedly between spinal segments C₄ and C₁. This lateral component of CTB-labeled afferents generates a dense meshwork of preterminal and terminal axons and varicosities of different sizes, with a predominance of small and medium-sized ones, in laminae III and IV of the spinal DH. Also, a narrow band of immunoreactivity labels lamina I that extends further caudally to segment C₆, and sparse fibers and varicosities appear scattered in laminae V and VI (Fig. 8). At segmental levels of the DH where CTB- and IB4-immunolabeling coexist, the inner one-half of lamina II displays a mixture of axons and terminals expressing either of the two tracers, and others expressing both (Fig. 7).

CTB-labeled fibers coursing caudalwards in the lateral parts of the DC from C₂ soon penetrate the DH to end as a few small terminal patches in laminae III-IV. No similar afferents are seen originating from C₃ dorsal roots. In contrast, rostrally directed axons in the DC are more abundant and distribute in a variety of terminal territories (Figs. 8, 9). These axons can be followed from their origin in the division of primary afferents, advancing near the medial surface of the upper cervical and caudal medullary DH with a slight dorsal shift until reaching the lateral surface of the cuneate nucleus. They then proceed into the wedge-shaped space that exists between the rostral and external cuneate nuclei and the medial edge of the trigeminal tract, where nucleus x, the dorsolateral reticular nucleus, and the ventral part
of the inferior (spinal) vestibular nucleus have been defined previously (Bermejo, Jiménez, Torres & Avendaño, 2003; Paxinos & Watson, 1986), and vanish in this region about 1.5 mm rostral to the obex. Along their whole course adjacent to the DH small bundles of axons descend and terminate at regular intervals in 10-12 restricted patches that apparently end in the most medial part of lamina III. None of them seemingly invades the internal basilar nucleus. At the level of the most rostral of these patches, about 1 mm caudal to the obex, a few scattered varicose fibers descend further towards the central canal, and some cross the midline dorsal to the canal within the commissural division of the nucleus of the solitary tract to end soon at the base of the contralateral DH. Other scattered fibers appear in deeper laminae (deep IV and V) from the caudal part of Sp5c through spinal segment C3 probably reaching, very sparsely, the central cervical nucleus and the dorsomedial part of the ventral horn. It could not be decided which of these deep afferents derive from the fibers coursing medially or laterally along the DH.

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

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

On a superficial observation, it was noticed that thin axons and small varicosities were dominant in lamina II and in the lateral portions of laminae III-IV in the DH and in Sp5i, while they were less prominent among relatively more abundant thicker axons and larger varicosities in the DCN, in the medial patches and at deeper levels of the DH, and in lamina I. Also, both terminal and *en passant* varicosities were found in all territories (Fig. 10).
Although no attempt was made to quantitate the different types of labeled axons and varicosities in this study, the overall volume of the innervated territories was estimated by stereological means. It was found that the volume of IB4-labeled tissue, nearly all of it in lamina II, covered 0.203±0.012 mm$^3$, which represents more than one-half of the overall volume of the territories innervated by CTB-labeled fibers (0.393±0.036 mm$^3$). And within the latter, more than three-quarters (0.303±0.022 mm$^3$) corresponded to laminae III-IV in the DH (Fig. 11).

**Retrograde labeling**

In all cases, motoneurons appeared retrogradely labeled by CTB or FB (but not IB4) in the medial division of the facial nucleus and in the ventral horn of spinal segments C1-C3. Their cell bodies and dendrites were strongly labeled, and their axons were readily followed along the central course of the facial nerve and the ventral roots. None or very few of the labeled primary afferents were traced to the vicinity of these neurons, and their axons were smooth and gave no collaterals that could be mistaken for primary afferents. This motoneuronal labeling also occurred in the sham-injected animals and was due presumably to contamination of local muscles or nerve twigs, not to the presence of skeletomotor fibers in the GON (see Discussion).

3.4. The primary sensory neurons in C$_2$ and C$_3$

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

Applying these criteria, A-cells only represented about 15% of all neurons in C₂ and C₃, B-cells stood out at 52%, and C-cells made up 33%. Although C₃ contained about 16% fewer neurons (mostly involving small cells) than C₂ this difference did not reach statistical significance, nor were there significant differences in neuron numbers between the right and left ganglia (Table 2). Significant lateral differences, however, were detected in the somatic volume of large neurons. A-cells were on average 16% larger in the right compared to the left ganglia, a difference that increased to 22% when only C₂ was considered. Lateral differences were not found for other cell types, nor for all neurons pooled (Fig. 12).

3.5. Light- and electron-microscopy of the GON

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

Stereological estimates of axon numbers (Table 3) showed a notable variability among the 11 GONs (5 on the left, 6 on the right) examined, with coefficients of variation ranging between 18% for MAx on the right to 53% for MAx on the left, and around 30% for UAx on either side. On average, MAx represented only 21% of all axons in the nerve. In several cases, an additional 1-2% of myelinated profiles apparently corresponded to degenerating MAx. Up to 20 UAx appeared in some cases ensheathed by a single non-myelinating Schwann cell; more commonly, these Remak bundles contained 8-10 UAx, while glial profiles ensheathing 1-2 UAx were rarely found. The degree of myelination of MAx assessed by the g-ratio was essentially identical on both sides, and correlated significantly with the size of MAx, the
thickest fibers exhibiting values twice as high as the thinnest (Fig. 14). Not a single MAx
displayed ChAT-immunolabeling, indicating that the GON does not have motor fibers at least
along its subcutaneous course. In contrast, about 20% UAx expressed TH-immunolabeling;
although not formally quantified this value represents a guesstimate based on the examination
of randomly sampled spots in adjacent ultrathin sections and immunoreacted semithin
sections of two GONs (Fig. 15).

4. DISCUSSION

The main goal of this study was to provide a comprehensive anatomical assessment of
the GON in the rat. The motivation for choosing this particular nerve was twofold: First, the
sensory innervation of the occipital region and posterior aspect of the neck was for a long time
an active area of research to understand the role of its extero- and proprioceptive contribution
to postural control and coordination of eye movements. Not surprisingly, the organization of
central projections from upper cervical spinal nerves was profusely investigated with every
anatomical or functional method that came to life along the last century (Bolton & Ray, 2000;
Escolar, 1948; Kerr, 1972; Kerr & Olafson, 1961; Neuhuber & Zenker, 1989; Pfaller &
Arvidsson, 1988; Yee & Corbin, 1939). Yet, only in two studies, using transganglionic
transport of HRP, were the GON and/or its central projections specifically examined
(Neuhuber & Zenker, 1989; Scheurer et al., 1983). And second, as the interest in the upper
cervical afferents in that context seemed to wane, the GON got renewed attention after
evidence accumulated on its involvement in the pathophysiology of referred pain in some
chronic headache disorders (Magis & Schoenen, 2012; Rodrigo, Acin & Bermejo, 2017; Tang
et al., 2017). Clinical findings were supported by the demonstration in different species of
overlapping projections of trigeminal and cervical afferents in the upper cervical and caudal
spinal trigeminal nucleus (Bartsch & Goadsby, 2002; Goadsby & Bartsch, 2010; Pfaller &
Arvidsson, 1988; Xiong & Matsushita, 2000). However, given the paucity of new anatomical
information on the GON in the rat, the time seemed ripe for a reappraisal of its structure and
connections using more sensitive and precise tracing and quantitative studies. The present
study provides a new and detailed qualitative and quantitative description of ganglia C₂ and
C₃, as well as of the ultrastructure of the GON and its central projection pattern and
innervation territories in the spinal cord and brainstem. The use of two neuron-specific tracers, CTB and IB4, has also made it possible to define subpopulations of the primary sensory neurons that subserve the GON, and recognize regional and laminar particularities in the central projections of putative myelinated and unmyelinated primary afferents coursing through the GON.

4.1. Comments on the tracing and labeling methods

The cervical ganglia contributing to GON

It was consistently found that ganglion C₃ displayed only a small fraction of labeled neurons after any type of tracer delivery to GON. Lacking a direct proof, it may be suggested that those neurons sent their axons (or axon collaterals) to GON through the communicating branch between the dorsal branches of the spinal nerves C₂ and C₃ (Fig. 2). In two cases ganglion C₄ was extracted but showed no labeled neurons. Ganglion C₁ was not identified during surgery, however, in two cases the thin dorsal root of the first cervical nerve was examined histologically. Since no ganglion neurons were found, no further attempt was made to dissect C₁. Ganglion C₁ is often absent in humans (reviewed in Campos et al., 2012), and rats (Neuhuber & Zenker, 1989), although in this species it may be represented by a group of scattered neurons in only a small percentage of cases (Pfaller & Arvidsson, 1988).

Spurious labeling following delivery of tracers to GON

In all cases that received intraneural injections of CTB labeled motoneurons were found in the facial nucleus and the spinal ventral horn at C₁-C₃ levels. Such labeling also occurred when, in the first cases injected, the area of injection was thoroughly rinsed with saline, and the nerve was carefully wrapped in Parafilm® to prevent leakage of the tracer. Moreover, similar retrograde labeling resulted when the GON was exposed in the same manner but left undamaged, and the same amount of tracer was instilled and left in place for the same time as during actual injections before rinsing and closing the wound. Since ChAT immunostaining failed to label myelinated axons in the GON, it could be ruled out that the GON contained motoneuron axons. It seemed, therefore, that some tracer was taken up by local muscles or muscle nerve twigs that resulted damaged during surgery. The topographical distribution of labeled motoneurons was consistent: in the facial nucleus they remained confined within its medial division, which innervates dorsal and posterodorsal superficial
auricular muscles (Friauf & Herbert, 1985; Watson, Sakai & Armstrong, 1982); in the ventral horn they were restricted to medial and central parts of this horn at caudal medullary and upper cervical levels, presumably corresponding to motoneurons of the spinal accessory nucleus innervating the clavotrapezius and sternocleidomastoid muscles (Kitamura & Sakai, 1982; Ullah, Mansor, Ismail, Kapitonova & Sirajudeen, 2007).

On the completeness of GON fiber labeling

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

It is more difficult to explain the notable mismatch between the number of UAx in the GON (about 3700 on the right side) and the number of IB4-labeled neurons in the (right) C2 and C3 ganglia (about 1300). Although, as mentioned above, it is not possible to rule out that some axons were not sufficiently exposed to the injected IB4, and that a subpopulation of
axons in the GON may not be able to take up the tracers, other explanations should be considered. For example, it was proposed that central or peripheral axons originating from ganglion cells bifurcate near the ganglion (Chung & Coggeshall, 1984; Langford & Coggeshall, 1981). More recent studies disproved this suggestion for the spinal dorsal roots (Tandrup, 1995) and the trigeminal root (L. Vazquez and C. Avendaño, Master Thesis, 2017, unpublished). It remains possible, however, that such bifurcations may take place in peripheral nerves farther away from the ganglion, at least for UAx. A second source of the high number of UAx is the population of visceromotor autonomic axons that is known to exist in peripheral nerves, and which after being removed by an extensive sympathectomy was estimated to account for 1/3 of all UAx in the sciatic nerve and its main branches (Schmalbruch, 1986). The proportion of putative autonomic axons found in the sciatic nerve was only 19% using the approach of counting TH-immunoreactive profiles on semithin axons (Castro et al., 2008). Although not quantified, the same procedure applied to the GON in the present study revealed frequent TH-immunoreactive profiles coincident with UAx profiles. These findings suggest that the numerical mismatch between the UAx in the GON and IB4-labeled ganglion neurons could be partly explained by the presence of autonomic motor axons in the nerve, and partly by an incomplete uptake of the tracer by UAx following intraneural tracer injection.

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

Our results show that GON afferents, including IB4- and CTB-labeled fibers, distribute in central territories similar to, but more extensive than those described in previous studies using more ‘classical’ techniques (reviewed in Neuhuber & Zenker, 1989; see also Scheurer et al., 1983). These territories coincide to a large extent with many of the structures innervated by fibers labeled by HRP, WGA-HRP or biotinylated dextran injected directly into spinal ganglia C2 and C3 (Neuhuber & Zenker, 1989; Pfaller & Arvidsson, 1988; Zhan, Pongstaporn & Ryugo, 2006), except for a lack of GON projections to vestibular, cochlear, perihypoglossal, Sp5c, the central cervical, and Clarke’s dorsal nuclei in thoracic segments, which were however innervated to various degrees following C2 and/or C3 injections. It was unsettled in previous reports whether the GON projected to the external cuneate nucleus, and whether GON afferents in the spinal DH remained within the first four cervical segments, or extended as far caudal as segments T3-T4. Our findings showed no more than a few terminal
fibers in the caudoventral region of the external cuneate, and failed to identify labeled fibers in the spinal cord caudal to segment C₆.

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

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

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

1. Overall, most GON projection territories also had variable amounts of trigeminal afferents, except for the lateral DH, whose laminae I-III were heavily innervated by the GON
between segments C₁ and C₆, but were essentially free from trigeminal afferents below C₂ (Jacquin, Semba, Egger & Rhoades, 1983; Marfurt & Rajchert, 1991; Pfaller & Arvidsson, 1988).

2. Trigeminal afferents to the lateral part of C₁ and or C₂, where a heavy GON projection distributes, originate from some, but not all, components of the ophthalmic division. By transganglionic tracing, afferents have been shown mainly in laminae III-IV from the frontal, supraorbital and anterior ethmoidal nerve (Panneton, Gan & Juric, 2006; Panneton, Hsu & Gan, 2010; Shigenaga et al., 1986; Takemura, Sugimoto & Shigenaga, 1991), and c-Fos expression was induced in laminae I-II at the same spot by painful thermal stimuli applied to the supraorbital region (Strassman & Vos, 1993). Afferents from the middle meningeal artery also had terminals in laminae I-IV in a similar region of the DH (Liu, Broman & Edvinsson, 2008). In addition, conjunctival, but not corneal, afferents distributed in a slightly more medial position in laminae I-III of segment C₁ (Panneton et al., 2010).

3. Afferents from the maxillary territory, including the whiskerpad, reached the rostral portions of C₁, but their location in the DH was at an intermediate position (Fernández-Montoya et al., 2017; Panneton et al., 2010; Shigenaga et al., 1986; Strassman & Vos, 1993), dorsal and lateral to the main focus of CTB- and IB4-labeled terminals from the GON.

4. The discontinuous patches of GON afferents in the medial DH are coincident with the central projections reported for the inferior alveolar and mylohyoid branches of the mandibular nerve (Jacquin et al., 1983; Strassman & Vos, 1993). The auriculotemporal nerve, another branch of the mandibular nerve whose cutaneous distribution is placed laterally and rostrally to that of the GON, innervates in C₁ a patch (Jacquin et al., 1983) that lies immediately lateral to the GON patches.

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

GON afferents consistently distribute in three regions outside the TCC, which also receive afferents from the trigeminal, intermediate, vagus and/or upper cervical nerves other than GON. First, the ventrolateral zone of the rostral cuneate nucleus, which receives dense CTB-labeled afferents from GON, also receives trigeminal innervation from the mandibular nerve (Jacquin et al., 1983). This region is also known to receive neck muscle and cutaneous
afferents (Abrahams et al., 1979; Bolton & Tracey, 1992; Edney & Porter, 1986), which likely arise from neurons in upper cervical ganglia (Imamura, Saunders & Keller, 1986; Bolton & Arvidsson, 1988), as well as fibers from periauricular structures innervated by the intermediate nerve (Arvidsson & Thomander, 1984), the auricular branch of the vagus nerve in cats (Nomura & Mizuno, 1984), and extraocular muscles in monkeys (Porter, 1986). Second, a moderate amount of CTB-labeled fibers from GON innervates a slender marginal band at the lateral surface of Sp5i, where trigeminal fibers end respecting a somatotopy whereby caudal parts of the face are represented more laterally (Jacquin, Stennett, Renehan & Rhoades, 1988; Jacquin et al., 1986), and where most of the nociceptive afferents in Sp5i preferentially distribute (Hayashi, Sumino & Sessle, 1984). Moreover, afferents from the palpebral and bulbar conjunctiva, but not the cornea, are known to terminate ventrolaterally in Sp5i in rabbits (Van Ham & Yeo, 1996) and rats (Panneton et al., 2010). It is noteworthy that extraocular muscle afferents (Buisseret-Delmas & Buisseret, 1990; Porter & Donaldson, 1991) and afferents from the territory of innervation of the auricular branch of the vagus nerve (Arvidsson & Thomander, 1984; Ichiyama et al., 1997; Nomura & Mizuno, 1984), similar to those projecting to the rostral cuneate, also innervate the marginal part of Sp5i.

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

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

These findings gave experimental support to the clinical practice of pharmacologically blocking the GON, which for many years was used to treat various headaches (reviewed in Young, 2010), and which has received renewed attention more recently as a proven therapy for refractory migraine and cluster headache (Cuadrado et al., 2017; Gul, Ozon, Karadas, Koc & Inan, 2017; Tang et al., 2017). Moreover, after a number of open-label clinical trials
reported beneficial effects of GON subcutaneous stimulation for various painful craniofacial conditions, a randomized, double-blind and controlled study (Saper et al., 2010) showed that GON stimulation provided significant improvement to medically intractable chronic migraine cases, including some who did not respond to GON block. Unsurprisingly, GON stimulation has been increasingly used along the last decade to treat refractory migraine and trigeminal autonomic cephalalgias (Dodick et al., 2015; Lambru et al., 2014; Lambru, Shanahan, Watkins & Matharu, 2014; Miller, Watkins & Matharu, 2016; Miller, Watkins & Matharu, 2017; Rodrigo et al., 2017).

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

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

REFERENCES


### TABLE 1

List of antibodies used in this study

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Immunogen</th>
<th>Manufacturer</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Anti-Griffonia Simplicifolia Lectin I</td>
<td>Lectin I purified from Griffonia (Bandeiraea) Simplicifolia</td>
<td>Vector Laboratories goat polyclonal, Cat no. AS2104 RRID: AB_2314660</td>
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<td>Anti-Cholera Toxin</td>
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<td>Rabbit Anti-Tyrosine Hydroxylase</td>
<td>Denatured TH from rat pheochromocytoma</td>
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<td>Biotinylated Rabbit Anti-Goat IgG</td>
<td>Purified goat IgG</td>
<td>Vector Laboratories, rabbit polyclonal, Cat no. BA5000, RRID:AB_2336126</td>
<td>1:250</td>
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<tr>
<td>Anti-Rabbit IgG Biotin Conjugate</td>
<td>Purified rabbit IgG</td>
<td>Sigma Aldrich, goat polyclonal, Cat no. B7389, RRID:AB_258613</td>
<td>1:500</td>
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</table>

### TABLE 2

Neuron number (N; mean±sd) and cell body volume (V; mean±sem) in the left (L, n=6) and right (R, n=8) DRG C2 and C3. *p=0.030 and *p=0.028 (R vs. L; Wilcoxon paired test)

<table>
<thead>
<tr>
<th>Ganglion</th>
<th>Side</th>
<th>N (x10^-3)</th>
<th>V (µm^3 x 10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>C2</td>
<td>L</td>
<td>1.49±0.6</td>
<td>6.16±3.1</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.25±0.6</td>
<td>4.16±0.8</td>
</tr>
<tr>
<td></td>
<td>L+R</td>
<td>2.87±0.7</td>
<td>10.67±3.2</td>
</tr>
<tr>
<td>C3</td>
<td>L</td>
<td>1.59±0.4</td>
<td>4.44±0.9</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.35±0.3</td>
<td>4.28±1.2</td>
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<tr>
<td></td>
<td>L+R</td>
<td>2.87±0.3</td>
<td>8.40±2.1</td>
</tr>
<tr>
<td>C2+C3</td>
<td>L</td>
<td>3.04±0.7</td>
<td>10.96±2.8</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2.69±0.7</td>
<td>8.65±1.4</td>
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</tbody>
</table>
TABLE 3

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

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th></th>
<th></th>
<th>R</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>sd</td>
<td>CV</td>
<td>Mean</td>
<td>sd</td>
<td>CV</td>
</tr>
<tr>
<td>N(Max)</td>
<td>825</td>
<td>441</td>
<td>0.53</td>
<td>975</td>
<td>173</td>
<td>0.18</td>
</tr>
<tr>
<td>N(UAx)</td>
<td>2913</td>
<td>958</td>
<td>0.33</td>
<td>3688</td>
<td>998</td>
<td>0.27</td>
</tr>
<tr>
<td>g-ratio (%)</td>
<td>47.2</td>
<td>2.4</td>
<td>0.05</td>
<td>47.5</td>
<td>4.0</td>
<td>0.08</td>
</tr>
<tr>
<td>A_{Mf} (%)</td>
<td>61.9</td>
<td>4.0</td>
<td>0.06</td>
<td>60.2</td>
<td>5.2</td>
<td>0.09</td>
</tr>
<tr>
<td>A_{Int} (%)</td>
<td>34.2</td>
<td>3.1</td>
<td>0.09</td>
<td>34.9</td>
<td>5.2</td>
<td>0.15</td>
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</tbody>
</table>
Figure 1. Illustration of a dorsal view of occipital and neck regions of the rat, with the skin removed to show the superficial muscles and the course of the GON and 3d occipital nerve. Only the muscles readily identifiable are depicted. On the left, the superficial fascia is removed; on the right the most superficial muscles are also partially removed to show more clearly the course of the nerves at deeper levels. Exposed nerves are drawn in solid white; their muscle-covered courses are marked as gray traces. 3dON, third occipital nerve; AS, m. auricularis superior; AT, m. acromiotrapezius; CT, m. clavotrapezius; FA, m. frontalis, pars auricularis; GON, greater occipital nerve; LAL, m. levator auris longus; SC, m. splenius capitis; T, m. temporalis.

Figure 2. Sketch of the dorsal roots, ganglia and spinal nerves C2 and C3, the communicating branches between adjacent spinal nerves and the distribution of the GON and 3dON. The loaded pipette points to the location of the tracer deposits in the GON before it divides into its two main terminal branches.

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

Figure 4. Examples of immunofluorescent neurons in sections of the right DRG C2 (A) and C3 (B). Magenta, green and blue channels are used to identify CTB-, IB4- and Bisbenzimide-labeled cell bodies and nuclei, respectively. Double-labeled neuronal perikarya appear in C2 in the merged image (panel on the right; see also Fig. 5). Scale bars 100 µm. Histograms show the estimations of the total number (mean+sd) of single- and double-labeled neurons in each ganglion.

Figure 5. High-power view of CTB- (magenta) and/or IB4-labeled (green) neurons in C2. While most CTB-labeled neurons are large or medium-sized, some are definitely small (B and C, thin arrows). Likewise, most IB4-labeled cells are small-to-medium sized, but a few examples occur of strongly labeled fairly large neurons (A, thick arrow). Most double labeled neurons belong within the small or medium-size range (C, arrowheads). Scale bar 50 µm.
Figure 6. Low-power confocal images of C2 (A) and C3 (B) ganglia sections displaying maximum retrograde labeling after a FB deposit in GON. Scale bar 200 µm. The inset shows the stereological estimate of total number (mean+sd) of FB-labeled neurons in these ganglia.

Figure 7. A: Low-power view of IB4-immunofluorescent terminal field in lamina II under confocal microscopy on a horizontal section of the upper cervical spinal cord. Arrows point to needle holes that were made midway between the insertion on the spinal cord of dorsal roots C2-C3 and C3-C4 before extracting the tissue after perfusion to serve as fiducial marks. B: Higher-power detail of the nearly complementary laminar terminal pattern of CTB- (magenta) and IB4-immunofluorescent (green) central projections of the GON in the DH of the spinal cord. While IB4-labeled fibers and varicosities are largely restricted to the outer two-thirds of lamina II, CTB-labeled profiles are present in lamina I and lamina III-IV, and also appears in the deeper part of lamina II, intermingling to some extent with IB4 labeling. Where there is topographical overlap, also a fraction of the terminals show co-localization of both tracers (not shown). Short vertical lines (top right corner) mark laminar boundaries, from the pial surface (right) to laminae II-III border (left). Scale bars 500 µm (A) and 100 µm (B).

Figure 8. Distribution of CTB-immunolabeled afferent projections from the GON in the cervical spinal cord and caudal brainstem. A: Series of eight DAB-reacted horizontal sections arranged dorso-ventrally (‘a’ through ‘h’, from left to right), with a 240 µm separation between adjacent sections. Sections are aligned rostro-caudally to preserve relative locations of labeling at different depths. Arrowhead in section ‘a’ marks the approximate location of the obex, and thick arrows in section ‘d’ point to the center of C2 and C3 dorsal roots, as indicated by the nearby reference needle holes (see Fig. 7). Contrast and brightness were adjusted to enhance visualization. B: Close up pictures of the labeled territories numbered in A (long arrows): 1 and 6, uneven projections at dorsal and ventral parts of the outermost region of Sp5i; 2 and 3, dense labeling in laminae III and IV of the DH, that extends from segmental level C1, where spinal and medullary DH merge, to segment C6; 4, separate patches of dense terminal fields that rostrally directed GON afferents leave medially in upper cervical and medullary DH; 5, in contrast, caudal divisions of GON afferents entering through C2 dorsal root (top) leave a few small and discrete dense terminal patches more laterally in the DH. Short arrows in A indicate the dense bundle of labeled fibers placed at more dorsal and
rostral levels (‘a’), strongly labeled C₂ rootlets (‘b’), and a few labeled fibers in the solitary complex crossing to the contralateral side (‘d’). Sp5c, caudal trigeminal nucleus; Sp5i, interpolar trigeminal nucleus; Cu, cuneate nucleus; DC, dorsal column; Gr, gracile nucleus; LC, lateral column. Scale bars 1 mm (A) and 50 µm (B).

**Figure 9:** Distribution of CTB- (magenta) and IB4-labeled (blue) GON afferents in spinal cord and brainstem territories in one case cut on the coronal plane. Labeled fibers and boutons in selected sections at the indicated levels were digitally enhanced and superimposed on attenuated greyscale images of adjacent Nissl-stained sections. Relevant nuclear or laminar boundaries are outlined. Anatomical terms follow Paxinos & Watson (1986) and Bermejo et al (2003): Bi, nucleus Bischoff; C₁-₄, spinal segmental levels; CuE, external cuneate nucleus; CuM, cuneate nucleus, medial division; CuR, cuneate nucleus, rostral division; Gr, gracile nucleus; NBi, internal basilar nucleus; Pa₅, paratrigeminal nucleus; Sol, solitary nucleus; Sp5cc, caudal trigeminal nucleus (caudal level); Sp5cr, caudal trigeminal nucleus (rostral level); Sp5i, interpolar trigeminal nucleus; x, nucleus x; z, nucleus z; I-V, dorsal horn or Sp5c laminae; XII, hypoglossal nucleus. Scale bar 500 µm.

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

**Figure 11:** Volume of central structures innervated by GON primary afferents in millions of cubic micra (mean+sem). In “Sp5i” it is included the territory of innervation in the paratrigeminal nucleus. “DCN” includes terminals in the rostral and external cuneate nuclei and less well defined adjacent cell groups, such as nucleus x or the dorsolateral reticular
nucleus. And “DH-deep” refers to all labeled fibers and varicosities in or deeper to inner part of lamina IV.

**Figure 12:** A: Distribution of perikaryal volumes of all neuron types in ganglia C2 and C3 pooled, plotted as frequencies over a log-transformed size scale (n = 10 rats). No statistical differences exist between right and left sides. B: Differences in cell body size exist, however, between the large, A-type cells in the left and right ganglia. As shown in the inset, the volume of A-type cells is significantly higher on the right side with respect to the left (p=0.028, Wilcoxon signed-rank test).

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

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

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