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Original Article

Newly identified axon types of the facial nerve unveil supplemental neural pathways in the innervation of the face

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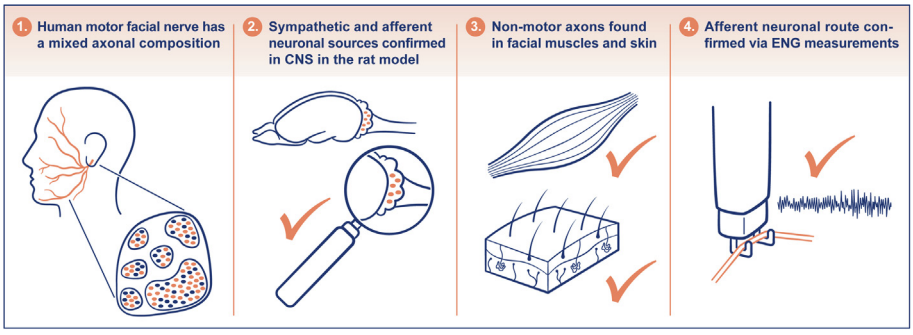
HIGHLIGHTS

- The extracranial facial nerve is believed to be a pure motor nerve.
- We demonstrated a mixed axonal composition of the motor facial nerve in human.
- Animal experiments revealed sympathetic and afferent neuronal sources of the motor facial nerve in the CNS.
- Sympathetic and afferent innervation pathways were confirmed in muscle and skin biopsies.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Neuromuscular control of the facial expressions is provided exclusively via the facial nerve. Facial muscles are amongst the most finely tuned effectors in the human motor system, which coordinate facial expressions. In lower vertebrates, the extracranial facial nerve is a mixed nerve, while in mammals it is believed to be a pure motor nerve. However, this established notion does not agree with several clinical signs in health and disease.

Objectives: To elucidate the facial nerve contribution to the facial muscles by investigating axonal composition of the human facial nerve. To reveal new innervation pathways of other axon types of the motor facial nerve.

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Facial palsy
Facial muscles
Sympathetic fibers
Hemifacial spasm
sensory feedback
proprioception

Methods: Different axon types were distinguished using specific molecular markers (NF, ChAT, CGRP and TH). To elucidate the functional role of axon types of the facial nerve, we used selective elimination of other neuronal support from the trigeminal nerve. We used retrograde neuronal tracing, three-dimensional imaging of the facial muscles, and high-fidelity neurophysiological tests in animal model.
Results: The human facial nerve revealed a mixed population of only 85% motor axons. Rodent samples revealed a fiber composition of motor, afferents and, surprisingly, sympathetic axons. We confirmed the axon types by tracing the originating neurons in the CNS. The sympathetic fibers of the facial nerve terminated in facial muscles suggesting autonomic innervation. The afferent fibers originated in the facial skin, confirming the afferent signal conduction via the facial nerve.
Conclusion: These findings reveal new innervation pathways via the facial nerve, support the sympathetic etiology of hemifacial spasm and elucidate clinical phenomena in facial nerve regeneration.

Introduction

The highly specialized facial neuromuscular system is capable of a vast number of functions: ingestion, breathing and tactile sensation [1,2]. Compared to other mammals, the human face enables unique facial expressions, which play a pivotal role in both verbal and non-verbal human interactions and physical as well as emotional well-being [3–5]. Nevertheless, the complex nature of motor control of facial expressions is still poorly understood [6]. This system lacks both visual and goniometric proprioceptive feedback, which supports control in other skeletal muscles. Therefore, the facial muscles must possess an alternate potent proprioceptive control system. Furthermore, muscle spindles are absent within the facial muscles [7] and previous studies on the axonal composition of the facial nerve in mammals have concluded that the extracranial facial nerve is a "pure motor" nerve [8]. The nature of sensory feedback from the facial neuromuscular system therefore remains unclear [8–10]. Although positive Tinel sign of regenerating axons from the facial nerve via the cross-face nerve graft or disturbed sensibility of the face in focal facial nerve injuries suggest afferent involvement of the facial nerve in the innervation of facial muscles or cutaneous mechanoreceptors, a detailed analysis of its axonal composition remains elusive [8–10].

Several studies investigating the axonal composition of the facial nerve speculated about the presence of other fiber types in the extracranial facial nerve [11–14]. Besides the controversial findings, the methodology used in these studies for differentiation of axonal types was inconclusive due to lacking data on the extent of the nerve's injury for retrograde labeling. In these studies, retrograde labeling of the rat's facial nerve was performed using immersion of the proximal stump of the facial nerve and no intramuscular application. No sympathetic or afferent co-innervation via the facial nerve was demonstrated using intramuscular application of a retrograde tracer in the aforementioned studies. Moreover, special anatomical features of the facial nerve differ between rodents and humans, thus must be considered in the interpretation of data from the rodent model. Especially, axonal exchange between different cranial nerve branches represents an essential obstacle in evaluation of the facial nerve's axonal composition and its contribution to the innervation of the face [15].

The importance of the facial nerve is evident in patients with facial palsy, hemifacial spasm or major facial injuries, as the conditions greatly affect social interaction, emotional well-being and personal identity, leading to enormous suffering [16–20]. Interestingly, the etiologies of the most common facial paralysis (idiopathic Bell's palsy with lifetime risk of 1 in 60) and hemifacial spasm remain unknown [18,21,22]. Better understanding of the facial nerve's contribution to the afferent and efferent innervation of the face would improve our understanding of the pathophysiology of these clinical conditions and, conclusively, may lead to optimized treatments.

In this study, we performed a multi-level evaluation of the facial nerve's contribution to the innervation of the human and rat's face using immunohistochemical analysis, whole-mount staining with 3D reconstructions via confocal microscopy and electrophysiological tests. Here, a previously unknown mixed composition of the extracranial facial nerve was identified in humans. Moreover, the facial nerve in rodents showed a composition of somatic efferent, sympathetic, and afferent fibers. In the rat, the origin of different axonal types was determined in the central nervous system (CNS). After surgical deafferentation by axotomizing all cutaneous branches of the trigeminal nerve, facial muscles showed sympathetic innervation of the vibrissae via the facial nerve in a rat model. Moreover, immunohistochemical and electrophysiological assessment of the facial nerve showed afferent innervation of a local skin region containing genal vibrissae in the rat. This reveals new functions of the facial nerve and explains possible pathophysiological mechanisms involved in facial palsy, hemifacial spasms and clinical outcomes after facial transplantation.

Material and methods

For a more detailed description of materials and methods, reference SI Appendix.

Harvesting facial nerve samples in human organ donors

Five heart-beating human organ donors were used in this study to validate the experimental data obtained in the animal model. The main trunk of the extracranial facial nerve was harvested for the evaluation of the axon composition. Approval for the trial with organ donors was granted by the ethics committee of the Medical University of Vienna (reference number EK Nr: 1213/2012).

Study design and animals

Thirty-six Sprague-Dawley rats (male, aged 8–10 weeks, weighting approximately 350 g) were allocated in three groups to investigate axonal mapping, central representation of the facial nerve and innervation of the facial structures (reference SI appendix, Table 2). For the axonal mapping, the facial nerve and its distal branches were harvested in the first procedure (n = 12). At the same time, the main trunk of the facial nerve was exposed to a retrograde tracer for the evaluation of the facial nerve's components represented in the central nervous system. For the identification of the afferent and sympathetic components, the cranial ganglia (geniculate, superior cervical and trigeminal ganglia) were harvested after the retrograde labeling. For the investigation of the facial muscles' innervation patter (levator auris longus, levator labii superioris and dilator nasi muscles), 14 rats were allocated for the deafferentation procedure (see SI appendix, Table 2).

Surgical procedures in the rat

For the complete axonal cartography of the facial nerve and its distal branches, nerves were harvested using a surgical approach as previously described [23]. In brief, the main trunk of the facial nerve and all distal branches were exposed by a preauricular incision, followed by the identification of the posterior auricular nerve right at the emergence from the stylomastoid foramen.

Immunofluorescence staining and quantification of nerve cross-sections

A detailed staining procedures can be found in extended material and methods, refence SI appendix.

Muscle and skin staining

Muscles were stored at 4 °C in PBS containing 0.05% sodium azide to avoid bacterial contamination and underwent whole-mount preparation. Skin preparations were dehydrated in graded solutions of sucrose, embedded in cryomatrix and stored in a fridge at −80 °C degree according to the procedure described previously [24]. Whole mounts of facial muscles and facial skin were processed for immunofluorescence staining. A detailed staining procedures can be found in extended material and methods, reference SI appendix.

Three-dimensional imaging and data analysis of immunofluorescence

Fluorescently labeled specimens were analyzed with a confocal laser scanning microscope [CLSM (Olympus FV3000, Olympus Europa SE & Co. KG, Hamburg, Germany)]. A series of virtual CLSM sections between 1 and 2 μm thickness were cut through the structures of interest. Each section was photo-documented with a 1024x1024 pixel resolution and 3D projections were rendered using Image J software (NIH, Bethesda, MA, USA). Double-colored images were generated using lasers with excitation wavelength 488 and 568 nm and triple-colored images using additionally a laser with excitation wavelength 633 nm. In some cases, bright-field images were recorded in the CLSM to correlate immunolabeling to morphological structures.

Retrograde labeling

The central representation of the extracranial facial nerve was assessed and quantified using retrograde labeling. A detailed description can be found in extended material and methods, refence SI appendix.

Electrophysiologic analysis

Functional assessment of the facial nerve's involvement in the innervation of the dermato-muscular complex was performed using ENG (Fig. 7A). Measurements were performed in rats without surgery prior to the measurements (n = 6) and in rats two weeks after the deafferentation procedure (n = 4) to exclude involvement of cutaneous branches in signal conduction. A detailed description can be found in extended material and methods, refence SI appendix.

Statistical analysis

A detailed description can be found in extended material and methods, refence SI appendix.

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethics committee of the Medical University of Vienna and the Austrian Ministry for Research and Science (reference numbers: BMWF-66.009/0302-V/3b/2019 and 2021–0.155.204). Approval for the trial with human organ donors was granted by the ethics committee of the Medical University of Vienna (reference number EK Nr: 1213/2012).

Results

Mixed axonal composition of the human extracranial facial nerve

To investigate if a mixed nerve fiber composition is also present in humans, facial nerves were harvested from five heart-beating organ donors to assess their axonal composition (see Supplementary Table 1). In detail, the main trunk of the facial nerve was harvested from all organ donors within 12 h postmortem. An axonal analysis using double immunofluorescent staining revealed a population of non-cholinergic axons in the main trunk of the extracranial facial nerve (Fig. 1A, B). Mean overall axon count of 12,343 ± 1,231 nerve fibers was comprised only of 85% cholinergic axons (Fig. 1A). The non-cholinergic fibers of the human facial nerve are gathered in clusters within the complete cross section of the nerve (Fig. 1B, dashed line).

Mixed neuronal components of the extracranial facial nerve in rodents

Extracranial facial nerve contains motor, afferent and sympathetic axons

We performed an axonal analysis of the main trunk and all distal branches of the extracranial facial nerve in rats. A complete cartography of cholinergic and noncholinergic components within the extracranial facial nerve was established (see Supplementary Fig. 1). Immunofluorescent staining identified a total of 5083 ± 127 axons within the main trunk of the rat facial nerve, of which 310 ± 29 (6.1 ± 0.32%) were non-cholinergic axons, indicating a mixed axonal composition of the facial nerve. The non-cholinergic fibers are distributed diffusely within the facial nerve's cross-section.

Likewise, the facial nerve's distal branches contained approximately 7% non-cholinergic fibers, confirming the contribution of these mixed nerve fibers to the innervation of the face (Fig. 2). In detail, the temporal branch contained 395 ± 45, the zygomatic branch 274 ± 38, the buccal branch 1437 ± 201, the marginal mandibular branch 2240 ± 328, the cervical branch 367 ± 59 and the auricular posterior branch 1623 ± 201 fibers (Fig. 2A). These distal branches showed similar patterns of noncholinergic axons with 4.6 ± 1.2% in the temporal branch, 5.2 ± 1.6% in the zygomatic branch, 5.9 ± 1.8% in the buccal branch, 6.9 ± 2% in the marginal mandibular branch, 12.9 ± 5.6% in the cervical branch and 28.2 ± 9% in the auricular posterior branch (Fig. 2C). The high non-cholinergic axon number in the auricular posterior branch has been previously known to be due to its partly sensory innervation of the external ear. The higher overall axonal load in the buccal and marginal mandibular branches results from their responsibility for motor innervation of the whisker pad.

Furthermore, the specific type of non-cholinergic axons within the main trunk of the facial nerve was determined using antibodies against tyrosine hydroxylase (TH) for sympathetic and calcitonin gene-related peptide (CGRP) for afferent fibers. Here, the main trunk of the facial nerve contained both CGRP-positive (afferent) as well as TH-positive (sympathetic) axons (Fig. 3). The main trunk

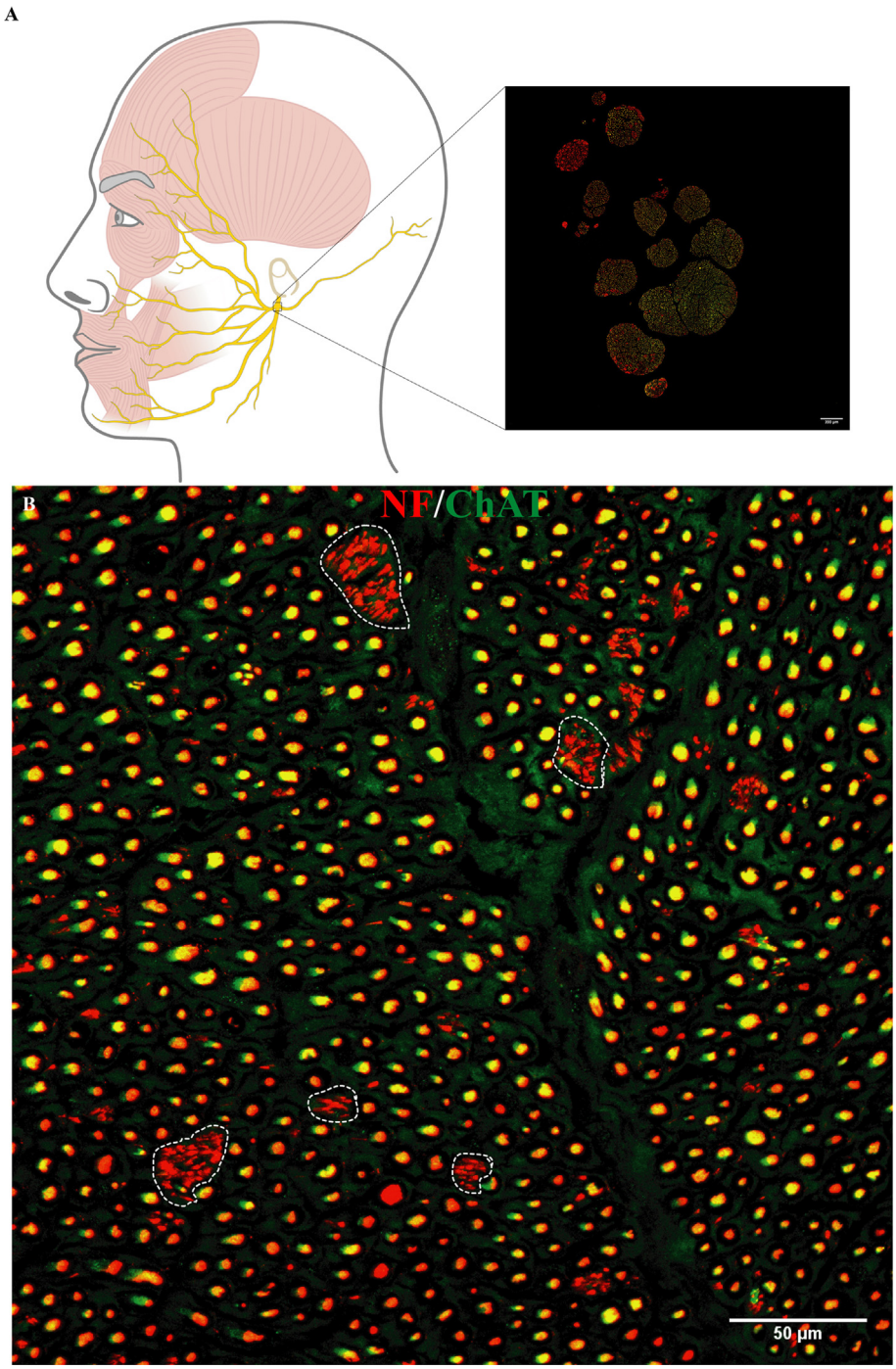

Fig. 1. Mixed axonal population within the human extracranial facial nerve. A. Schematic illustration of the harvesting site for the extracranial facial nerve. Cross-sections of the extracranial facial nerve are stained using antibodies against neurofilament (red) and antibodies against choline acetyltransferase (green). Axons displaying a double signal (anti-NF and anti-ChAT) demonstrate the predominant motor axon population. Axons with only an anti-NF signal are non-cholinergic. The overall axon number within the main trunks was 12,343 ± 1231, whereby 1622 ± 453 (15%) are noncholinergic. **B.** The magnified image (x60 magnification) of the cross-section of the facial nerve demonstrates clustered non-cholinergic fibers (dashed line), which are seen resembled in every single fascicle of the extracranial facial nerve. The image represents an overlapping green (anti-ChAT) and red (anti-NF) signals.

contained 4.4 ± 1.7% of nonmyelinated CGRP-positive (afferent) fibers and 2.4 ± 0.8% of TH-positive (sympathetic) in the main trunk of the facial nerve (Fig. 3).

Origin of noncholinergic nerve fibers identified in sensory and sympathetic ganglia

Using retrograde tracers, the facial nerve's axons of different types were traced to their originating cell bodies in the CNS in the rat (Fig. 4A). Here, the origin of all axons was located in the neuron cell bodies within the central nervous system (CNS). Although most motor fibers within the facial nerve were identified in the facial nucleus, stained cell bodies were also located in the ipsilateral geniculate and cervical superior ganglions. This confirmed the mixed axonal composition of the extracranial facial nerve. No labeled neurons were identified in the ipsilateral trigeminal or contralateral geniculate or cervical superior ganglions.

A

	Total	Cholinergic		Non-cholinergic	
	Mean ± SD	Mean ± SD	%	Mean ± SD	%
Main trunk	5083 ± 126.5	4773 ± 112.2	93.9%	310.1 ± 28.8	6.1%
Temporal branch	394.9 ± 45.37	376.6 ± 42.72	95.4%	18.3 ± 5.81	4.6%
Zygomatic branch	274.1 ± 37.76	259.9 ± 36.51	94.8%	14.2 ± 5.57	5.2%
Buccal branch	1437 ± 201.2	1353 ± 192.7	94.2%	84.3 ± 28.67	5.9%
Marginal mandibular branch	2240 ± 328.3	2085 ± 333.9	93.1%	155.3 ± 42.61	6.9%
Cervical branch	367.3 ± 59.02	320.1 ± 47.96	87.1%	47.2 ± 27.25	12.9%
Auricular posterior branch	1623 ± 200.8	1165 ±151.7	71.8%	457.5 ± 185.5	28.2%

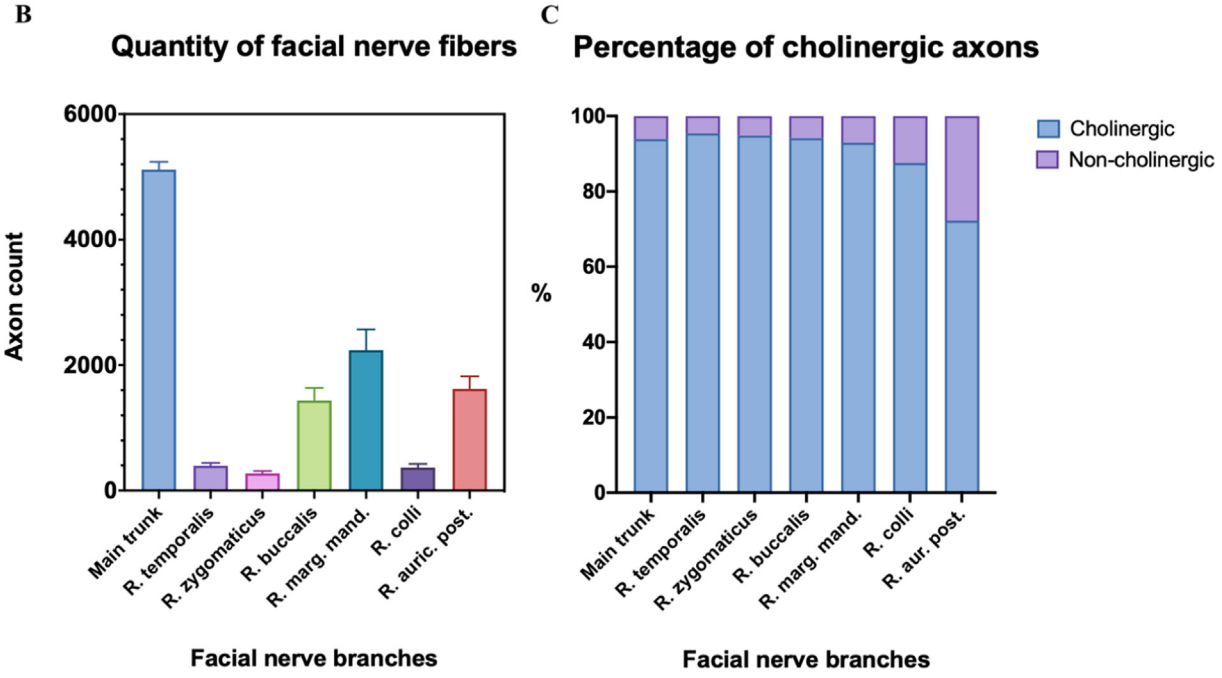

Fig. 2. Axon quantification of the facial nerve and its distal branches in the rat. (A, B) Absolute numbers of the cholinergic and noncholinergic axons within the facial nerve and its respective branches (n = 12). **(C)** Quantification of axons within the facial nerve and its distal branches from twelve animals. The axons within cross-sections of the nerves stained with antibodies against NF/ChAT were quantified, whereby noncholinergic axons were labeled only with anti-neurofilament and cholinergic axons showed double signal with anti-NF and anti-ChAT. The number of noncholinergic axons was almost equally distributed along all distal branches: 4.6 ± 1.2% in the temporal branch, 5.2 ± 1.6% in the zygomatic branch, 5.9 ± 1.8% in the buccal branch, 6.9 ± 2% in the marginal mandibular branch, 12.9 ± 5.6% in the cervical branch. Overall, the main trunk of the facial nerve consisted of 6.1 ± 0.32% noncholinergic fibers. The auricular posterior nerve, known for its partial afferent innervation of the auricula, contained 28.2±% of noncholinergic fibers, which were present in a separate nerve fascicle. Data is presented as means ± standard deviation.

The number of labeled cell bodies in the geniculate and superior cervical ganglions correspond to the amount of different axon types within the extracranial facial nerve. The quantitative analysis demonstrated clear correlation in the number of labeled cell bodies and different axon types (Fig. 4B, C and D). The total number of motor axons in the rat's facial nerve (4773 ± 112) corresponded to the number of motor neurons in the facial nucleus (4837 ± 227), the afferent axons (230 ± 34) with cell bodies in the geniculate ganglion (194 ± 50) and sympathetic fibers (126 ± 33) with (121 ± 34) cell bodies in the cervical superior ganglion.

Sympathetic muscle innervation via the facial nerve

The innervation pattern of the rat's facial muscles (levator labii superioris [LLS], dilator nasi muscles [MDN] and levator auris longus [LAL]) was investigated using an immunofluorescent whole-mount staining method (Fig. 5A). The potential involvement of the trigeminal nerve in the facial muscles' innervation was excluded by experimental denervation of the cutaneous branches of the trigeminal nerve [23]. Three weeks after the denervation, all harvested facial muscles were stained using antibodies against various fiber types: anti-NF for all axons, anti-ChAT for cholinergic fibers, anti-TH for sympathetic fibers and anti-CGRP for afferent fibers. All muscles showed a dense pattern of neuromuscular junctions, which were innervated by cholinergic fibers (Fig. 5B). All facial muscles contained identical mixed axonal distribution containing sympathetic and afferent fibers. CGRP-positive fibers were identified within the axonal bundles (Fig. 5C). Nevertheless, facial muscles did not show any CGRP-positive and thus afferent nerve fiber endings. Along with afferent axons, all muscles demonstrated vast number of sympathetic fibers along the blood vessels as well as within the axon bundles passing through the muscle (Fig. 5D).

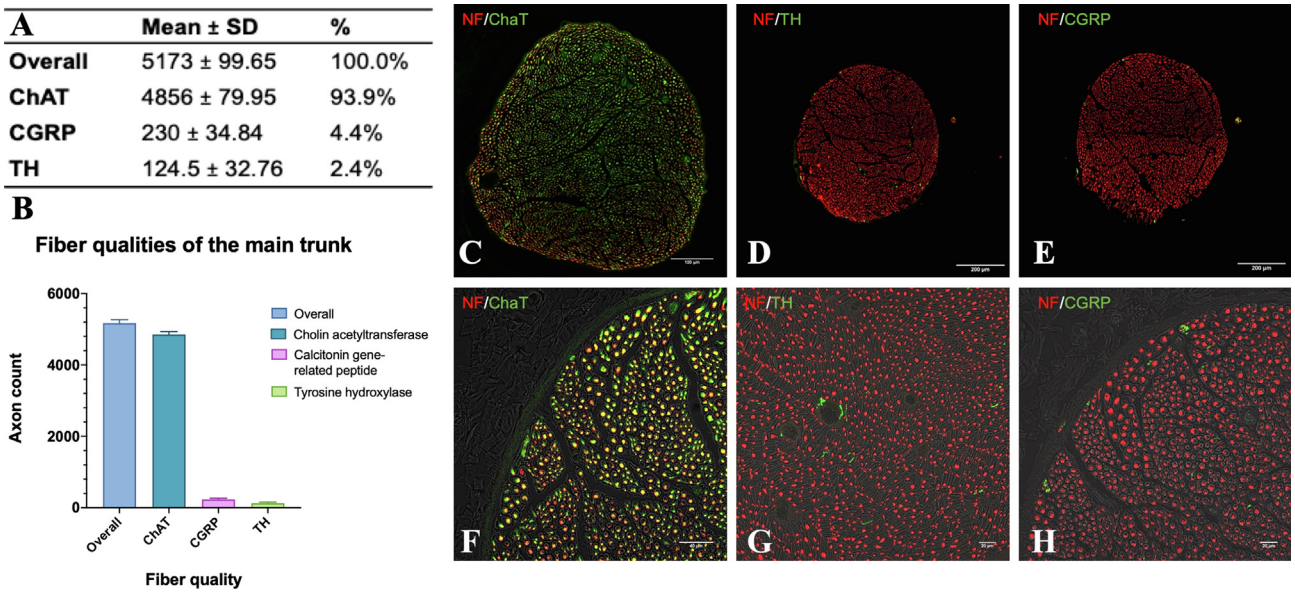

Fig. 3. Analysis of different axon types in the facial nerve. (A), (B) Quantitative analysis of the different axonal components in the facial nerve in the rat. ChAT + for somatic efferent axons, CGRP + for afferent axons and TH + for sympathetic axons. Data are presented as means ± standard deviation. Cross-sections of the main trunk of the facial nerve stained with anti-NF, anti-ChAT **(C)**, anti-NF and anti-TH **(D),** anti-NF and anti-CGRP antibodies **(E)**. Magnified images of the cross-sections using anti-NF, anti-ChAT and bright-field light **(F)**, anti-NF, anti-TH and bright-field light **(G)**, anti-NF and -CGRP antibodies **(H)** and bright-field light show that ChAT fibers form the majority within the facial nerve.

The unoperated facial muscles showed non-cholinergic fibers within the axonal bundles as well (see Supplementary Fig. 2A, B).

Intramuscular application of a retrograde tracer (2% Fast-Blue) in the levator labii superioris and dilator nasi muscles following deafferentation confirmed the sympathetic innervation of the facial muscles. The facial nucleus as well as the superior cervical ganglion demonstrated stained cell bodies indicating the sympathetic co-innervation of the facial muscles via the facial nerve (see Supplementary Fig. 2C). No signal was detected in the ipsilateral geniculate ganglion.

Facial nerve innervates facial skin and genal vibrissae

The involvement of the facial nerve in the afferent innervation of the facial skin was assessed in rodents after unilateral denervation of the cutaneous trigeminal branches [23]. No afferent fibers were found in the denervated perioral and periorbital skin regions or mystacial whisker pad. Using whole-mount staining, the contralateral skin region without deafferentation surgery demonstrated the same innervation pattern of the skin region with genal vibrissae (Fig. 6B, C). The skin region around the denervated genal vibrissae demonstrated abundant NF- and CGRP-positive fibers (Fig. 6D). The CGRP-positive fibers indicated the afferent nature of the axons, which were identified in the main trunk of the facial nerve as well. The TH-positive fibers were only identified accompanying the blood vessels within the denervated facial skin (Fig. 6E).

Facial nerve conducts afferent signals from the facial skin

Electrophysiological measurements of the facial nerve validated the afferent skin innervation. Touch stimuli applied to the genal vibrissae and to the vibrissae origination point revealed a measurable response via the buccal branch in all animals. Vibration evoked afferent responses in all animals, for both the vibrissae and the vibrissae originating point as well. To prevent possible efferent signals from the facial nerve caused by a trigemino-facial reflex [25], the facial nerve was transected at its emergence from the stylomastoid foramen, and the measurements were repeated resulting in no difference of the measured signals (see Supplementary Movie 1).

In the unoperated rats the average of the maximal root mean square (RMS) amplitudes were 0.86 ± 0.66 μV for touching the vibrissae, 1.96 ± 0.96 μV for touching the emergence point of the vibrissae, 1.69 ± 0.88 μV for vibrating the genal vibrissae and 3.87 ± 2.51 μV for vibrating the emerging point of the genal vibrissae. Following the deafferentation procedure, we recorded 0.76 ± 0.27 μV for touching the vibrissae, 1.49 ± 0.92 μV for touching the emergence point of the vibrissae, 1.24 ± 0.44 μV for vibrating the genal vibrissae and 2.29 ± 1.62 μV for vibrating the emerging point of the genal vibrissae. The noise-offset, which was subtracted from the filtered RMS signal, amounted to 1.95 ± 0.13 μV for the unoperated rats and 1.8 ± 0.08 μV for the rats after deafferentation (Fig. 7F).

No signals were evoked by applying stimuli to the ipsilateral mystacial whisker pad. Furthermore, stretching the dilator nasi muscle did not result in any proprioceptive signals via the buccal branch of the facial nerve (Fig. 7E). For the proprioceptive stimuli we recorded an average of the maximal RMS amplitudes of 0.12 ± 0.02 μV, while the maximum amplitude was 0.09 μV nociceptive stimuli. Signals for both, proprioception, and nociception, can be considered a random fluctuation of filtered ENG-signal. Pricking the dilator nasi muscle did not show any nociceptive response, indicating no involvement of CGRP-positive fibers in the muscular co-innervation (Fig. 7E).

Discussion

Our findings provide evidence of new axon populations and functions of the extracranial facial nerve. The facial nerve is, in fact, not a pure motor nerve, as previously thought, but is responsible for the autonomic muscle co-innervation as well as afferent innervation of genal region of the skin and tactile organs in the rat. This mixed axonal composition of the facial nerve was also confirmed in humans. These findings shed light on the pathophysiology of different clinical conditions (hemifacial spasm and orofacial pain) and may optimize surgical treatment modalities for facial paralysis

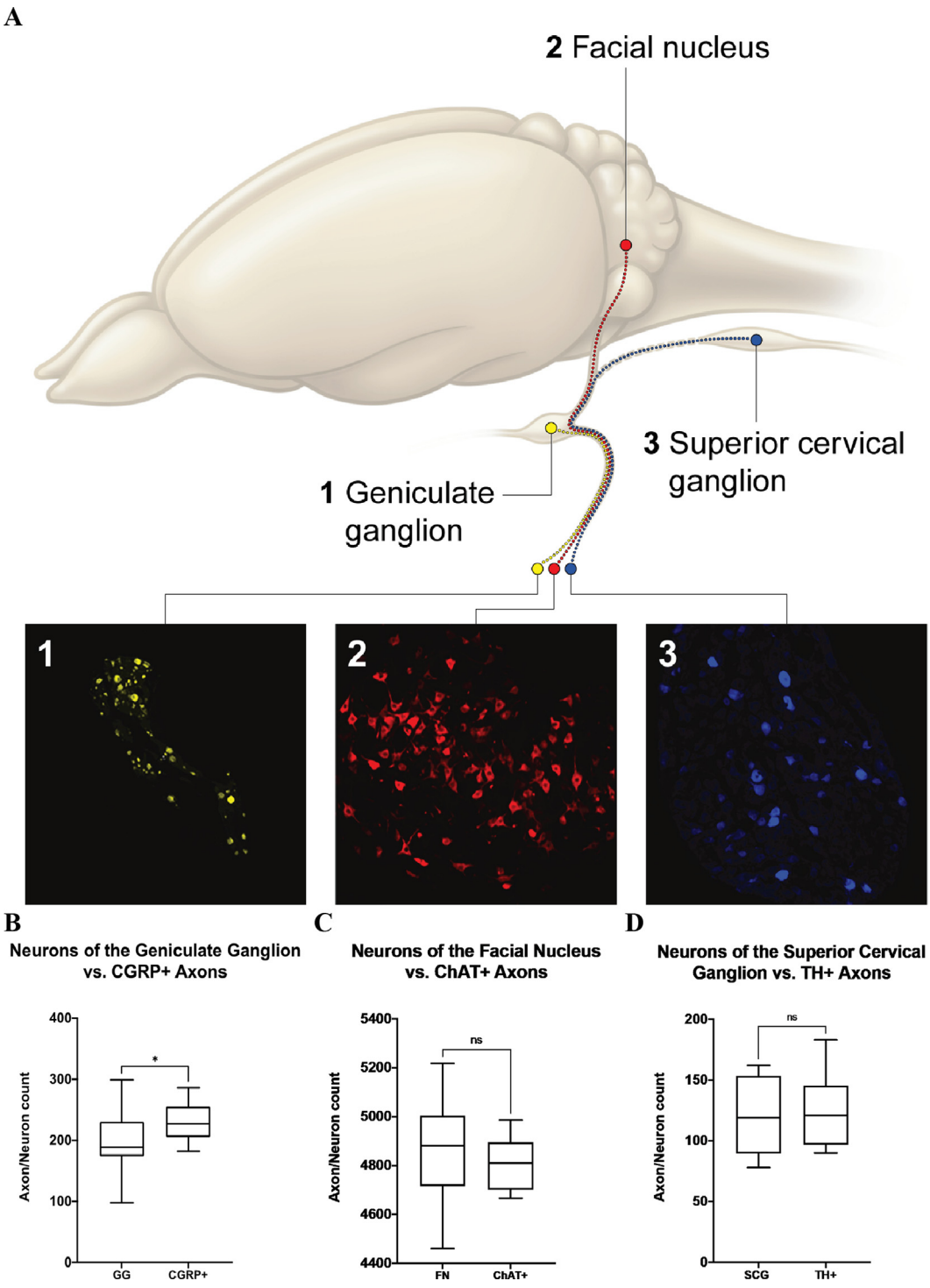

Fig. 4. New paradigm for the central representation of the facial nerve. (A) Schematic interpretation of the central origins for different fiber types in the facial nerve. Three different fiber types from the facial nerve were traced back to their neural sources. The facial nucleus is the origin of the somatic efferent axons innervating the facial muscles. The geniculate ganglion is the origin of the afferent fibers from the facial nerve. The superior cervical ganglion is the origin of the sympathetic fibers. **(B)** The overall number of the sympathetic fibers and labeled cell bodies in the superior cervical ganglion did not statistically differ: 124 ± 49 vs. 120 ± 30 respectively (paired *t*-test, p = 0.18, n = 5). **(C)** Number of anti-ChAT positive axons and the labeled motoneurons in the facial nucleus did not statistically differ: 4803 ± 100 somatic efferent axons vs. 4837 ± 218 motoneurons (paired *t*-test, p = 0.48, n = 14). **(D)** The number of anti-CGRP positive fibers within the facial nerve corresponded with the number of cell bodies labeled within the geniculate ganglion but showed a difference with statistical significance: 230 ± 81 vs. 194 ± 48 (paired *t*-test, p = 0.035, n = 5).

and facial transplantation. These findings demonstrate that the role of the facial nerve is far beyond pure efferent innervation of the facial muscles. Hence, there is a need for reassessment of its clinical significance in various pathological conditions of the face.

Sensory feedback from the facial muscles

Proprioceptive control is mediated by a variety of sensors within the neuromuscular system and several other sensory affer-

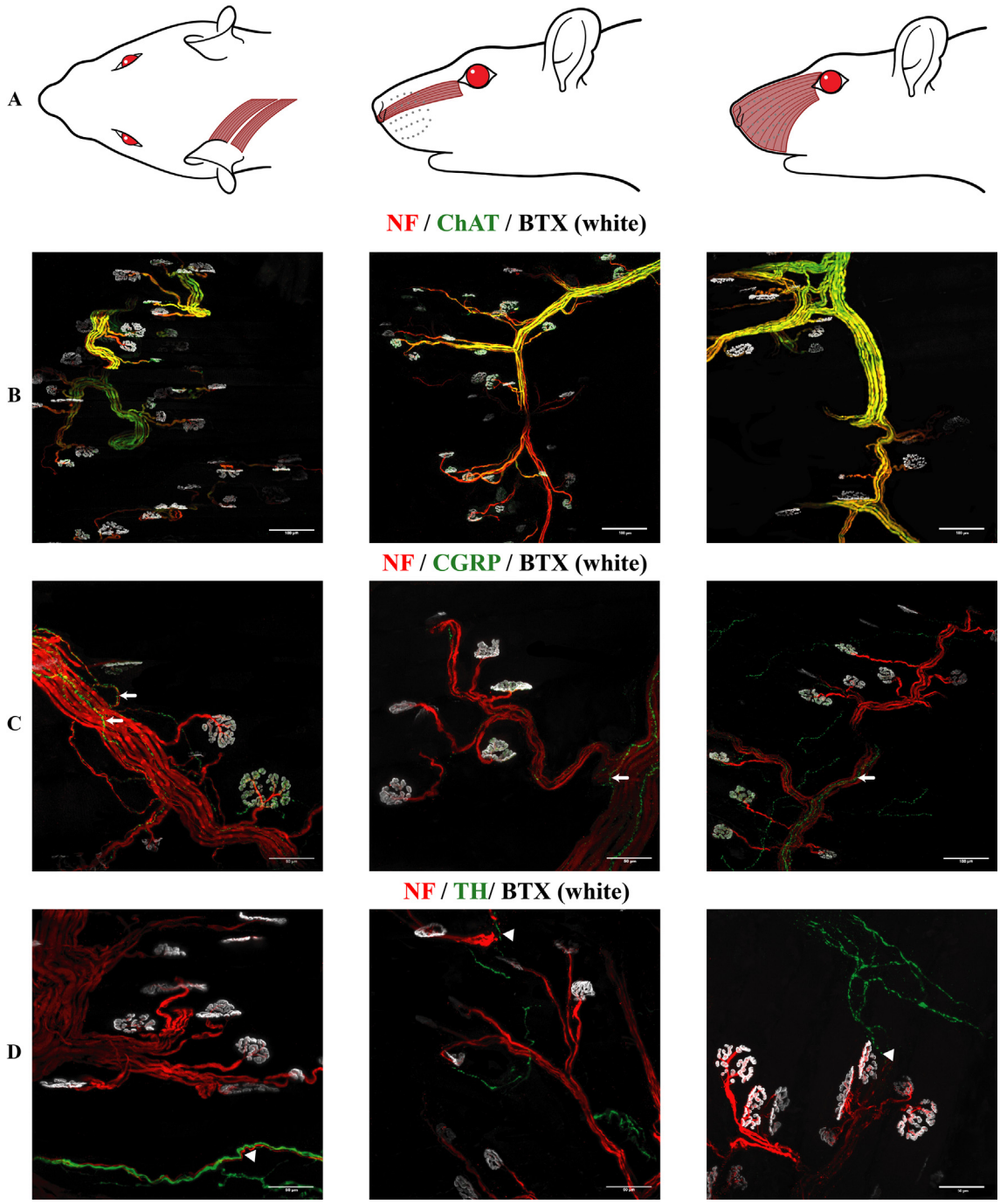

Fig. 5. Innervation pattern of the facial muscles via the facial nerve. (A) Illustration of the harvested muscles. Left: levator auris longus (LAL); middle: dilator nasi (MDN); right: levator labii superioris (LLS) muscles. The facial muscles were harvested in nine rats for the whole-mount staining after deafferentation procedure. **(B)** Immunofluorescent images of the facial muscles using anti-ChAT, anti-NF and alpha-bungarotoxin. Anti-ChAT and alpha-bungarotoxin signals demonstrate the cholinergic nature of the neuromuscular innervation. **(C)** Immunofluorescent images using the antibodies anti-NF, anti-CGRP and alpha-bungarotoxin in LAL, LLS and MDN muscles harvested after deafferentation. All muscles show CGRP-positive signals within the axonal bundles (arrows). **(D)** Immunofluorescent images of LAL, LLS and MDN muscles after deafferentation using anti-NF, anti-TH antibodies and alpha-bungarotoxin. Whole-mounts indicated the presence of the TH-positive fibers within all three facial muscles (arrowheads).

ents as an integrative multimodal system. Depending on the specific need or circumstance, feedback and thus control can be provided by intramuscular afferents, musculotendinous sensors such as Golgi-apparatus, the numerous mechanoreceptors (e.g., Meissner's corpuscles, Merkel disk receptors or Pacinian corpuscles) within the skin overlying the muscle providing a feedback matrix. In some special scenarios, such as in our fingertips, when playing an instrument or using our vocal cords, sound may provide the most sensitive and accurate feedback.

The face and its numerous layers of muscles are one of the most finely tuned neuromuscular systems. Facial expressions, verbal, and non-verbal communication as well as perioral and periorbital control require detailed high-fidelity proprioceptive feedback. Considering that two of the most important additive control ele-

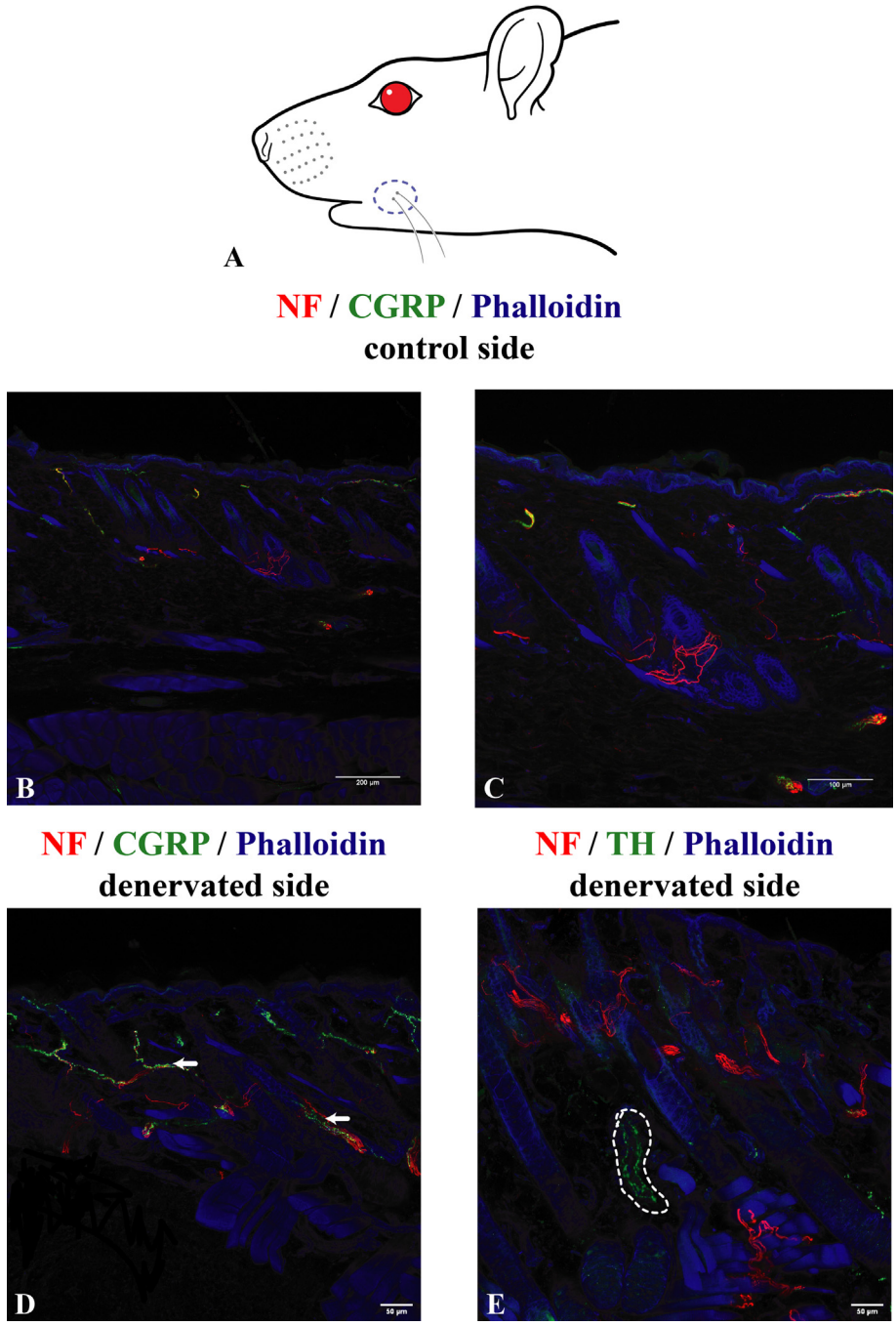

Fig. 6. Innervation of the skin and genal vibrissae via the facial nerve. **(A)** Schematic illustration of the harvesting region of the facial skin containing genal vibrissae. **(B, C)** Immunofluorescent images of the skin harvested on the unoperated side using anti-NF, anti-CGRP and phalloidin at 10x **(B)** and 20x **(C)** magnification. The images show CGRP-positive axonl bundles within the skin indicating their afferent nature. **(D, E)** Immunofluorescent images of the facial skin harvested from the denervated side. **(D)** Denervated skin show a dense innervation pattern of CGRP-positive axons after the deafferentation procedure (arrows). **(E)** Immunofluorescent staining using anti-NF, anti-TH and phalloidin antibodies show only a few TH-positive axons witihin the skin vessels, suggesting sympathetic innervation of the vessels (dashed line).

ments - joint movement and visual control - are missing in facial control, one would expect an intricate network of intramuscular afferents. Alas, facial muscles apparently do not provide this architecture and the VIIth cranial nerve is described as a "pure motor" nerve [26]. Nonetheless, the advanced control capacity of the CNS in relation to face movements suggests a sensory componentry of the extracranial facial nerve. This contradiction has triggered the present investigation with the purpose of providing a detailed description of the axonal fiber composition of the facial nerve and potentially add to our knowledge of how proprioceptive control is achieved in the facial neuromuscular system. The study has been performed with both rat and human samples.

The findings from the animal model raise questions whether the findings of this study also apply to the human facial nerve. Due to obvious ethical limitations, it is not possible to reproduce the performed methods in humans. Post-mortem retrograde labeling is technically not feasible, however, using a double immunofluorescent staining we demonstrated the heterogenous axonal composi-

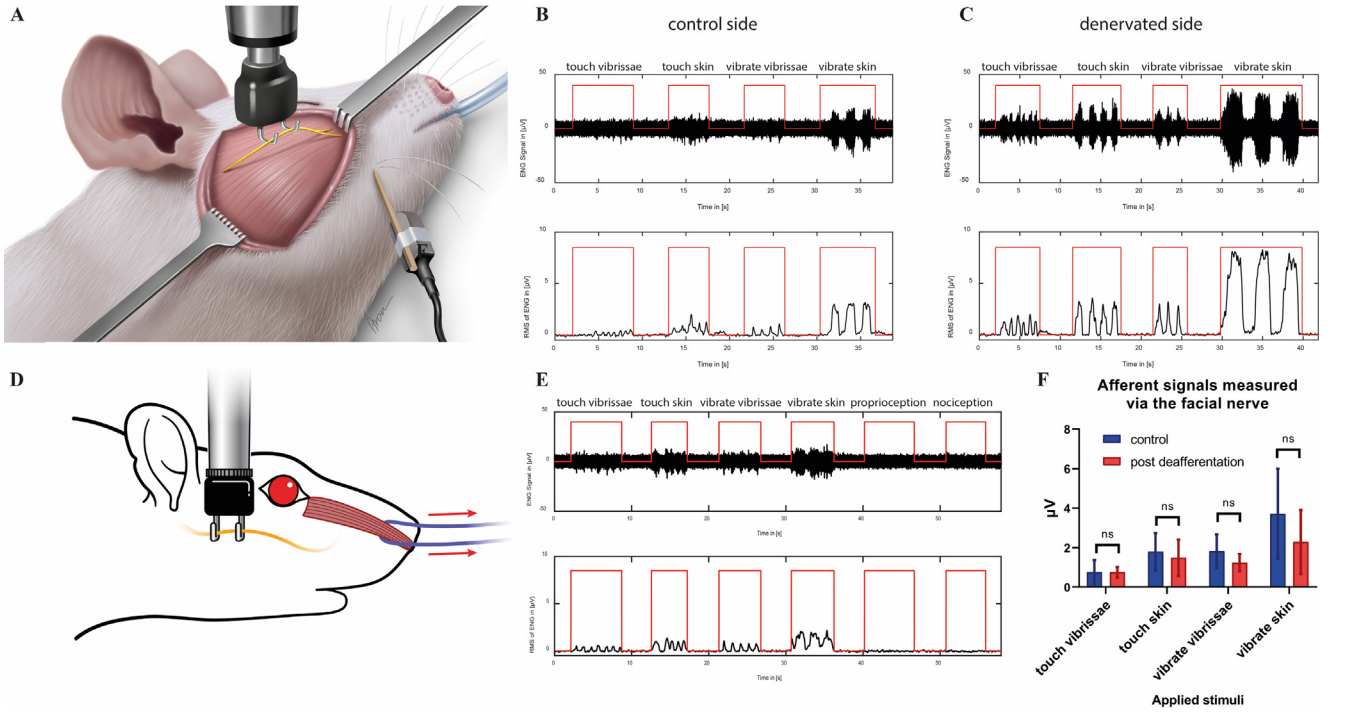

Fig. 7. Facial nerve conducts afferent signals from the genal vibrissae (A) Illustration of the experimental setting for electrophysiological measurements. The buccal branch of the facial nerve was exposed and placed onto the hook-electrode. The genal vibrissae and the surrounding skin was stimulated by slight touch of vibration. The measurements from the unoperated **(B)** and operated **(C)** sides after applying stimuli to the ipsilateral genal vibrissae in the following order: touch vibrissae, touch skin, vibrate vibrissae, vibrate skin. **(D)** Illustration of applying a stretch stimulus to the dilator nasi muscle: after the complete isolation of the muscle from the surrounding tissue, the tendinous muscle part of the dilator nasi muscle was looped using a vessel loop. Sharp and short stretches were applied to the vessel loop to induce a stretch response. **(E)** No afferent signals were obtained from the buccal branch either after applying the stretch or nociceptive stimuli. The average amplitudes measured in the unoperated rats and rats after deafferentation did not statistically differ **(F):** 0.86 ± 0.66 μV and 0.76 ± 0.27 μV respectively for touching the vibrissae (p = 0.61; Mann-Whitney test), 1.96 ± 0.96 μV and 1.49 ± 0.92 μV for touching the emergence point of the vibrissae (p = 0.62; unpaired T-test), 1.69 ± 0.88 μV and 1.24 ± 0.44 μV for vibrating the genal vibrissae (p = 0.25; unpaired T-test), 3.87 ± 2.51 μV and 2.29 ± 1.62 μV for vibrating the emerging point of the genal vibrissae (p = 0.35; Mann-Whitney test).

tion in the main trunk of the extracranial facial nerve. Quantitative analysis revealed that only 85% of all axons are cholinergic, indicating a similar axon distribution pattern as in the rat (Fig. 1, see Supplementary Fig. 1). Besides the highly specialized anatomy of the facial muscles, the human face lacks specialized perioral sensory hairs, such as the vibrissae. This may complicate the interpretation of the animal findings in humans, but clinical phenomena may shed light on the afferent role of the facial nerve in the facial innervation.

Multilevel mapping of sympathetic and afferent components within the facial nerve did, however, not yet elucidate their role within the dermato-muscular complex. Although sporadic histological findings suggested the presence of proprioceptive organs within the facial muscles [27,28], the majority of studies do not support these findings [7,8]. One of the possible explanations for the absence of proprioception in the facial muscles may be their essential role in the expression of emotions, which underlies direct cortical control [6,29].

Role of the sympathetic fibers in the facial nerve

Our findings regarding the innervation of facial muscles may reveal several insights into facial muscle control via the facial nerve. In our study neither histological nor electrophysiological evidence of proprioceptive feedback from facial muscles (LLS, MDN) was found (Fig. 7E). Abundant sympathetic muscle innervation after deafferentation revealed a sympathetic co-innervation of the facial muscles (Fig. 5D). These findings were supported by the presence of labeled neurons in the superior cervical ganglion after intramuscular retrograde labeling. However, intramuscularly labeled small neuronal population in the superior cervical ganglion can represent sympathetic fibers accompanying muscular vessels as well. Nevertheless, the TH-positive axons within the cross-section of the facial nerve indicate sympathetic contribution via the facial nerve to the muscular innervation. Sympathetic fibers are not only responsible for the modulation of universal nociception [30], but are also involved in the modulation of muscular pain [31,32]. Although the sympathetic contribution to the pathophysiology of the myofascial pain syndrome (MPS) was presumed in previous studies [33], our findings showed the sympathetic innervation of the facial muscles directly via the facial nerve. This indicates the need to investigate possible causality of facial nerve abnormalities causing damage of the sympathetic fibers and MPS symptoms.

Beside the involvement of the sympathetic fibers in the modulation of sensory perception, there is robust evidence of sympathetic contribution to motor control. Although sympathetic innervation of neuromuscular junctions was described approximately a century ago [34], essential sympathetic contribution to the maintenance and modulation of neuromuscular junctions was demonstrated only recently [35,36]. One of the hypotheses for the pathophysiology of the hemifacial spasm suggests a sympathetic involvement [37,38]. This hypothesis proposes a transmission of ectopic action potentials from sympathetic nerve endings coming from the adventitia of surrounding blood vessels to the damaged facial nerve fibers. Hence, the evidence of sympathetic axons travelling via the facial nerve to the facial muscles suggests several modifications to a recent "sympathetic hypothesis" for the pathogenesis of the hemifacial spasm. Our findings highlight the possible involvement of sympathetic fibres travelling within the

facial nerve in abnormal activation of neuromuscular junctions in the facial muscles [39]. This hypothesis supports clinical data on the abrupt symptom cessation of the hemifacial spasm after the microvascular decompression of the facial nerve [40]. Moreover, it explains the anatomical course of the postganglionary sympathetic fibers to the extracranial facial nerve. Sympathetic fibers may travel from the superior cervical ganglion through the adventitia of a vessel of the posterior circulation to the extracranial facial nerve.

The evidence of the mixed axonal composition of the facial nerve may elucidate differences in the postoperative outcome in facial palsy patients after using different donor nerves for muscle reinnervation [41,42]. The muscle strength and resting tone of the reinnervated muscle are the most significant criteria for the clinical outcome. It has been shown that sympathetic activation modulates muscle contractility by fastening its twitch force [43]. When muscle contraction is about to reestablish in patients with recovering facial palsy, an excessive muscle tonus at rest and uncontrolled/exuberant excursions for voluntary movements are commonly observed symptoms and might be explained by a yet imperfect sensory-motor interplay of regenerating facial nerve fibers at the site of the muscle organ. Against the background of our results, it is exactly this ensemble acting of different fibers of the facial nerve that is trained by profound pan-facial physiotherapy to regain highly coordinated, symmetrical movements. This may be regulated by the co-modulation of the neuromuscular junctions by sympathetic fibers. These findings shed light on the pathophysiology of different clinical conditions (hemifacial spasm and orofacial pain) and can optimize surgical treatment modalities for facial paralysis and facial transplantation.

Role of the afferent fibers in the facial nerve

Another insight of this study reveals afferent skin innervation via the extracranial facial nerve. As the "pure motor" nature of the facial nerve is widely accepted in humans, the lower vertebrates (amphibians, reptiles etc.) are known for a high proportion of sensory components within the facial nerve, which is notably reduced in the ascending evolutionary scale of vertebrates [44]. This is mainly due to the differentiation of the facial innervation in highly developed species: predominant innervation of the cutaneous sensory area via the trigeminal nerve and motor innervation of the facial muscles via the facial nerve [45]. Nevertheless, many speculations regarding deep afferent sensations via the facial nerve in humans were proposed but never rigorously verified [45–47]. Interestingly, tiny unmyelinated CGRP-positive axons were identified in cross-sections of the facial nerve of the rat, whereby no thick myelinated afferent fibers have been found. Thus, these axons can represent evolutionary remnants of sensory fibers from lower vertebras. Our electrophysiological findings showed specific regional cutaneous and vibrissal afferent innervation via the facial nerve for the first time after deafferentation of the rat's face (Fig. 6C). The genal vibrissae represent a highly complex sensory-motor organ and a scarcely explored entity of the tactile sensing system in mammals [48,49]. Interestingly, despite the well-explored anatomy of vibrissal innervation, no afferent twigs branching from the infraorbital nerve were found innervating the genal vibrissae in the literature [49]. Our electrophysiological findings are supported by the immunofluorescent staining of the deafferentiated facial skin, mixed axonal composition and retrogradely labeled neurons within the geniculate ganglion.

One may speculate the originally significant involvement of the facial nerve in the tactile perception on the lower evolutionary stages is remained as a vestige in humans due to the high morphological specialization. Due to the absence of specialized tactile organs like vibrissae in the human face, the exact role of the afferent components is difficult to interpret. Nevertheless, recent anecdotal findings showed presumably modified mechanosensitive structures in the human facial muscles [50]. This may point out an adaptation modality of afferent cutaneous fibers travelling within the facial nerve, making them more an exaptation then a vestige in the human's face.

Various disorders and injuries of the facial nerve remain a therapeutic challenge. Most treatment approaches are based on the assumption that the facial nerve is a "pure" somatic motor nerve, however, many clinical phenomena cannot be explained if this were the case. For example, to assess axonal growth in the regenerating facial nerve in patients after a reconstructive surgery, tactile sensations are triggered by tapping the skin area over the regenerating nerve (Tinel's sign) [42,51]. Moreover, numerous victims of Bell's palsy report that a prodrome of pain, dull aching, or pulling discomfort behind the ear and/or across the affected hemiface precedes the full spectrum of symptoms [52]. Likewise, Ramsay Hunt syndrome – a late reactivation of the dormant Varicella-zoster virus migrating along the facial nerve via the geniculate ganglion – typically includes pain along the ipsilateral side of the face [53]. Our findings elucidate these persistent clinical observations and indicate the presence of afferent axons within the human facial nerve. Moreover, abundant literature demonstrated the evidence of enigmatic changes in cutaneous sensibility of the face in patients suffering exclusively from facial palsy irrespective of the etiology [54,55]. On the contrary, the remaining tactile perception after either complete denervation [46] or anesthesia [45] of the cutaneous trigeminal branches was described decades ago [47]. These observations suggest the hitherto elusive involvement of the facial nerve in tactile perception of the face, which was confirmed by our results from the animal model. However, anatomy of axonal communications between the trigeminal and the facial nerve branches is shifted in the rodents' face much more rostral compared to human [15,23]. Thus, the caution is needed in extrapolation of animal data to clinical phenomena. Due to different topographical patterns of axonal communications between cranial nerve, some neurobiological phenomena in rodents do not necessarily reflect clinical phenomena in humans.

Nowadays, extensively disfiguring injuries of the face with consequent patient's social withdrawal or death may be surgical addressed by face transplantation [56,57]. Despite its increasing feasibility, the neurobiological mechanisms in the motor and sensory reinnervation of the transplanted face are still scarcely explored [58]. One of the surgical challenges is achieving complete reinnervation of the dermato-muscular matrix of the face [59]. Our findings contribute to a better understanding of the spontaneous sensory recovery in facial grafts without coaptation of all sensory nerves [19,60,61]. This may be explained by the aberrant sprouting of afferent fibers from the facial nerve to contribute to cutaneous reinnervation of the facial graft. Moreover, the abundance of sympathetic fibers within the facial nerve may be responsible for the restoration of cutaneous thermoregulation in the facial graft [62]. These findings may highlight the prioritization of the coaptation of all distal branches of the facial nerve in the face transplantation surgery due to the motor and possible sensory recovery of the facial graft.

This multi-level investigation of the facial nerve demonstrated a heterogenous axonal composition consisting of about 85% somatic efferent fibers in both rat and human samples. In the rat the remaining fibers were distributed equally to 7% sympathetic and roughly 8% afferent axons. We could further demonstrate that the sympathetic fibers contributed to the innervation of muscle as well as afferent innervation of the facial skin, which was electrophysiologically confirmed. These results are concordant with the pathophysiology of different clinical conditions (facial palsy, hemi-

facial spasm, myofascial pain syndrome and facial transplantation) and may contribute to novel therapeutic strategies.

Compliance with Ethics Requirements

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5).

CRediT authorship contribution statement

Vlad Tereshenko: Conception and design, Surgical procedure, Imaging analysis, Electrophysiological analysis, Analyses and interpretation of data, Drafting of the article. **Udo Maierhofer:** Conception and design, Surgical procedure, Imaging analysis, Analyses and interpretation of data. **Dominik C.Dotzauer:** Surgical procedure, Imaging analysis, Electrophysiological analysis. **Gregor Laengle:** Surgical procedure, Electrophysiological analysis, Analyses and interpretation of data, Drafting of the article. **Martin Schmoll:** Electrophysiological analysis, Analyses and interpretation of data, Drafting of the article. **Christopher Festin:** Electrophysiological analysis, Drafting of the article. **Matthias Luft:** Surgical procedure. **Genova Carrero Rojas:** Imaging analysis. **Olga Politikou:** Imaging analysis, Analyses and interpretation of data. **Laura A.Hruby:** Surgical procedure, Imaging analysis, Analyses and interpretation of data, Drafting of the article. **Holger J.Klein:** Analyses and interpretation of data, Drafting of the article. **Steffen U.Eisenhardt:** Analyses and interpretation of data, Drafting of the article. **Dario Farina:** Analyses and interpretation of data, Supervision, Drafting of the article. **Roland Blumer:** Conception and design, Imaging analysis, Analyses and interpretation of data, Supervision, Drafting of the article. **Konstantin D.Bergmeister:** Conception and design, Surgical procedure, Imaging analysis, Analyses and interpretation of data, Supervision. **Oskar C.Aszmann:** Conception and design, Surgical procedure, Imaging analysis, Electrophysiological analysis, Analyses and interpretation of data, Supervision, Drafting of the article.

All authors critical revision and final approval of the version to be published.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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