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Connections between postparotid terminal branches of the facial nerve: An immunohistochemistry study

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Abstract

It has been assumed that connections between the postparotid terminal branches of the facial nerve are purely motor. However, the nature of their fibers remains unexplored. The aim of this study is to determine whether these connections comprise motor fibers exclusively. In total 17 connections between terminal facial nerve branches were obtained from 13 different facial nerves. Choline acetyltransferase antibody (ChAT) was used to stain the fibers in the connections and determine whether or not all of them were motor. All connections contained ChAT positive and negative fibers. The average number of fibers overall was 287 (84–587) and the average proportion of positive fibers was 63% (37.7%–91.5%). In 29% of the nerves, >75% of the fibers were ChAT+ (strongly positive); in 52.94%, 50%–75% were ChAT+ (intermediately positive); and in 17.65%, <50% were ChAT+ (weakly positive). Fibers traveling inside the postparotid terminal cranial nerve VII branch connections are not exclusively motor.

KEYWORDS

choline acetyltransferase, facial nerve, facial nerve motor disorders, immunohistochemistry, nerve fibers

1 | INTRODUCTION

Connections between the five terminal branches of the facial nerve (cranial nerve [cn] VII) have been described since the second half of the nineteenth century. They constitute a structure with multiple connected branches called the "subparotid plexus" (Hovelaque, 1927; Sappey, 1879) or "parastenon plexus" (Pons-Tortella, 1947). Most authors have used this description and it has served as a basis for different proposed facial nerve classifications (Alomar, 2021; Bernstein & Nelson, 1984; Davis et al., 1956; Katz & Catalano, 1987; Kitamura & Yamazaki, 1958; Martínez Pascual et al., 2019; Myint et al., 1992; Tzafetta & Terzis, 2010).

Connections between facial nerve branches have been found more frequently in the temporofacial division (TF). This has been attributed to its greater number of branches and its plexiform nature; the cervicofacial division (CF) supplies fewer branches, so connections between them are less common (Davis et al., 1956; Diamond et al., 2011; Lineaweaver et al., 1997; Martínez Pascual et al., 2019; Pons-Tortella, 1947; Salame et al., 2002; Tansatit et al., 2015).

It has been assumed that the fibers within these connections are motor because the five terminal branches of the facial nerve supply the mimic muscles, and the sensory innervation of the face depends on the trigeminal nerve (cn V) (Shoja et al., 2014). However, branches of cn VII also communicate with terminal ramifications of cn V in the face: the auriculotemporal (Kwak et al., 2004; Namking et al., 1994; Tansatit et al., 2015), supraorbital (Hwang et al., 2005; Li et al., 2009; Martínez Pascual et al., 2019), infraorbital (Hu et al., 2007; Hwang et al., 2004; Martínez Pascual et al., 2019; Tansatit et al., 2016), and mental (Hwang et al., 2007; Kim et al., 2009; Martínez Pascual et al., 2019; Touré et al., 2019) nerves, or the well-known connection between the lingual nerve and the chorda tympani conveying the sense of taste (Dixon, 1899; Kwak et al., 2004; Hwang et al., 2007; Diamond et al., 2011; Shoja et al., 2014; Takezawa & Kageyama, 2015).

Different types of fibers have been proposed to constitute the VII-V connections: autonomic (Bowden & Mahran, 1960; Lewy et al., 1938; Tansatit et al., 2015), motor (Conley, 1964; Martin & Helsper, 1957; Odobescu et al., 2012) or sensory (Baumel, 1974; Cobo, Abbate, et al., 2017; Cobo, Solé-Magdalena, et al., 2017; Odobescu et al., 2012; Yang et al., 2013). Thus, non-motor fibers of VII-V connections could continue to travel through the postparotid facial connections, so not all the fibers inside those connections are necessarily motor type. However, all these studies of the VII-VII and V-VII connections are based on anatomical dissection, which does not reveal the real functions of the component fibers.

Therefore, the goal of our study is to determine, using specific immunohistochemical techniques, whether the connections between the terminal branches of the facial nerve are purely motor or whether they also carry other types of fiber.

2 | MATERIALS AND METHODS

The study was carried out on 13 hemiheads from embalmed adult Caucasian bodies (seven men, five women) from the Body Donations and Dissecting Rooms Centre of the Complutense University of Madrid. The average age of the cadavers was 83 years (range 75–90 years) at the time of death.

The authors state that every effort was made to follow all local and international ethical guidelines and laws that pertain to the use of human cadaveric donors in anatomical research (Iwanaga et al., 2022).

2.1 | Microdissection

The extrapetrous course of 13 facial nerves (seven right, six left) was dissected from proximal to distal using microsurgical forceps and scissors with the help of surgical loupes (2.5x) (Optimedic®). The facial nerve was dissected and the postparotid terminal facial-facial connections were identified, sectioned, and extracted for processing.

2.2 | Immunohistochemistry

Immunohistochemistry was performed in the Section of Anatomy from Department of Neuroscience at the University of Padova and the Department of Immunology, Ophthalmology and ENT at the Complutense University School of Medicine in Madrid. The connections were processed and embedded in paraffin blocks. Transverse sections (6 μm thickness) were cut with a microtome and mounted on slides, deparaffinized, and rehydrated before staining. Antigen retrieval was performed with sodium citrate (pH 6.1) for 20 min at 95°C. The sections were then washed in phosphate-buffered saline (PBS) and placed in 1% hydrogen peroxide (H_2O_2) in PBS for 10 min at room temperature, and then put into blocking buffer (0.2% bovine serum albumin in PBS) for 1 h at room temperature. After washing with PBS, they were incubated for 24 h at 4°C with the primary antibody anti-choline acetyl transferase (ChAT) (Gene Tex® [N1N3]; 1:800). A negative control was performed for each different sample. The slides were washed with PBS and incubated with secondary antibody (goat anti-rabbit, Jackson Immunoreserch®; 1:300) in blocking buffer for 1 h at room temperature. After further washing, color was developed with DAKO chromogen for 30 s. The samples were washed with distilled water and finally counterstained with hematoxylin, dehydrated and mounted.

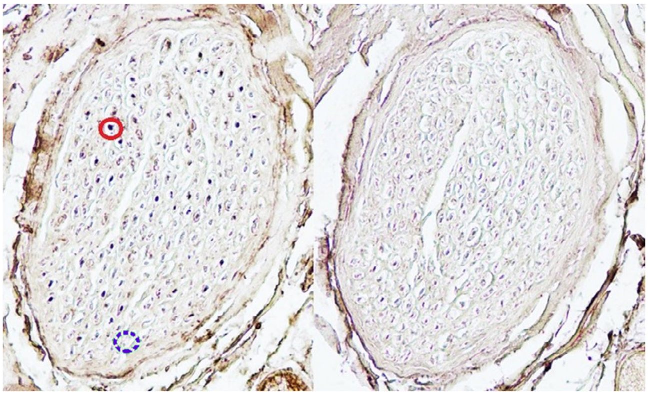

FIGURE 1 Example of images used for counting axons. The continuous circle shows a ChAT+ motor fiber. The discontinuous circle shows a ChAT− fiber.

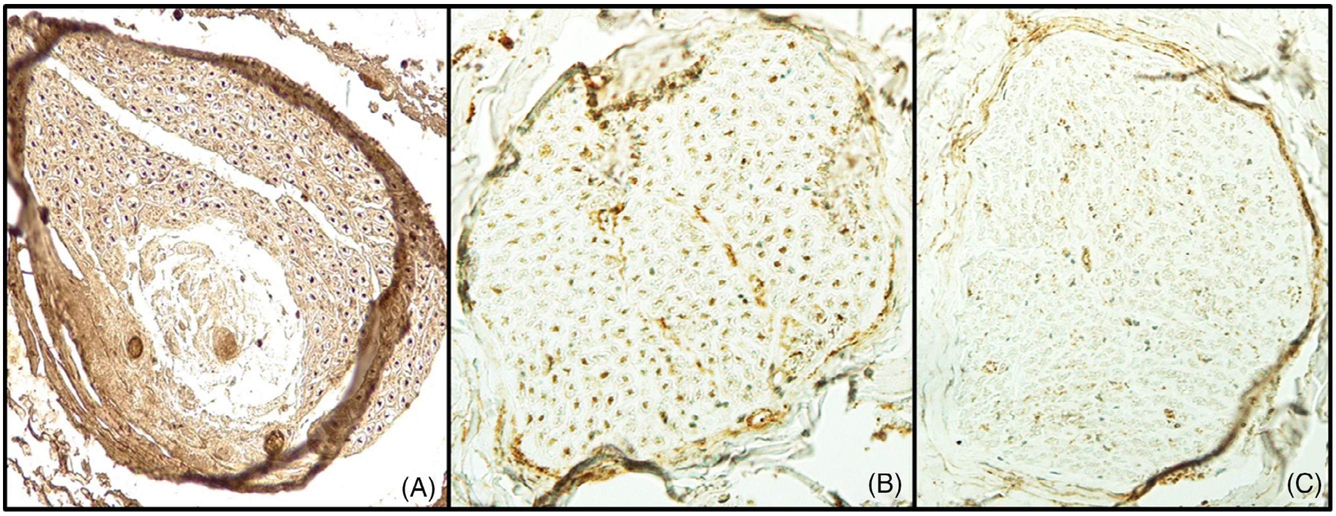

FIGURE 2 Classification of facial nerve connections based on number of motor fibers. (A) Strongly positive connection (>75% of ChAT+); (B) Intermediately positive (50%–75% of ChAT+); (C) Weakly positive (<50% of ChAT+).

2.3 | Images analysis

The immunohistochemical images were photographed under a microscope (Nikon E800M). Motor axons (ChAT positive) (Figure 1) were counted in every sample using ImageJ Fiji 1.52p software (National Institutes of Health®) with 20× magnification. The connections were classified on the basis of number of motor fibers: strongly positive (>75% ChAT+ fibers), intermediately positive (50%–75% ChAT+ fibers) and weakly positive (< 50% ChAT+ fibers) (Figures 2A–C).

2.4 | Statistical analysis

Both descriptive and analytical statistics were used; percentages, means, ranges, and standard deviations were collected and compared. A one-factor experimental design was used to detect significant differences in the average number of fibers with respect to side. The Kolmogorov–Smirnov Test was used to determine the normality of the underlying data distribution. A significance level alpha = 0.05 was used for all tests. SPSS software version 22 (IBM Corporation, Armonk) was used for the analyses.

3 | RESULTS

A total of 17 VII-VII connections were analyzed. These connections were more frequent on the left side (10 connections) than on the right (seven). Nine of the connections were in male specimens while the other eight were in female cadavers. Tables 1 and 2 show the distribution of connections by side and sex respectively.

The different types of connections are reported first, followed by a global overview.

3.1 | Temporo-temporal

A temporo-temporal (tt) connection was found in one case. It had 84 fibers, 76.2% (64/84) of them ChAT positive (ChAT+) and the other 23.8% (20/84) ChAT negative (ChAT−). Therefore, this connection was strongly positive (Figure 3A).

3.2 | Temporo-zygomatic

Two temporo-zygomatic (tz) connections were found. The number of fibers in them totalled 469, and the average was 234 (range 134–335); 46.1% (216/469) were ChAT+ while 53.9% (253/469) were ChAT−. Both tz connections were weakly positive (Figure 3B).

3.3 | Zygomatic-zygomatic

There were two zygomatic-zygomatic (zz) connections. The number of fibers in them totalled 685, and the average was 343 (259–426); 69.8% (478/685) were ChAT+ and 30.2% (207/685) were ChAT−. One connection was classed as strongly positive while the other was intermediately positive (Figure 3C).

3.4 | Zygomatic-buccal

The zygomatic-bucal (zb) connection was the most frequent, being found in six cases. Five of the buccal branches (b) arose from the TF division and one from the CF division. The total number of fibers was 1871 and the average was 312 (144–587); 53.9% (1009/1871) were ChAT+ and 46.1% (862/1871) were ChAT−. Five were intermediately positive and the other was weakly positive (Figure 2B).

TABLE 1 Distribution of facial nerve connections by side.

Name of connection ($n = 17$)	Average N° fibers	Right (7)		Left (10)		Total %	
		ChAT+	ChAT−	ChAT+	ChAT−	ChAT+	ChAT−
Temporo-temporal ($n = 1$; 1 R)	84	76.2% (64/84)	23.8% (20/84)	—	—	76.2% (64/84)	23.8% (20/84)
Temporo-zygomatic ($n = 2$; 2 L)	234 (134–355)	—	—	46.1% (216/469)	53.9% (253/469)	46.1% (216/469)	53.9% (253/469)
Zygomatico-zygomatic ($n = 2$; 1 R, 1 L)	343 (259–406)	56.6% (241/426)	43.4% (185/426)	91.5% (237/259)	8.5% (22/259)	69.8% (478/685)	30.2% (207/685)
Zygomatico-buccal ($n = 6$; 2 R, 4 L)	312 (144–587)	60.1% (345/574)	39.9% (229/574)	51.5% (664/1271)	48.5% (633/1271)	53.9% (1009/1871)	46.1% (862/1871)
Bucco-buccal ($n = 1$; 1 R)	115	80% (92/115)	20% (23/115)	—	—	80% (92/115)	20% (23/115)
Bucco-mandibular ($n = 3$; 1 R, 2 L)	286 (259–322)	65.2% (167/256)	34.8% (89/256)	69.3% (417/602)	30.7% (185/602)	68.1% (584/858)	31.9% (274/858)
Mandibulo-cervical ($n = 2$; 1 R, 1 L)	399 (232–566)	75% (174/232)	25% (58/232)	64.1% (363/566)	35.9% (203/566)	67.3% (537/798)	32.7% (261/798)

Abbreviations: L, left; n, number of samples; R, right.

TABLE 2 Distribution of facial nerve connections by sex.

Name of connection ($n = 17$)	Average N° fibers	Male (9)		Female (8)		Total %	
		ChAT+	ChAT−	ChAT+	ChAT−	ChAT+	ChAT−
Temporo-temporal ($n = 1$; 1 M)	84	76.2% (64/84)	23.8% (20/84)	—	—	76.2% (64/84)	23.8% (20/84)
Temporo-zygomatic ($n = 2$; 2 M)	234 (134–355)	46.1% (216/469)	53.9% (253/469)	—	—	46.1% (216/469)	53.9% (253/469)
Zygomatico-zygomatic ($n = 2$; 1 M, 1 F)	343 (259–406).	56.6% (241/426)	43.4% (185/426)	91.5% (237/259)	8.5% (22/259)	69.8% (478/685)	30.2% (207/685)
Zygomatico-buccal ($n = 6$ 3 M, 3 F)	312 (144–587)	55% (639/1161)	45% (522/1161)	52.1% (370/710)	47.9% (340/710)	53.9% (1009/1871)	46.1% (862/1871)
Bucco-buccal ($n = 1$; 1 F)	115	—	—	80% (92/115)	20% (23/115)	80% (92/115)	20% (23/115)
Bucco-mandibular ($n = 3$; 2 M, 1 F)	286 (259–322)	75.3% (435/578)	24.7% (143/578)	53.2% (149/280)	46.8% (131/280)	68.1% (584/858)	31.9% (274/858)
Mandibulo-cervical ($n = 2$; 2 F)	399 (232–566)	—	—	67.3% (537/798)	31.7% (261/798)	67.3% (537/798)	31.7% (261/798)

Abbreviations: F, female; L, left; M, male; n, number of samples; R, right.

3.5 | Bucco-buccal

We found just one bucco-buccal (bb) connection. Both b branches belonged to the TF division. It had 115 fibers, 20% (23/115) of them ChAT− and 80% (92/115) ChAT+, so it was a strongly positive connection (Figure 3D).

3.6 | Bucco-mandibular

There was a bucco-mandibular (bm) connection in three facial nerves. Two b branches came from the TF division and the other from the CF. The total number of fibers was 858 and the average was 286 (259–322), 68.1% (584/858) of them ChAT+ and 31.9% (274/858) ChAT−. Two connections were intermediately positive while the other was strongly positive (Figure 3E).

3.7 | Mandibulo-cervical

There was a mandibulo-cervical (mc) connection in two facial nerves. These had the highest number of fibers, averaging 399 (232–566) with a total number of 798, 32.7% (261/798) being ChAT− and 67.3% (537/798) ChAT+. One connection was strongly positive and the other intermediately positive (Figure 3F).

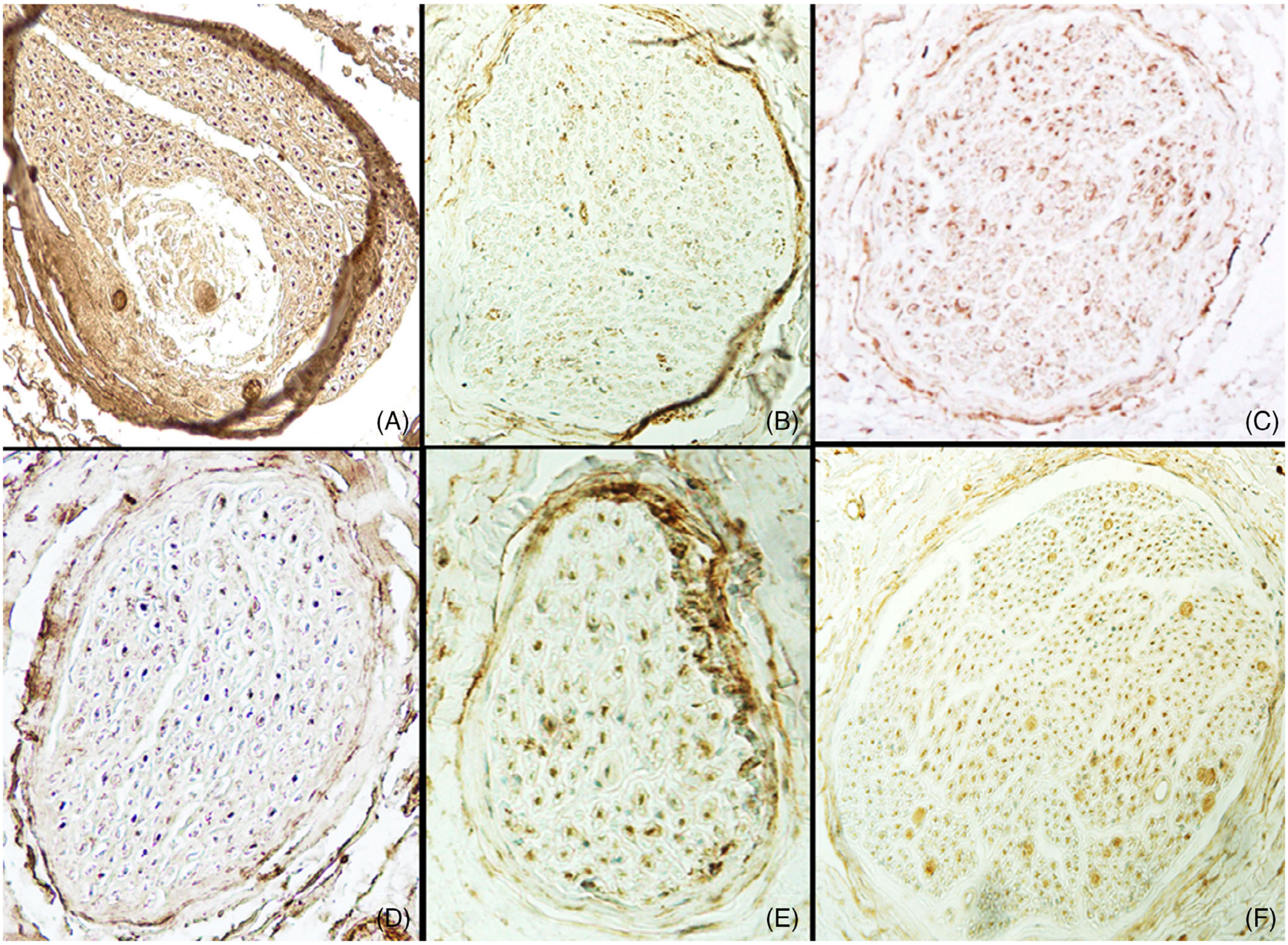

FIGURE 3 Different types of terminal facial nerve connections. (A) Temporo-temporal (strongly +); (B) Temporo-zygomatic (weakly +); (C) Zygomatic-zygomatic (intermediately +); (D) Bucco-buccal (strongly +); (E) Bucco-mandibular (intermediately +); (F) Mandibulo-cervical (strongly +).

3.8 | Global view

The average number of fibers in the VII-VII connections overall was 287 with a standard deviation of 145.46 (range 84–587), and the average proportion of positive fibers was 63% with a standard deviation of 15% (range 37.7%–91.5%).

The average number of fibers on the left side was 319 with a standard deviation of 146.49 (range 134–587), and the average proportion of positive fibers was 58.9% with a standard deviation of 17.2% (range 37.6%–91.5%). In contrast, the average number of fibers in the right side was 241 with a standard deviation of 141.5 (range 84–430), and the average proportion of positive fibers was 68.9% with a standard deviation of 9.82% (range 55.6%–80%) (Figure 4).

The average number of fibers in males was 302 with a standard deviation of 164.81 (range 84–587) and the average proportion of positive fibers was 61.21% with a standard deviation of 14.03% (range 43.3%–83.2%). The average number of fibers in females was 270 with a standard deviation of 129.3 (range 115–566) and the average proportion of positive fibers was 64.9% with a standard deviation of 17.1% (range 37.6%–91.5%) (Figure 4).

The distributions of both ChAT+ and ChAT− fibers was normal (Kolmogorov–Smirnov Test *p*-values = 0.821 and 0.871, respectively) and the ANOVA table associated with the one factor experimental design corroborated the hypothesis of equity between the average numbers of positive fibers by side (*p*-value = 0.429) and of negative fibers by side (*p*-value = 0.273).

Similar results were obtained for sex (*p*-values = 0.926 for ChAT + fibers and 0.483 for ChAT− fibers). Therefore, there were no statistically significant side or sex differences.

Strongly positive ChAT+ connections were found in 29% of the nerves in the sample (five cases in 17), intermediately positive in 52.94% (nine cases in 17) and weakly positive in 17.65% (three cases in 17).

4 | DISCUSSION

Previous studies of VII-VII connections were based on anatomical dissection, so the real functions of those fibers could not be established. No other immunohistochemical studies of the types of fibers in the

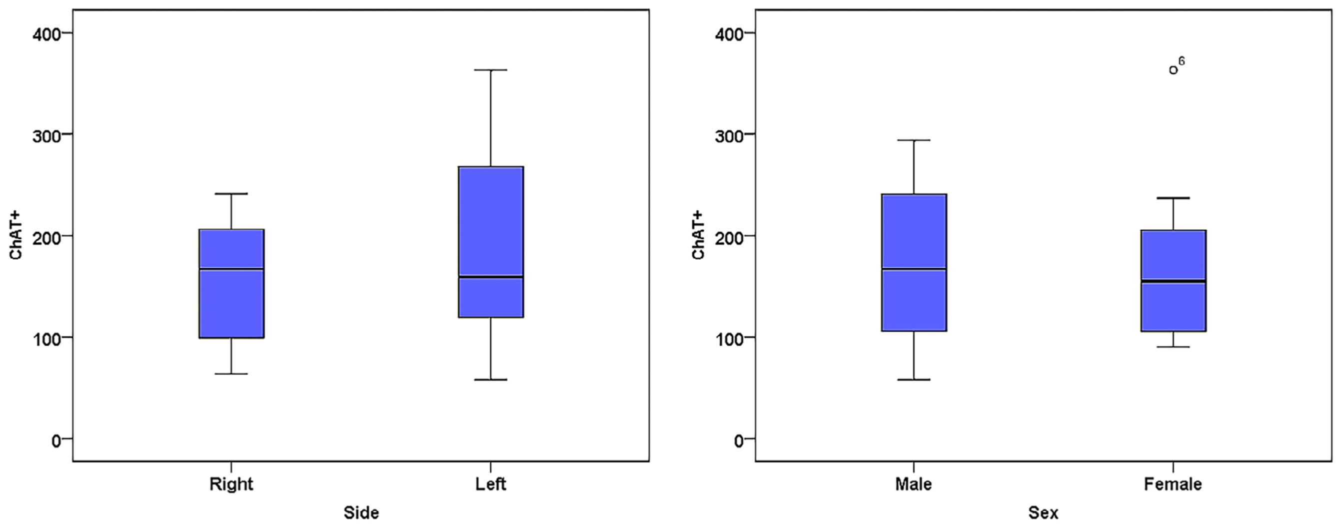

FIGURE 4 Box plot diagram of ChAT+ fibers by side and sex. The number of ChAT+ fibers was more variable on the left side than the right, and more variable in males than females, but these differences were not statistically significant.

postparotid facial connections in humans were found in the literature studied, so our results cannot be compared with others.

ChAT antibody has been proved specific/selective for motor axons in peripheral nerves (Castro et al., 2008; Courties et al., 2020; Kim et al., 2018; Lago et al., 2007; Lago & Navarro, 2006; Zhou et al., 2021), even after nerve injury (Castro et al., 2008; Kim et al., 2018; Lago et al., 2007; Lago & Navarro, 2006; Zhou et al., 2021). Therefore, it was chosen in this study to establish whether or not the VII-VII connections are exclusively motor. The results showed that every connection had ChAT+ and ChAT− fibers, so they all contained both motor and non motor fibers.

The connection with the highest percentage of ChAT− fibers was the temporo-zygomatic (53.9%), while the bucco-buccal had the highest percentage of ChAT+ fibers (80%). This could be because the buccal branches innervate the middle and lower facial thirds, where there are more muscles (Le Louarn, 2009) than in the upper third (Abramo, 2018; Abramo et al., 2016); bucco-buccal connections could therefore carry a greater motor axonal load.

Connections between the sensory terminal ramifications of cn V in the face and the terminal branches of the extrapetrous cn VII have already been described with the auriculotemporal (Kwak et al., 2004; Namking et al., 1994; Tansatit et al., 2015), supraorbital (Hwang et al., 2005; Li et al., 2009; Martínez Pascual et al., 2019), infraorbital (Hu et al., 2007; Hwang et al., 2004; Martínez Pascual et al., 2019; Tansatit et al., 2016), and mental (Hwang et al., 2007; Kim et al., 2009; Martínez Pascual et al., 2019; Touré et al., 2019) nerves. The nature of those connections has been discussed by many authors. Some believe they can be autonomic (Bowden & Mahran, 1960; Lewy et al., 1938; Tansatit et al., 2015), some motor (Conley, 1964; Martin & Helsper, 1957; Odobescu et al., 2012) and some sensory (Baumel, 1974; Cobo, Solé-Magdalena, et al., 2017; Odobescu et al., 2012; Yang et al., 2013). Thus, assuming that these fibers continue to travel from the V-VII connection through the rest of the extrapetrous facial nerve, it follows that the terminal branches of the facial nerve and their connections also have non-motor fibers (Cattaneo & Pavesi, 2014; Cobo, Solé-Magdalena, et al., 2017).

Facial muscles typically lack proprioceptors, and facial proprioceptive impulses travel via branches of the trigeminal nerve to the central nervous system (Cattaneo & Pavesi, 2014; Cobo, Solé-Magdalena, et al., 2017). Those propioceptive fibers from the trigeminal nerve in facial-trigeminal connections could also be conveyed in VII-VII connections and could innervate sensory structures in facial muscles that substitute for typical muscle spindles in facial proprioception (Cattaneo & Pavesi, 2014; Cobo, Abbate, et al., 2017).

These findings could explain some of the feelings experienced by a patient with facial palsy after partial recovery, such as painful pressure at some points of the face (e.g. the zygomatic muscles, chin, forehead) (Valls-Solé, 2013). Furthermore, muscle mass contraction or synkinesis, both observed in facial palsy patients, could also be related to this mixture of fibers traveling through the multiple connections in the face (Raslan et al., 2020; Valls-Solé, 2013). Indeed, some authors explain synkinesis in terms of activation of latent nervous circuits preexisting in the healthy subject (Ton Van & Giot, 2021). We believe that these preexisting neural circuits can be conveyed by VII-VII connections.

Therefore, we can affirm that the nerve fibers traveling inside the postparotid terminal facial branch connections are not exclusively motor. The nature of the fibers that do not stain for ChAT still needs to be studied using antibodies specific/selective for different types of sensory and autonomic nerves.

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