



## Expression of Choline Acetyltransferase of the Peripheral Type in the Primary Sensory Neurons of the Guinea Pig Trigeminal Ganglion

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### Key words

Trigeminal ganglion; Choline acetyltransferase, Substance P; Calcitonin gene-related peptide; Protein gene product 9.5.

### ABSTRACT:

The fact that the splice variant form of choline acetyltransferase (pChAT) is expressed in peripheral organs, including sensory ones, preferentially than the common type (cChAT) is well known. In the current study the possible functional significance of this variant in sensory neurons has been characterized immunohistochemically by investigating the pChAT-immunoreactivity (IR) in the trigeminal ganglia (TG) of the guinea pig. We documented an almost uniform distribution and a considerable number of pChAT-immunoreactivity of all trigeminal neurons. The size of pChAT-IR neurons varied from small to medium-size, although large-sized neurons also observed. Most pChAT reactivity was mainly in the cytoplasm with few number of pChAT-IR neurons had nuclear staining. Double immunofluorescent study showed that a great proportion of substance P (SP)- and calcitonin gene-related peptide (CGRP)-positive trigeminal cells showed pChAT-immunoreactivity, although those with SP outnumbered those with CGRP. The intracellular expression of pChAT (which differs from that of cChAT) probably reflecting a difference in the physiological roles between pChAT and cChAT in ACh production in distinct intracellular compartments. The present data suggest also that pChAT may play roles other than nociception and may be involved in the sensory functions of the TG neurons.

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### 1-Introduction

The trigeminal ganglion (or Gasserian ganglion or semilunar ganglion) is a sensory ganglion of the trigeminal nerve that occupies a cavity in the dura mater, covering the trigeminal impression near the apex of the petrous part of the temporal bone. From its convex border, three large nerves emerges; the ophthalmic, maxillary, and mandibular. In rodents, the trigeminal ganglion is important, as it is the first part of the pathway from the whiskers to the brain. It lies at the base of the skull and projects to trigeminal brain stem areas (Ziyal et al., 2004; Leston, 2009; Bathla and Hegde, 2013). (Tooyama and Kimura, 2000) cloned a splice variant of cDNA of acetylcholine synthesizing enzyme, ChAT, lacks exons 6–9 and called its protein product, pChAT. This was because pChAT immunohistochemistry readily revealed peripheral cholinergic structures and it is widely accepted now to be one of ACh synthesizing enzymes too (for review see Bellier and Kimura, 2011). Immunohistochemistry using antibody against pChAT preferentially reveals peripheral cholinergic structures including the trigeminal ganglia of rat but not guinea pig (Nakajima et al., 2000; Chiocchetti et al., 2003; Yasuhara et al., 2004; Yasuhara et al., 2007 and 2008). In addition pChAT immunostaining could reveal neurons and nerve

fibers that have not previously been accepted as cholinergic such as magnocellular neurons in the tuberomammillary nucleus of the posterior hypothalamus (Kanayama et al., 2003) and in some retinal ganglion cells (Yasuhara et al., 2003). The assumption that there is cholinergic sensory neurons was confirmed after localization of pChAT-IR neurons in dorsal root ganglia (Bellier and Kimura, 2007), nodose ganglia (Nakanishi et al., 1999; Okano et al., 2006), trigeminal ganglia of rat (Yasuhara et al., 2004; 2007 and 2008) and rat cochlea (Kitanishi et al., 2013). Using Western blotting, RT-PCR and ChAT enzyme assay, (Yasuhara et al., 2004; Bellier and Kimura, 2007) confirmed the cholinergic nature of some primary sensory neurons.

Substance P (SP) and calcitonin gene-related peptide (CGRP) are found mainly in small to medium-sized ganglion cells of primary sensory neurons in TG and DRG, which are neurotransmitters for nociceptive sensory neurons (Tervo et al., 1981; Wisenfeld-Hallin et al., 1984; Lee et al., 1985a; Skofitsch and Jacobowitz, 1985; Matsuyama et al., 1986; Yasuhara et al., 2007 and 2008). Colocalization of these two peptides with pChAT has never been studied before in guinea pig TG and so the possible function of pChAT in TG neurons need

to be elucidated. Moreover pChAT-immunoreactivity has been studied well and characterized in detail in rat trigeminal ganglia (Yasuhara et al., 2004; 2007 and 2008) and non-human primate (Koga et al., 2013) indicating that pChAT play roles in nociception. However, such positive neurons were not identified in guinea pig TG before. In the present study, therefore, we further characterized pChAT-IR trigeminal neurons in the guinea pig by investigating cell size, distribution and colocalization with other chemical markers such as SP, CGRP, and a general neuronal marker, protein gene product 9.5 (PGP 9.5) in a trial to know in which sensory modality pChAT may play a role.

## 2-Materials and methods

Male Hartley guinea pigs weighing 300-450 gm were used in the current study. Procedures involving animals and their care were conducted in conformity with the standards for animal experiments and comply with the NIH Guide for the Care and Use of Laboratory Animals, US (1996). Under pentobarbital anesthesia (50-80 mg/kg, i. p.), each animal was perfused on crushed ice through the ascending aorta with 10 mM phosphate-buffered saline (PBS; pH 7.4), followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, the trigeminal ganglion was dissected out from each guinea pig and then immersed overnight in the same fixative at 4 °C. After cryoprotection by immersion for 48 h in 0.1 M PB containing 15% sucrose at 4 °C, the tissues were frozen in OCT compound using compressed CO<sub>2</sub> and cut into 14-µm-thick serial sections in a cryostat, and collected in 0.1 M PBS containing 0.3% Triton X-100 (PBST). Some paraffin sections (5-7 µm-thicks) were prepared and stained by hematoxylin and eosin (H and E) for histological observations.

### 2.1. Single immunohistochemistry in the guinea pig trigeminal ganglion

Free floating TG sections were incubated for a period indicated bellow with a primary antibody (as indicated in table 1), for 1 h with biotinylated secondary antibodies of an appropriate species (diluted 1:2,000; Vector, USA) at room temperature, and for 1 h with avidin-biotinylated peroxidase complex (diluted 1:2,000; ABC Elite, Vector) at room temperature. Primary antibodies used were, rabbit antiserum to pChAT (diluted 1:100,000; Tooyama and Kimura, 2000), mouse monoclonal antibody to PGP 9.5 (diluted 1:2,000; UltraClone, UK), goat antiserum to CGRP (diluted 1:5,000; Biogenesis, UK), and mouse monoclonal antiserum to SP (diluted 1:10,000; Peninsula, USA). With antibodies to pChAT, sections were incubated for 3-4 days at 4°C, while with antibodies to PGP 9.5, CGRP and SP sections were reacted overnight at room temperature. Dilution of the reagents and washing sections between each step were done with PBST. Color was developed by treating the sections for 10 min with a mixture containing 0.02% 3,3'-diaminobenzidine, 0.0045% H<sub>2</sub>O<sub>2</sub> and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The stained sections were mounted on gelatin-coated glass slides, air-dried, washed in tap water, dried through a graded series of alcohol, cleared with xylene, and cover-slipped with Entellan (Merck, Germany). Immunohistochemical controls, where each primary, secondary antiserum or the ABC reagent was omitted, gave no positive staining.

### 2.2. Double fluorescent immunohistochemistry in the guinea pig TG

To study the colocalization of pChAT with that for PGP 9.5, CGRP and SP, sections from trigeminal ganglia were double immunostained for pChAT and each of these markers using fluorescence-labeled secondary antibodies. The sections were incubated overnight at room temperature with a mixture of pChAT antiserum plus an antiserum to PGP 9.5, CGRP or SP. After washing, the sections were reacted for 3-6 hours at room temperature with Alexa Fluor

**Table 1. Details of the primary antibodies used in the present study**

Antigen	Type of antibody	Dilution	Reference or source
pChAT	Rabbit polyclonal	1:100,000	Tooyama and Kimura, 2000
SP	Mouse monoclonal	1:10,000	Peninsula Lab, USA
CGRP	Goat polyclonal	1:5,000	Biogenesis, UK

Antigen	Type of antibody	Dilution	Reference or source
PGP9.5	Mouse monoclonal	1:2000	UltraClone, UK

594-conjugated donkey anti-rabbit IgG (for pChAT) mixed with either Alexa Fluor 488-conjugated goat anti-mouse IgG (for PGP 9.5), Alexa Fluor 488-conjugated donkey anti-goat IgG (for CGRP), or Alexa Fluor 488-conjugated goat anti-mouse IgG (for SP). All the reagents of Alexa Fluor 488 (Molecular Probes, USA) were used at dilution 1:2,000. Dilution of the reagents and washing tissue specimens between each step were done with PBS. After washing, the corneal sections were mounted on glass slides, cover-slipped with buffered glycerin, and observed under a confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY). Low and high magnification images of a single optical slice were acquired using 20X and 40X objective lens.

### 3. Results

The gross anatomy of the guinea pig TG showed that it is occupied a cavity in the dura mater of the cranium, covering the trigeminal impression near the apex of the petrous part of the temporal bone. From its convex border, three large nerves emerges; the ophthalmo-maxillary and mandibular branches (Fig. 1). The H and E stained sections showed that the trigeminal ganglia had three large nerve divisions; the ophthalmic, maxillary, and mandibular branches (Fig 2). As sensory ganglia, the trigeminal ganglionic neurons appeared round- or oval in shape, and varied in size from small, medium to large cells. The cytoplasm of most of the neurons was basophilic and contained Nissl's granules and the nuclei (large ones) of some cells showed prominent nucleoli. Satellites cells surrounded the neurons in an almost complete layer around each cell (Fig. 2).

PChAT-positive cells distributed regularly throughout the trigeminal ganglion. Few numbers of immunoreactive fibers located in the ophthalmic, maxillary and mandibular nerve trunks (Fig. 3A and B). The majority of pChAT-positive ganglionic neurons were of small- to medium-size, although there were also very few large-sized cells stained for pChAT in this study. These pChAT- positive neurons varied from being; rounded, ovoid, or polyhedral in shape. CGRP-immunoreactivities were observed also in small to medium-sized neurons of guinea pigs trigeminal ganglion in its either divisions (Figs. 3C and D). Most of the CGRP-immunoreactive

neurons showed granular appearance of the cytoplasm.

We examined, by double fluorescent immunostaining, the co-expression of pChAT with other neuronal markers to get information about the nature and characteristics of pChAT-immunoreactive neurons in the trigeminal ganglion. pChAT-immunoreactivity was observed mainly in PGP9.5-positive neurons those were of small to medium size (Figs. 4A-C). The expression of pChAT was mainly intracytoplasmic and rarely seen intranuclear, while the PGP9.5 expression was usually in both locations (Fig. 4D-F). About half of PGP9.5-neurons showed pChAT-immunoreactivity. Since PGP9.5 is a general neuronal marker and the present study showed that all trigeminal ganglionic neurons were PGP9.5-immunoreactive, therefore, about half of total neurons in the trigeminal ganglion were pChAT-immunoreactive.

With double immunostaining, some of pChAT-immunoreactive neurons showed also coexpression with that of CGRP-immunoreactive neurons (Figs. 5A-C). Few large trigeminal ganglionic neurons expressed both pChAT and CGRP. This expression was substantially intracytoplasmic and rarely seen both intracytoplasmic and intranuclear (Fig. 5D-F).

With double immunostaining, some of pChAT-immunoreactive neurons showed also coexpression with that of SP-immunoreactive neurons (Figs. 6A-C). The colocalization of pChAT and SP was only intracytoplasmic. Analysis of colocalization showed that most of SP-immunoreactive neurons exhibited pChAT-immunoreactivity, while less than the half of pChAT-immunoreactive cells contained SP-immunoreactivity. On the other side, a large proportion of the SP-immunoreactive neurons exhibited pChAT-immunoreactivity, while about half of the pChAT- immunoreactive neurons expressed SP immunoreactivity too. At high magnification of the same proteins the expression of pChAT only, without co-expression of SP, was notice in some neurons while there was a colocalization with SP in few numbers of large neurons.

### 4. Discussion

The present study describes, for the first time, some characteristics of guinea pig trigeminal ganglia and document the presence of cholinergic pChAT- positive cells distributed all over its regions. The guinea pig trigeminal ganglia locates in the Meckel's cave and shows three large divisions; the ophthalmic, maxillary, and mandibular branches. Similar results have been documented in many animal species including rat (Yasuhara et al., 2007 and 2008), rabbit (Kolesar et al., 2006), cat (Feher et al., 1981; Oyagi et al., 1989), Mongrel dogs (Esteves et al., 2009) and human (Leston, 2009; Ziyal et al., 2004; Yousry et al., 2005; Bathla and Hedge, 2013; Wu et al., 2013). In the present work, the trigeminal ganglionic pseudounipolar neurons possesses round or oval shaped neurons with variable size from small to large cells. This results was in agreement with (Hassanali et al., 1999; Kobash et al., 2005; Kolesar et al., 2006; Yasuhara et al., 2007 and 2008; Koga et al., 2013). These pseudounipolar neurons have basophilic cytoplasm and rounded nuclei with prominent nucleoli in most of them and were surrounded by satellite cells in an accord with what is mentioned by (Moses, 1967; Krastev et al., 2007; Ray et al., 2010; Wu et al., 2013).

In the current study, pChAT-positive cells distribute uniformly throughout the trigeminal ganglion of g. pig. We confirmed that they are neurons, where all pChAT-positive cells show immunoreactivity for PGP9.5, a general neuronal marker. In addition, it is also documented here that about half of all trigeminal neurons are pChAT-immunoreactive and most of them are of small to medium-sized cell bodies. It also important to mention that, pChAT-immunoreactivity colocalizes, in various degrees, with SP-, CGRP- immunoreactivity in the g.pig TG neurons. (Yasuhara et al., 2007 and 2008) documented, in rat TG, similar distribution pattern and immunoreactivity for pChAT as well as colocalization of pChAT and SP, CGRP in the TG neurons.

Primary sensory neurons in the trigeminal ganglion were classified into three groups in the present study; small, medium-sized, and large according to previous studies of the trigeminal ganglion (Ichikawa and Sugimoto, 2001; Yasuhara et al., 2007 and 2008) and the dorsal root ganglia (Lee et al., 1985b; Ju et al., 1987; Aimi et al., 1991). Moreover (Harper and Lawson, 1985) mentioned that small to medium-sized ganglionic cells possess thin un-myelinated C-fibers or fine myelinated A- $\delta$ -fibers and these

types of fibers are accepted to mainly mediate nociception. (Cabanes et al., 2003) studied the effects of temperature on membrane properties and excitability in sensory neurons of the intact guinea pig trigeminal ganglion (TG) maintained in vitro and found some of them are thermosensitive. Since most of pChAT-positive cells are small to medium-sized in the trigeminal ganglion, and only few of pChAT-positive cells are large, it is likely that many of pChAT-positive ganglionic cells participate in nociception (Yasuhara et al., 2007 and 2008) others may be thermosensitive. Meanwhile, (Hoheisel and Mense, 1986; Lee et al., 1986a) deny any correlation between cell size and function in primary sensory neurons. In addition, (Porter et al., 1983) reported that proprioceptive neurons were confined to the trigeminal ganglion the, although small in numbers.

Double immunostaining studies used here confirmed the involvement of pChAT in nociception in g. pig TG. CGRP and SP are two neuropeptide transmitters known to have a neuromodulatory role on nociception and temperature sensitivity (Tervo et al., 1981; Wisenfeld-Hallin et al., 1984; Lee et al., 1985a; Skofitsch and Jacobowitz, 1985; Matsuyama et al., 1986; Ray et al., 2010; Yasuhara et al., 2008). In previous immunohistochemical studies, 35–50% of rat trigeminal neurons contained CGRP immunoreactivity, while 10–20% was SP-immunoreactive (Lee et al., 1985a; Terenghi et al., 1985). It was also shown that all SP-positive cells contained CGRP-immunoreactivity (Lee et al., 1985a; Terenghi et al., 1985). Thus, CGRP-immunoreactive neurons were either small to medium-sized neurons containing SP in the same soma or large neurons lacking SP (Matsuyama et al., 1986).

In the present study, almost all SP-positive trigeminal cells and the majority of CGRP-positive cells showed pChAT-immunoreactivity. In the same time, pChAT-positive cells outnumbered the SP-positive or CGRP-positive cells. In parallel (Yasuhara et al., 2007) assumed that there are three types of pChAT-positive neurons in the trigeminal ganglion, 1) SP-positive/CGRP-positive, 2) SP negative/CGRP-positive, and 3) SP-negative/CGRP-negative. They added the first group of pChAT-positive neurons, which must be nociceptors containing both SP and CGRP. The other two groups of neurons may be involved in other sensory functions than nociception may be thermo sensitivity. In the current work, as well as (Yasuhara et al., 2007), more than half of

pChAT-positive trigeminal neurons are supposed to be non-nociceptors. Therefore, the significance of colocalization of pChAT with other neuropeptides in sensory neurons of g. pig TG also remains uncovered and need more investigations.

In summary, pChAT-immunoreactivity in the present study, and in rat TG (Yasuhara et al., 2007), is present not only in most of SP- and CGRP-positive nociceptors, but also in SP negative non-nociceptive neurons which are either CGRP positive or -negative. The cell populations of pChAT positive non-nociceptive cells outnumber positive nociceptive cells in the g.pig TG. It is known that most cell bodies of proprioceptors are located in the mesencephalic trigeminal nucleus (Zhang et al., 2005), and only a small proportion of them are distributed in the trigeminal ganglion (Porter et al., 1983; Ichikawa et al., 1996). The present finding indicates that pChAT may not associate with proprioceptors, but most likely associate with other sensory cells such as mechanoreceptors mediating tactile sensation and thermoreceptors in the trigeminal ganglion. (Yasuhara et al., 2004 and 2007; Sann et al., 1995; Nakanishi et al., 1999; Tata et al., 2004) have proposed the concept of the cholinergic sensory system which is mostly predicted here in the TG of g.pig. Until recently, it was suggested that pChAT has a low ChAT enzyme activity (Yasuhara et al., 2004). Nevertheless, (Bellier and Kimura, 2007) in their study on preparations purified from the rat dorsal root ganglia showed a significant enzyme activity, therefore, the pChAT function in nociception and other sensory functions is relevant. This may lead to therapeutic intervention of sensory dysfunction targeting the cholinergic sensory system will be possible in future.

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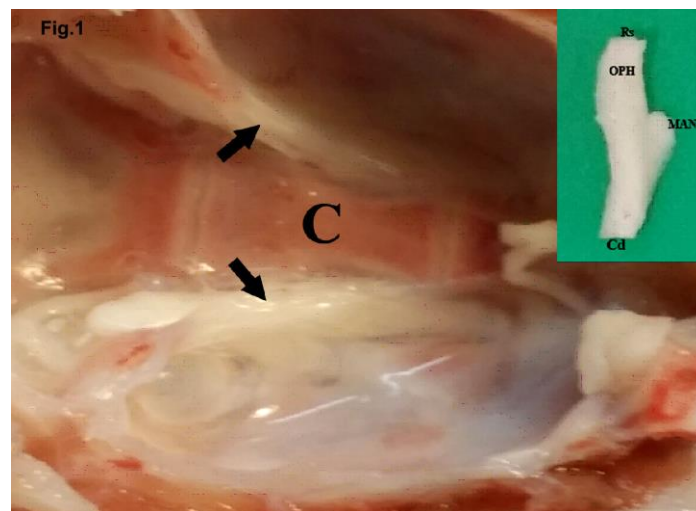
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**Fig.1:** Photomicrograph of TG showing the location of the ganglia (arrows) in cavities in the cranium (C) and the inset shows the ophthalmo-maxillary branch (OPH) and mandibular branch of the g. pig TG; Rs, rostral and Cd, caudal.

Fig.2

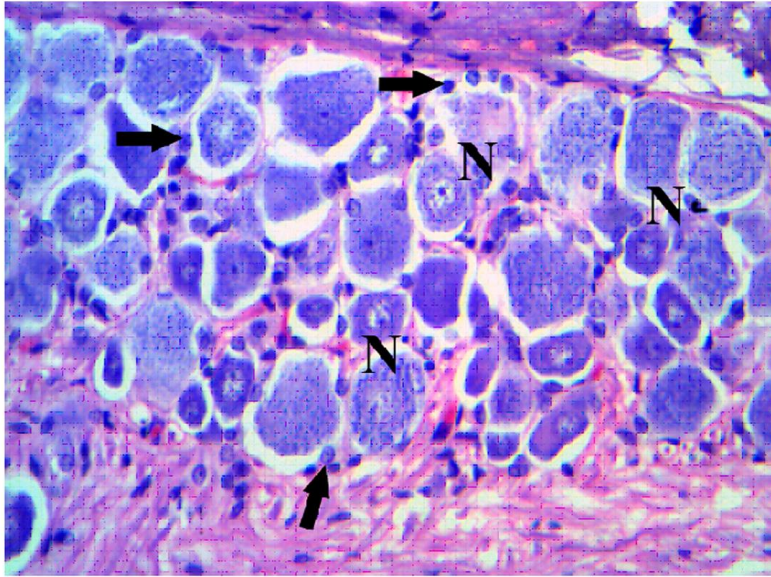


Fig. 2: Photomicrograph of TG showing, the neurons (N), with Owl's eye nucleus, distinct Nissl's granules in their cytoplasm and surrounded by satellite cells (arrows). H and E stain X40.

Fig.3

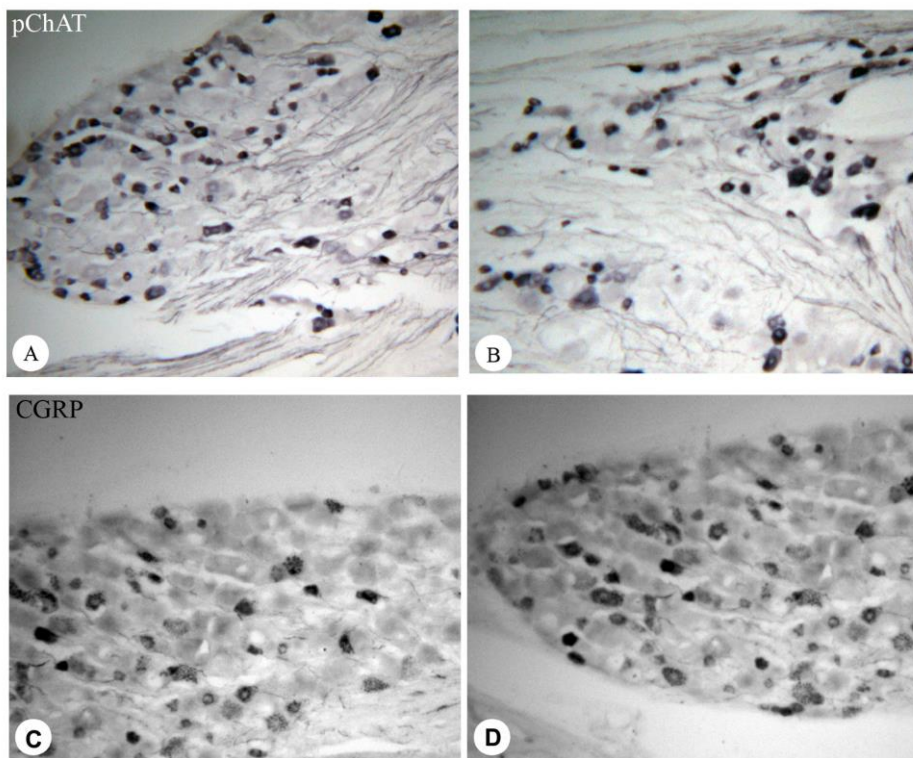
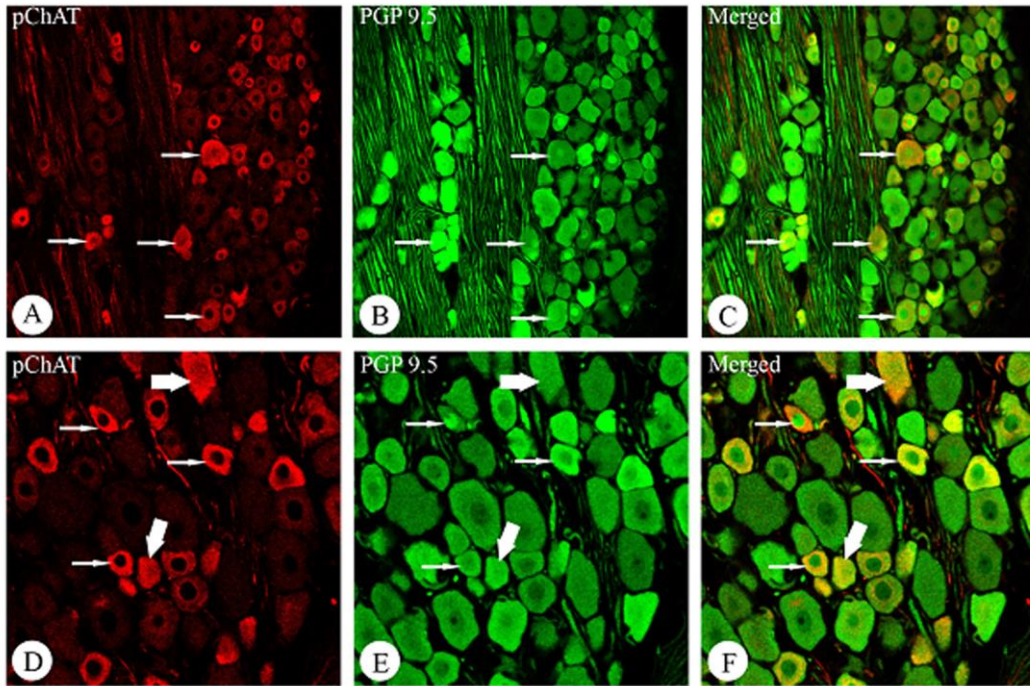


Fig. 3: Panel A-B showing the pChAT-positive neurons. Panel C-D showing CGRP-positive neurons in the trigeminal ganglion of the g. pig. A: Showing the mandibular division. B: Showing the ophthalmic division of g. pig trigeminal ganglion with pChAT-positive cells and few positive fibers run in either division. X10  
C: Showing the ophthalmic division. D: Showing the of mandibular division g. pig trigeminal ganglion. Some CGRP positive neurons had granular appearance in their cytoplasm with few positive fibers in either division. X10

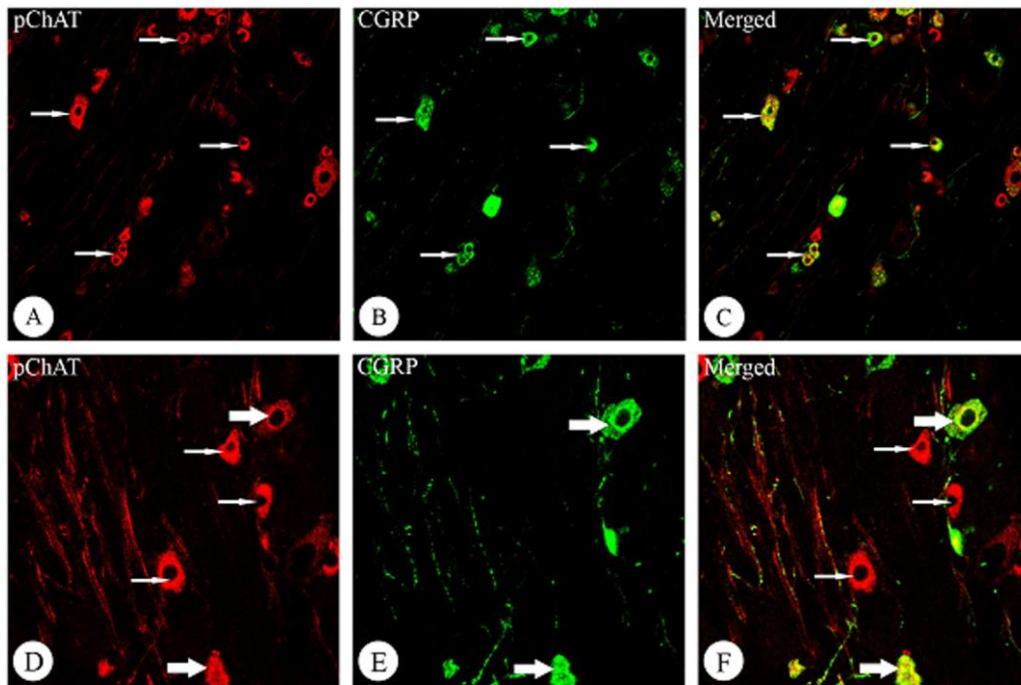


**Fig.4**



**Fig. 4:** Microphotographs showing the double immunofluorescence for pChAT and PGP 9.5 in the g. pig trigeminal ganglion. A–C: Low magnification of pChAT (A), PGP9.5 (B) and a merged image of A and B (C), showing pChAT-immunoreactivity in small to medium-sized PGP9.5-positive neurons and sometimes large sized one. D-F: High magnification of the same proteins showing the expression of pChAT intra cytoplasmic (thin arrows) and intranuclear (broad arrows). X20 and X40.

**Fig.5**



**Fig. 5:** Microphotographs showing the double immunofluorescence for pChAT and CGRP in the g. pig trigeminal ganglion. A–C: Low magnification of pChAT (A), CGRP (B) and a merged image of A and B (C), showing pChAT-immunoreactivity in small to medium-sized CGRP-positive neurons. D-F: High magnification of the same proteins showing the expression of pChAT only (thin arrows) in some neurons and colocalization with CGRP in large neurons (broad arrows). X20 and X40.

**Fig.6**

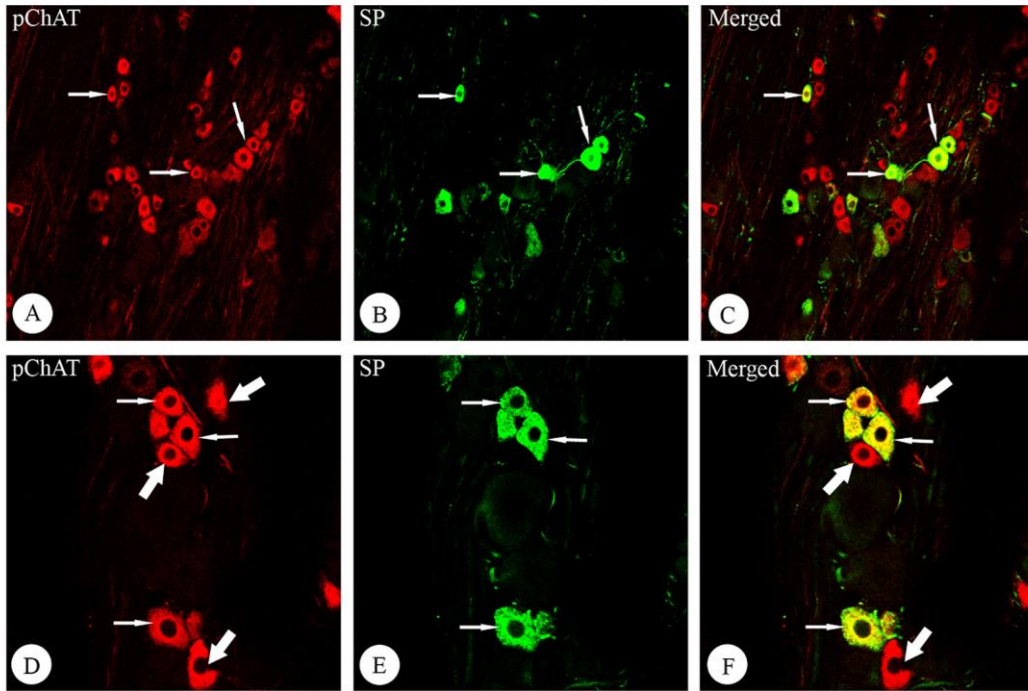


Fig. 6: Microphotographs showing the double immunofluorescence for pChAT and SP in the g. pig trigeminal ganglion. A–C: Low magnification of pChAT (A), SP (B) and a merged image of A and B (C), showing pChAT-immunoreactivity in small to medium-sized SP-positive neurons. D–F: High magnification of the same proteins showing the expression of pChAT only (broad arrows) in some neurons and colocalization with SP in large neurons (thin arrows). X20 and X40.