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Immunoreactivity for Choline Acetyltransferase of Peripheral-Type (pChAT) in the Trigeminal Ganglion Neurons of the Non-Human Primate *Macaca fascicularis*

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Transcripts of the choline acetyltransferase (ChAT) gene reveal a number of different splice variants including ChAT of a peripheral type (pChAT). Immunohistochemical staining of the brain using an antibody against pChAT clearly revealed peripheral cholinergic neurons, but failed to detect cholinergic neurons in the central nervous system. In rodents, pChAT-immunoreactivity has been detected in cholinergic parasympathetic postganglionic and enteric ganglion neurons. In addition, pChAT has been observed in non-cholinergic neurons such as peripheral sensory neurons in the trigeminal and dorsal root ganglia. The common type of ChAT (cChAT) has been investigated in many parts of the brain and the spinal cord of non-human primates, but little information is available about the localization of pChAT in primate species. Here, we report the detection of pChAT immunoreactivity in trigeminal ganglion (TG) neurons and its co-localization with Substance P (SP) and/or calcitonin gene-related peptide (CGRP) in the cynomolgus monkey, *Macaca fascicularis*. Neurons positive for pChAT were observed in a rather uniform pattern in approximately half of the trigeminal neurons throughout the TG. Most pChAT-positive neurons had small or medium-sized cell bodies. Double-immunofluorescence staining showed that 85.1% of SP-positive cells and 74.0% of CGRP-positive cells exhibited pChAT immunoreactivity. Most pChAT-positive cells were part of a larger population of neurons that co-expressed SP and/or CGRP.

Key words: acetylcholine, choline acetyltransferase, immunohistochemistry, sensory ganglion, trigeminal nerve

I. Introduction

Choline acetyltransferase is the enzyme that catalyzes the biosynthesis of acetylcholine (acetyl-CoA, choline-*O*-acetyl-transferase, ChAT; EC 2.3.1.6). In previous studies, protein expression of ChAT could be detected by immunohistochemical staining on almost all of the cholinergic neurons in the central nervous system, but not on parasympathetic postganglionic neurons or intramural ganglionic neurons of the enteric nervous system [3]. Therefore, conventional ChAT was considered a partially ineffective marker of cholinergic neurons. In 2000, Tooyama and Kimura cloned a splice variant of ChAT from rat pterygopalatine ganglion that lacks exons 6–9 of the ChAT cDNA [21]. Because of its predominant localization in peripheral neurons, a protein product of the mRNA variant was designated ChAT of a peripheral type (pChAT). The conventional ChAT protein, found in both central and peripheral neurons, was named ChAT of the common type (cChAT). An antibody directed against pChAT failed to stain any known central cholinergic neurons in the central nervous system, but did stain physiologically identified peripheral cholinergic structures that were not stained with various antibodies against cChAT [4, 14, 15, 24, 25]. In addition to cholinergic neurons in the peripheral nervous

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system such as parasympathetic postganglionic neurons and intramural ganglionic neurons of the enteric nervous system, expression of pChAT protein has been identified in neurons not previously classified as cholinergic. These include neurons in the tuberomammillary nucleus of the posterior hypothalamus [8] and primary afferent neurons such as the dorsal root ganglion cells [2], some retinal ganglion cells [23], and trigeminal ganglion (TG) neurons [26] of rats. However, pChAT expression has not been previously investigated in primate species.

Primary afferent neurons in the dorsal root ganglia (DRG) and TG are classified into several subpopulations on the basis of their neurochemical profiles. Substance P (SP) and/or calcitonin gene-related peptide (CGRP) are found mainly in small to medium-sized ganglion cells, and are assumed to be neurotransmitters for nociceptive sensory neurons [11, 12, 17, 19, 22]. Previously, pChAT immunoreactivity was also identified in small to medium-sized neurons in the rat TG, indicating that pChAT plays a role in nociception [24], although these pChAT-positive neurons were not characterized in detail in primate. In the present study, we characterized pChAT-positive neurons in the TG of a non-human primate, the cynomolgus monkey *Macaca fascicularis*, by analyzing cell size distribution and colocalization with SP and CGRP.

II. Materials and Methods

Animals and tissue preparations

Specimens of brain tissue were obtained from three female cynomolgus monkeys (10–11 years old) that were killed under a deep anesthesia with an intravenous injection of sodium pentobarbital. These animals had been used previously for other purposes by other researchers. Since these animals were used only for the development of new laparoscope, TGs in the monkeys seemed unaffected by the experiments. The TGs were removed from the animals and immediately fixed in 4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 days. The fixed tissue was then cryoprotected by immersion in 15% sucrose, 0.1 M phosphate buffer (pH 7.4) containing 0.1% sodium azide. The sucrose solution was changed daily for 4 d and the tissue was then stored in sucrose solution at 4°C until sectioning.

Cynomolgus monkeys were used with permission of the Shiga University of Medical Science Animal Experiment Committee and Biosafety Committee. The animals were used in accordance with Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science.

Western blot analysis

Since the TG in *Macaca fascicularis* is very small, we used the placenta for western blotting. A frozen specimen of placental tissue from *Macaca fascicularis* was processed in 50 mM Tris-HCl, pH 7.4, containing a protease inhibitor cocktail (Complete mini, Roche). The homogenate was centrifuged for 45 min at 15000×*g* at 4°C and the supernatant was collected as the soluble fraction of the lysate. Aliquots containing approximately 50 μg of protein were electrophoretically separated on a 5–20% sodium dodecyl sulfate-polyacrylamide gel (WAKO superSepAce, WAKO Pure Chemicals, Japan) under reducing conditions, and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). Nonspecific binding to the membrane was blocked by soaking for 1 hr in 5% skim milk in 50 mM Tris-buffered saline, pH 7.4, (TBS) at room temperature. The membranes were then incubated overnight with pChAT antiserum (rabbit polyclonal, made in house) diluted at 1:10000 in TBS containing 0.05% Tween-20 (TBST) at 4°C.

The production and characterization of the pChAT antiserum has been described previously [21]. Prior to its use in western blotting, the pChAT antiserum was incubated overnight with 10 volumes of normal monkey serum to inhibit nonspecific immunoreactivity. After rinsing with TBST, the membranes were incubated at room temperature for 1 hr with a secondary peroxidase-labeled anti-rabbit antibody. Chemiluminescence signals were obtained using the Chemi-Lumi One system (Nacalai tesque, Japan) and were imaged by LAS4000 (Fuji Film, Japan).

Immunohistochemical staining

Cryostat sections of the TG were incubated at 4°C overnight with the primary antibody at the following working dilutions: cChAT, 1:10000; pChAT, 1:100000. The tissues sections were incubated at room temperature for 2 hr with biotinylated secondary anti-rabbit antibody (Vector, Burlingame, CA; diluted 1:1000), and then at room temperature for 1 hr with the avidin-biotinylated peroxidase complex (ABC Elite, Vector; diluted 1:2000). PBST was used to dilute the reagents and for the washing of tissue sections between steps. The sections were stained for 15 min with a solution containing 0.02% 3,3-diaminobenzidine, 0.0045% H_2O_2, and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The stained sections were mounted on glass slides, air dried, washed in tap water, dried through a graded series of ethanol, cleared with xylene, and cover slipped with Entellan (Merck, Darmstadt, Germany).

Immunofluorescence staining

Immunofluorescence histochemistry was employed according to previous reports [5, 18]. In brief, cryostat sections were incubated at 4°C overnight with a mixture of primary antibodies at respective working dilutions. The antibodies used in this study are summarized in Table 1. The combinations of the antibodies examined were rabbit anti-pChAT serum and guinea pig polyclonal anti-SP or guinea pig polyclonal anti-CGRP. After washing, the sections were incubated at room temperature for 2 hr in a mixture of secondary antibodies of Alexa 594-labeled

donkey anti-rabbit IgG (for pChAT) and Alexa 488-labeled donkey anti-guinea pig IgG (for SP and CGRP) (Molecular Probes Inc., Eugene, OR; diluted 1:500). PBST was used to dilute the reagents and for the washing of tissue sections between steps. After washing, the sections were mounted on glass slides, cover slipped with glycerin, and then imaged on a confocal laser scanning microscope, LSM510, equipped with an argon laser (458/488/514 nm) and a green helium/neon laser (543 nm, Carl Zeiss, Oberkochen, Germany). Single optical slice images were taken using ×10, ×20, or ×40 Plan-Neofluor dry objective lenses.

Imaging data evaluation

Horizontal cryostat sections of TG were cut and collected in PBST. Five cryostat sections were randomly selected and stained with immunofluorescence histochemistry. Double-staining immunofluorescent analysis was performed by confocal laser-scanning microscopy. A guide square of 460.68×460.68 μm was imaged by confocal laser microscopy and the data were transferred to a computer. Three areas per section were analyzed. The images were adjusted to contrast pChAT-, CGRP-, and SP-positive cells by Zeiss image browser (Carl Zeiss, Oberkochen, Germany). The brightness and contrast of the final images were adjusted and neurons were counted using Image J 1.46r (NIH) software. Five, randomly selected guide squares were imaged per section. Finally, five squares in five sections were analyzed by the naked eye.

III. Results

Specificity of the pChAT polyclonal antibody

Western blot analysis demonstrated that the polyclonal anti-pChAT antibody detects a single band with a molecular weight of approximately 55 kDa (Fig. 1A). This size is consistent with the previously reported molecular weight of rat pChAT [2, 23–25].

The Ab144P, generated using full-length recombinant cChAT, detected two bands at molecular weights of approximately 68 kDa (arrowhead in Fig. 1) and 55 kDa (arrow in Fig. 1). These sizes correspond to the molecular weights of cChAT and pChAT, respectively.

Immunohistochemical localization of pChAT in the trigeminal ganglion

Immunohistochemical staining for pChAT was identified in neurons in the TG as well as in DRG neurons and some fibers (Fig. 2A). The neurons were grouped into three classes of size according to a previous paper [9]. The pChAT immunoreactivity was particularly prominent in small (less than 30 μm, * in Fig. 2B) and medium-sized (30–50 μm, ¶ in Fig. 2B) neurons, but was absent in large neurons (50–80 μm, § in Fig. 2B).

Double immunofluorescence identified some pChAT-positive neurons that also expressed CGRP (Fig. 3A, B and C) or SP (Fig. 3D, E and F), although the number of pChAT-positive neurons was larger than the number of CGRP-positive or SP-positive neurons. Semi-quantitative analysis showed that 85.1% of SP-positive neurons and 74.0% of CGRP-positive neurons co-expressed pChAT (Table 2).

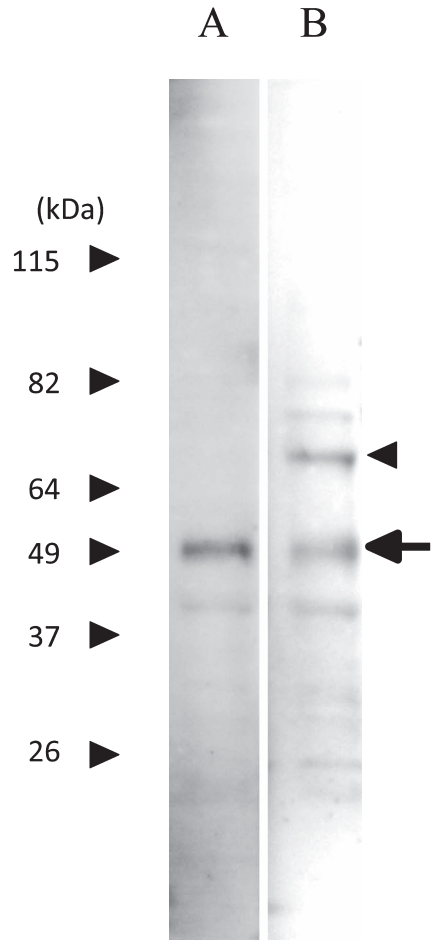

Fig. 1. Western blot analysis of placental extracts from *Macaca fascicularis* probed with pChAT antibody (**A**) and AB144p (**B**). The pChAT antibody detected a single band of approximately 55 kDa, which is in agreement with the molecular weight of pChAT. AB144p detected two bands of approximately 68 kDa (arrowhead) and 55 kDa (arrow) that correspond to the molecular sizes of cChAT and pChAT, respectively.

IV. Discussion

The present study demonstrated pChAT expression in a subpopulation of TG cells in the cynomolgus monkey. These observations are in accordance with previous studies that identified pChAT, but not cCHAT, in rat sensory neurons including the TG, DRG, and nodose ganglion by immunostaining [1, 13, 16], despite cCHAT being classed as cholinergic-specific by pharmacological and neurophysiological analyses [4, 7, 10, 20]. ChAT activity was detected in rat DRG, suggesting that pChAT in the DRG can synthesize acetylcholine. Thus, the existence of pChAT

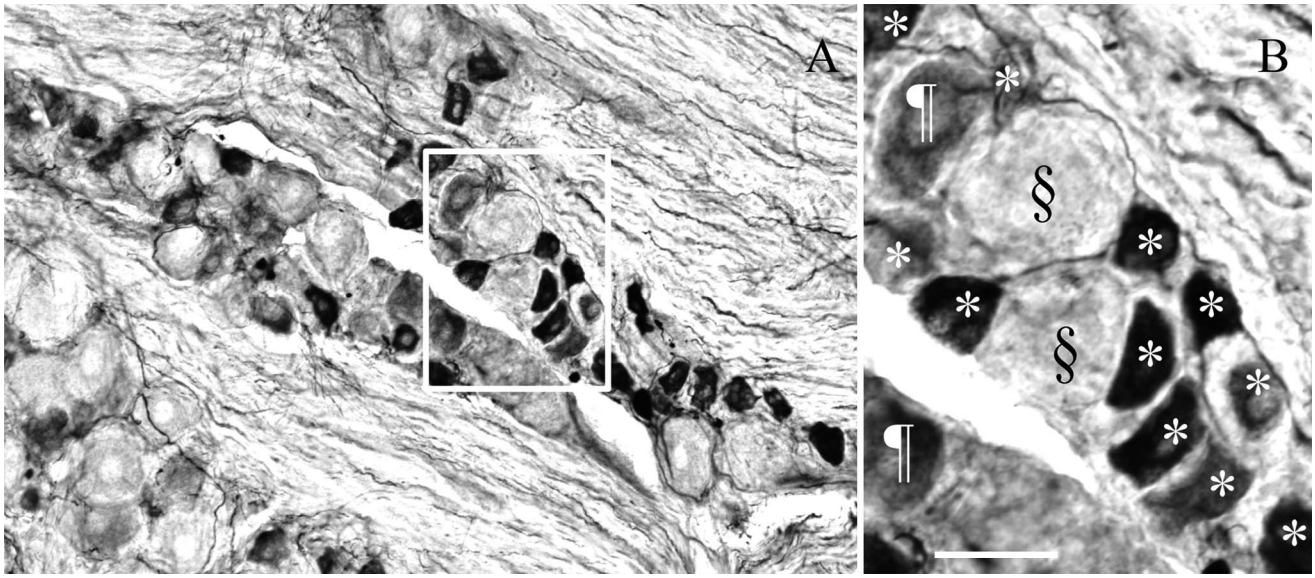

Fig. 2. Immunohistochemical staining of pChAT in the TG of *Macaca fascicularis*. **A**; Immunoreactivity for pChAT was seen in perikarya as well as some fibers. **B**; The pChAT immunoreactivity is particularly apparent in perikarya of small (<30 μm, *) and medium-sized (30–50 μm, ¶) neurons, but not in large neurons (50–80 μm, §). Some fibers are also positive for pChAT. Bar=50 μm.

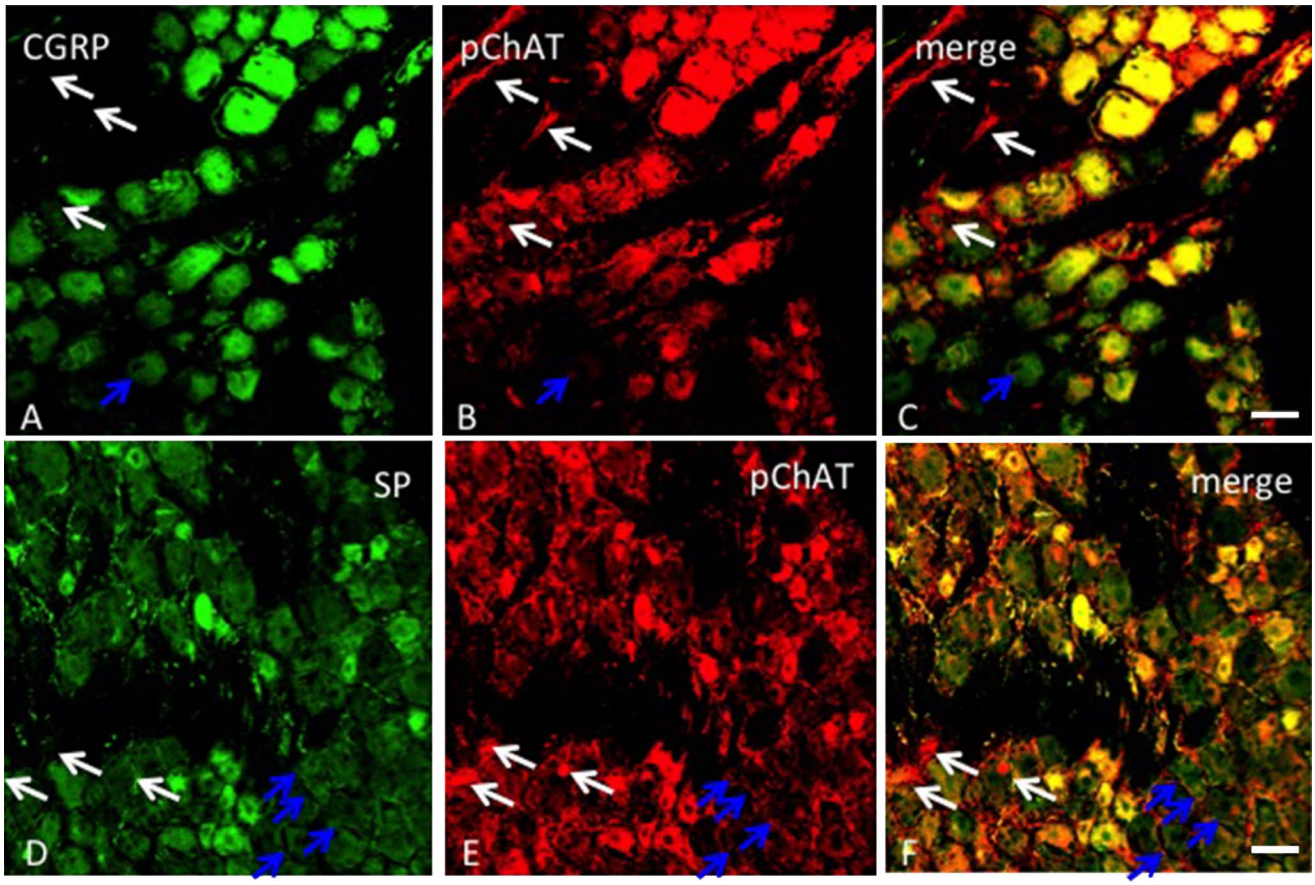

Fig. 3. Double-immunofluorescent staining for pChAT and CGRP (**A, B, C**) or SP (**D, E, F**) in the TG of *Macaca fascicularis*. A larger number of neurons are pChAT-positive than are CGRP-positive or SP-positive. Most of the CGRP-positive neurons and the SP-positive neurons co-express pChAT. White arrows indicate pChAT-positive and CGRP-negative neurons (**A–C**) or pChAT-positive and SP-negative neurons (**D–F**). Blue arrows indicate pChAT-negative and CGRP-positive neurons (**A–C**), or pChAT-negative and SP-positive neurons (**D–F**). Bars=20 μm.

Table 1. *Details of the primary antibodies used in the present study. pChAT antiserum was generated in our laboratory against a pChAT peptide (41 amino acids spanning over the splice joint between exons 5 and 10 of ChAT cDNA)*

Antigen	Type of antibody	Dilution	Reference or source
pChAT	Rabbit polyclonal (antiserum)	1:30000	Tooyama and Kimura, 2000
cChAT	Goat polyclonal (antiserum)	1:10000	Chemicon (Ab144P)
SP	Guinea pig polyclonal (affinity-purified)	1:5000	Bachem (T-5019)
CGRP	Guinea pig polyclonal	1:5000	Peninsula Lab. (T-5053)

Table 2. *Counts of substance P (SP) or CGR-positive neurons co-localized with and without pChAT in the trigeminal ganglion of cynomolgus monkey*

Immunoreactivity	Total	pChAT (+) (%)	pChAT (–) (%)
SP (+)	242	206 (85.1)	36 (14.9)
CGRP (+)	127	94 (74.0)	33 (26.0)

provides a clue to the missing link among cholinergic systems previously suggested by pharmacological and physiological analyses.

Small and medium-sized neurons in the monkey TG expressed pChAT in the perikaryon and dendrite, but not in large ganglion neurons. These TG neurons were located in the dorsomedial population, suggesting a role for pChAT in nociception, thermoreception, and proprioception in the dorsomedial population of TG neurons [8]. Double immunostaining revealed that 85.1% of SP-positive neurons and 74.0% of CGRP-positive neurons co-localized with pChAT-expressing neurons. The values are slightly less than those found in rat TG, wherein 90.3% of SP-positive neurons and 82.7% of CGRP-positive neurons also expressed pChAT. The expression of pChAT in peptidergic neurons supports the proposal that it could play a cooperative role in nociception, thermoreception, and proprioception in the TG.

This study was the first to demonstrate the immunohistochemical localization of pChAT in cynomolgus monkey tissues. Cross-reactivity to pChAT antiserum has been observed in a wide range of species from invertebrate mollusks including octopus, slug, and snail to mammals including mouse, guinea pig, pig, cow, sheep, and human [3]. This study of pChAT expression in monkey could provide further useful insights into the human nervous system than previous studies that have focused on the rodent nervous system, which is more divergent from humans than monkey.

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VI. References

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