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Expression and localization of pChAT as a novel method to study cholinergic innervation of rat adrenal gland

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ABSTRACT

Cholinergic innervation of the rat adrenal gland has been analyzed previously using cholinergic markers including acetylcholinesterase (AChE), choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT). In the present study, we demonstrate putative cholinergic neurons in the rat adrenal gland using an antibody to pChAT, which is the product of a splice variant of ChAT mRNA that is preferentially localized in peripheral cholinergic nerves. Most of the ganglionic neurons as well as small single sporadic neurons in the adrenal gland were stained intensely for pChAT. The density of pChAT-immunoreactive (IR) fibers was distinct in the adrenal cortex and medulla. AChE-, cChAT- and VAChT-immunoreactivities were also observed in some cells and fibers of the adrenal medulla, while the cortex had few positive nerve fibers. These results indicate that ganglionic neurons of the adrenal medulla and nerve fibers heterogeneously express cholinergic markers, especially pChAT. Furthermore, the innervation of the adrenal gland, cortex and medulla, by some cholinergic fibers provides additional morphological evidence for a significant role of cholinergic mechanisms in adrenal gland functions.

Introduction

The adrenal gland is an endocrine organ and its function is mediated through the secretion of hormones. It plays a vital role in both the acute and the prolonged mammalian stress responses (Di Curzio and Goldowitz, 2011). In all adult mammals, the adrenal gland comprises the adrenal medulla and three layers of the adrenal cortex that surround the medulla (Deschepper et al., 2004). These cortical regions include the *Zona glomerulosa*, *Zona fasciculata* and *Zona reticularis* (Di Curzio and Goldowitz, 2011). The adrenal medulla comprises two types of chromaffin cells, adrenaline (epinephrine) and noradrenaline (nor-epinephrine) cells. Furthermore, a few large ganglion cells are also present in the medulla (Oomori et al., 1994; Holgert et al., 1996a,b; Kato et al., 2014).

The cortex and medulla of the adrenal gland traditionally have been regarded as independent entities, and as such have been studied separately by either endocrinologists or neuroscientists. Regarding the adrenal gland innervation, it is generally accepted that the cells of the adrenal gland receive both an extrinsic and an intrinsic innervation. The majority of external fibers project to the adrenal travel via the splanchnic nerves. After penetrating the capsule they branch to form an extensive subcapsular network; from here fibers pass to both cortex and medulla to form further networks surrounding the cells of these regions. The intrinsic innervation arises from ganglion cells sparsely distributed throughout the gland in subcapsular, cortical and medullary regions (Coupland, 1965a,b; Lewis and Shute, 1969; Parker et al., 1993). The adrenal gland is supplied by sympathetic preganglionic cholinergic nerves, derived from neurons located in the intermediate gray matter of the spinal cord. However, a varying number of neurons project ipsilateral to the medulla synapse either in the ganglia of the sympathetic chain or in the suprarenal ganglion. These represent a relatively small number of postganglionic sympathetic fibers innervating the adrenal gland (Parker et al., 1993). The adrenal medulla receives a most dense nerve supply terminating on the postganglionic neurons and chromaffin cells (Kondo et al., 1985; Kesse et al., 1988). However, the adrenal cortex is also innervated, especially in the outer capsule and *z. glomerulosa* region (Watanabe et al., 1990; Janossy et al., 1998). Medullary neurons in the mammalian adrenal gland have been shown not only to innervate medullary chromaffin cells, but also to project to the cortex, approaching secretory cells and blood vessels. Such neurons were shown to contain catecholamines or acetylcholine (ACh). They were assumed to

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be targets of preganglionic nerve fibers from the spinal cord and from the parasympathetic dorsal nucleus of the vagus, respectively (Colombo-Benkmann et al., 1996).

The adrenal medulla, which ontogenetically develops into an endocrine organ from neural crest cells, is densely innervated with preganglionic cholinergic innervations (Coupland, 1965a,b; Edwards and Jones, 1993). The presence of cholinergic nerve fibers in the adrenal glands has been studied in rat (Purwar, 1978; Parker et al., 1993; Janossy et al., 1998; Murabayashi et al., 2009), mouse (Oomori et al., 2013), guinea pig (Parker et al., 1990), cow (De Diego, 2010), goat (Watanabe et al., 1990), pig (Tornøe et al., 2000), and human (Charlton et al., 1991) using different markers. Additionally the vagal innervation of the adrenal gland of guinea pig and rat (Coupland et al., 1989; Tomlinson and Coupland, 1990) and the observation of AChE- positive neurons of rat adrenal medulla (Coupland and Holmes, 1958; Coupland, 1965a; Tomlinson and Coupland, 1990) support the localization of cholinergic nerve fibers in the adrenal glands.

Acetylcholine (ACh) is the primary neurotransmitter mediating catecholamine secretion from the adrenal medulla as it activates three types of receptors on chromaffin cells. It stimulates and mutually facilitates the secretion of catecholamine by generating various second messengers (Malhotra et al., 1989; Tomlinson and Coupland, 1990; Iwasa et al., 1991; Tischler et al., 1999; Rosol et al., 2001; Hamelink et al., 2002). Four possible marker proteins have been used to reveal cholinergic nerves. They are: (1) the ACh degrading enzyme, acetylcholinesterase (AChE); (2) the ACh synthesizing enzyme, choline acetyltransferase (ChAT); (3) the vesicular ACh transporter (VAChT), which mediates transport of ACh to storage vesicles in nerve terminals; and (4) the high-affinity choline transporter (CHT), which transports choline into neurons for the synthesis of ACh. Previous attempts to label ACh-containing preganglionic neurons by demonstration of the AChE were unsatisfactory. This handicap was overcome by the immunohistochemical application of antibodies to ChAT (Colombo-Benkmann et al., 1995). ChAT is accepted to be a reliable marker for cholinergic structures, but it has been recognized that most ChAT antibodies fail to identify cholinergic nerves in peripheral tissues (Arvidsson et al., 1997; Hoover et al., 2004; Murabayashi et al., 2009). A novel splice variant of ChAT cDNA, which lacks exons 6–9 in the coding region, has been cloned from rat pterygopalatine ganglion (Tooyama and Kimura, 2000). Because of its predominant localization in peripheral neurons, its protein product was designated ChAT of a peripheral type (pChAT). The conventional ChAT protein was called ChAT of the common type (cChAT). Although the antibody against pChAT is capable of detecting some positive neurons in the central nervous system (Kanayama et al., 2003; Yasuhara et al., 2003), pChAT immunohistochemistry proves to be a powerful tool to visualize peripheral cholinergic structures (Nakanishi et al., 1999; Nakajima et al., 2000; Chiocchetti et al., 2003; Yasuhara et al., 2004, 2007, 2008) and it is accepted now to be one of the ACh synthesizing enzymes by Bellier and Kimura (2011) who suggested that pChAT may utilize a new catalytic center alternative to His^{334}. They added, all neurons contained pChAT, and extracted pChAT showed sufficient enzyme activity to produce physiological concentrations of ACh.

Because the adrenal gland shows functional diversity and multiple chemical coding (Richardson et al., 2003), it is of interest to examine the expression of pChAT, as a novel cholinergic marker, in the adrenal gland. We noticed that previous studies have reported the expression of AChE (Allen et al., 1958), ChAT (Henion and Landis, 1990; Holgert et al., 1994; Kato et al., 2014), and VAChT (Arvidsson et al., 1997) in the adult adrenal gland. Moreover, the distribution of AChE in the developing adrenal gland of rat and mouse (Holgert et al., 1994; Iwasa et al., 1991) has been documented. However, details regarding the localization of the cholinergic markers; pChAT, cChAT, VAChT, and AChE, has never been studied in comparison and is a matter of debate. It is possible that heterogeneity in expression of pChAT in adrenal glands may contribute to functional diversity of such an organ. In the present study, therefore, we address the possible expression and distribution pattern of pChAT in the rat adrenal gland. We aimed also to re-evaluate the immunohistochemical distribution of other cholinergic markers and to compare their expression with that of pChAT-IR structures in the adrenal gland of rat.

Materials and methods

Fifteen male Wistar rats, weighing 250–350 g were used in the current study. 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). The adrenal gland was dissected out and immersed for 2 days in the same fixative at 4 °C, and then cryoprotected by replacing in 0.1 M PB containing 15% sucrose at 4 °C for 24–48 h. 14–20 μm-thick sections were cut using a cryostat. The sections were collected separately in 0.1 M PBS containing 0.3% Triton-X 100 (PBST) to be used for histochemical and immunostaining. Transverse paraffin wax sections 5–8 μm-thick were prepared from the adrenal glands and mounted on gelatin-coated glass slides to be used for light microscopic observations, using hematoxylin and eosin stain (H&E). Procedures involving animals and their care were conducted in conformity with the standards for animal experiments and are in compliance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

For AChE histochemistry, the sections were reacted by the Cu-acetylthiocholine method (Karnovsky and Roots, 1964) for 20–30 min at 378 °C. To verify the specificity of the reaction product, control incubations were carried out (a) in the absence of substrate, (b) in the presence of the cholinesterase inhibitor physostigmine (10 μM), or (c) in the presence of the pseudocholinesterase inhibitor, iso-OMPA tetraisopropylpyrophosphoramide (10 μM) and no positive staining could be seen.

Immunostaining was performed using the avidin–biotin complex (ABC) technique. Specificity, working dilution, and sources of the primary antibodies used in this work are summarized in Table 1. Free-floating cryostat sections of the adrenal glands were treated for 30 min with 0.3% hydrogen peroxide in methanol at room temperature, to eliminate endogenous peroxidase activity, and then incubated for a period, indicated below, with a primary antibody. The biotinylated secondary antibodies of an appropriate species (diluted 1:2000; Vector Laboratories, Burlingame, CA, USA) were then applied for 1 h at room temperature. Later on ABC complex (diluted 1:2000; ABC Elite, Vector) was used for 1 h at room temperature. Sections were incubated for 3–4 days at 4 °C

Table 1
Primary antibodies used.

Primary antibodies	Species	Type	Source	Dilution
Choline acetyltransferase of peripheral type (pChAT)	Rabbit	Polyclonal	Kind gift of H. Kimura	1:50,000
Choline acetyltransferase of common type (cChAT AB144p)	Goat	Polyclonal	Chemicon, USA	1:500
Vesicular acetylcholine transporter (VAChT)	Goat	Polyclonal	Chemicon, USA	1:10,000
Tyrosine hydoxylase (TH)	Mouse	Monoclonal	Chemicon, USA	1:2000

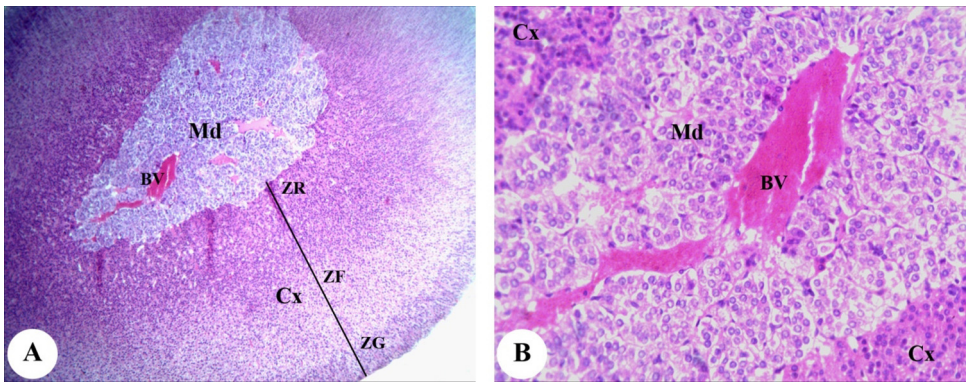

Fig. 1. Light photomicrograph of rat adrenal gland. (A) the cortex (Cx) consists of three zones, *Zona granulosa* (ZG), *Zona fasiculata* (ZF) and *Zona reticularis* (ZR) surrounding the adrenal medulla (Md) which contains a large blood vessel (BV). (B) higher magnification of the cortex (Cx) and medulla (Md) with its chromaffin cells in clusters and large blood vessel (BV) in between them. H&E stain (A = ×10 objective; B = ×40 objective).

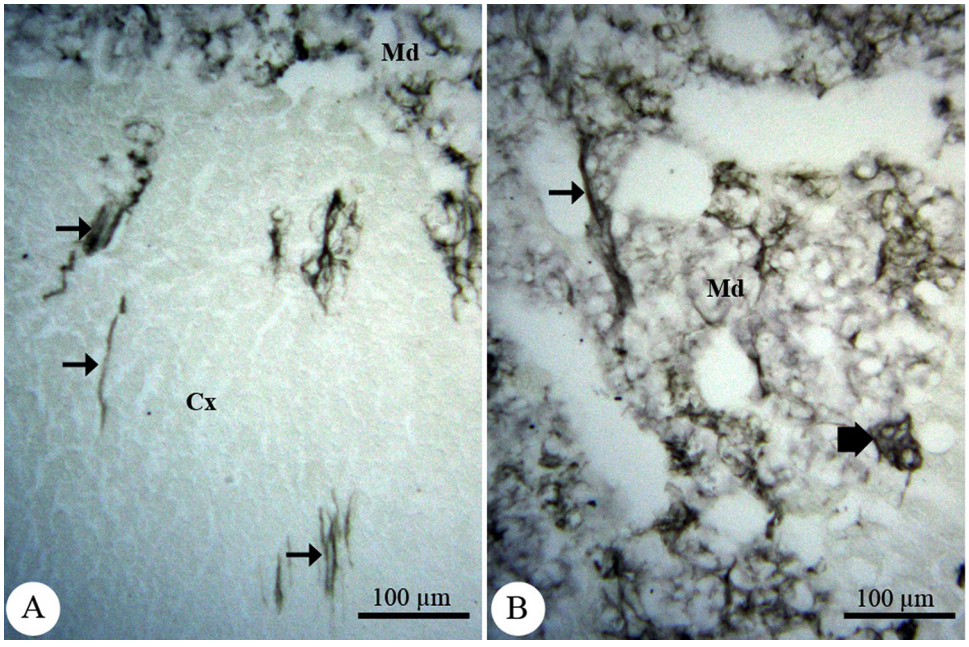

Fig. 2. Light micrograph of AChE activity in the rat adrenal gland. (A) shows strong AChE activity in the nerve bundles (arrows) of the cortex (Cx), while AChE activity is weakly stained and diffuse in the medullary (Md). (B) shows some AChE-positive nerve fibers (arrow) in adrenal medulla (Md) and strong activity in a ganglion cell (broad arrow).

with antibodies to pChAT, cChAT and VAChT, while with antibody to TH 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 (DAB), 0.0045% H_2O_2 and 0.3% nickel ammonium sulfate in 50 ml Tris buffer (pH 7.6). The stained sections were dehydrated by alcohol, cleared by xylene, and cover-slipped by Entellan®. For an immunohistochemical control, no positive staining could be seen when a pre-immune serum of the rabbit that produced the specific antiserum against pChAT was used. Additionally, when primary, secondary antiserum or the ABC reagent was omitted, no positive staining was also observed.

Results

H&E

The rat adrenal gland in light microscopic observation was seen to consist of two distinct regions; adrenal cortex and medulla. The cortex showed three zones namely the *Zona granulosa*, *Zona fasiculata*, and *Zona reticularis* surrounded the adrenal medulla (Fig. 1A). The adrenal medulla consisted of polyhedral chromaffin cells, randomly clustered, and a little amount of connective tissue in between. There was also a large blood vessel in between the adrenal chromaffin cells (Fig. 1B).

AChE

AChE-activity was seen strongly stained in a few large ganglion cells, and weak in clusters of chromaffin cells, but negative in other medullary and cortical cells (Fig. 2A). AChE- positive chromaffin cell islands were also seen. Several thick or thin bundles of AChE-positive nerve fibers ran through the cortex directly into the medulla and ramified numerous thinner nerve bundles and fibers in the course. They closely contacted chromaffin cells and a few ganglion cells, but not cortical cells. These AChE-positive varicose nerve fibers ran among the chromaffin cells rather than along the blood vessels in the medulla. Sometimes basket-like network of

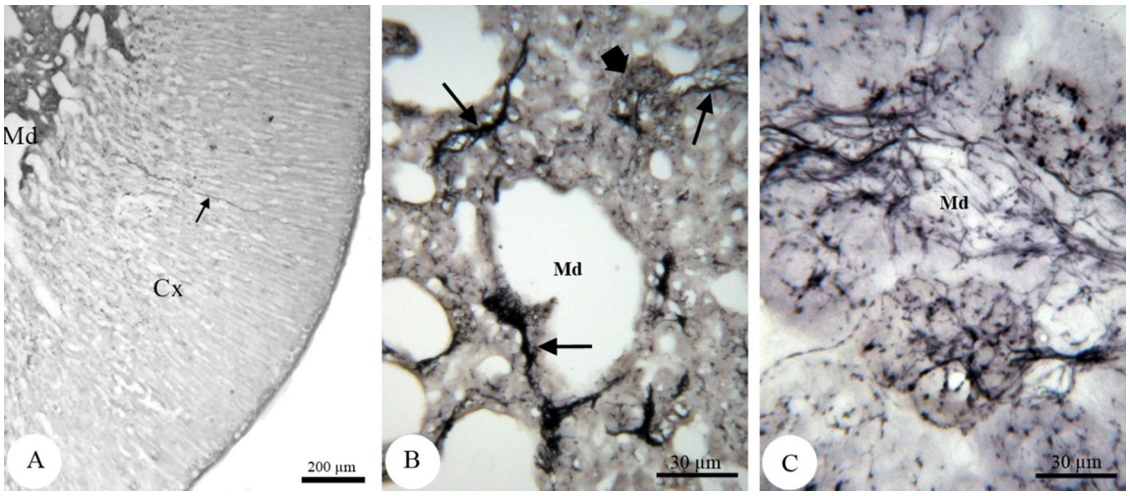

Fig. 3. Light micrograph of cChAT immunoreactivity in the rat adrenal gland. (A) the cChAT-IR thick nerve bundles (arrow) run through the cortex (Cx) into the medulla (Md). (B) and (C) abundant thin and thick cChAT-IR nerve bundles and fibers (arrows) are seen in the medulla (Md). These nerve fibers sometimes appear to surround groups of medullary cells (broad arrow in B).

AChE- positive varicose nerve fibers encircled groups of several chromaffin cells (Fig. 2B).

cChAT

Fig. 3 shows cChAT immunoreactivity in the thick nerve bundles penetrating the adrenal cortex and traversing the cortical septa without substantial ramifications and entering the medulla (Fig. 3A). A dense meshwork of cChAT-IR varicose and non-varicose nerve fibers interlaced the entire adrenal medulla. Some cChAT-IR nerve fibers surrounded some clusters of medullary cells. No cChAT-IR cells could be seen in the adrenal cortex or in the medulla.

pChAT

The current study showed for the first time pChAT-IR nerve fibers in the capsule, and cortex region. In addition strongly pChAT-positive nerve fibers and cells were located in the adrenal medulla. In the cortex, fine and thick pChAT-IR nerve bundles and fine fibers were seen crossing radially through the capsule and the cortex region into the medulla (Fig. 4A–C). The adrenal medulla showed strong pChAT-IR nerve bundles and fibers (Fig. 4D and E). Ganglionic cells, either grouped or single, showed very strong reactivity to the pChAT antibody. The axons of the pChAT-IR cells at the periphery of adrenal medulla entered into the cortex (Fig. 4F–H).

VAChT and TH

VAChT immunoreactivity was observed as nerve terminal in the form of small puncta that had penetrated through the cortex into the medulla. Relatively numerous VAChT-IR nerve terminals were seen around single and clusters of chromaffin cells in the medulla. However, no VAChT immunoreactivity was observed in the cortical and medullary cells (Fig. 5A). TH immunoreactivity was observed only in the chromaffin cells of the adrenal medulla in the form of very strong reaction (Fig. 5B). We could not find TH immunoreactivity in the cortex or the capsule.

Discussion

The adrenal gland of rat was organized into discrete cell complexes arranged into an outer cortex, consisting of three zones and the inner medulla surrounded by sparse connective tissue. This was in agreement with what is reported in many animal species including rat (Rosol et al., 2001; Wan Ezumi et al., 2007) and mouse (Di Curzio and Goldowitz, 2011; Monfared et al., 2014). Although physiological studies have shown that the secretion of adrenal chromaffin cell is mediated by cholinergic agonists (Douglas and Poisner, 1965; Unger and Philips, 1983; De Diego, 2010), attempts to develop immunohistochemical markers for the identification of cholinergic neurons have presented several difficulties. Several antibodies to ChAT have been generated; however, most ChAT antibodies fail to identify cholinergic nerves in peripheral tissues since they are cytosolic proteins that are not specifically concentrated at nerve terminals (Weihe et al., 1996; Arvidsson et al., 1997; Hoover et al., 2004). Since pChAT preferentially localized in the peripheral cholinergic neurons (Tooyama and Kimura, 2000) and is capable of detecting some positive neurons in the central nervous system (Kanayama et al., 2003; Yasuhara et al., 2003, 2007, 2008), so it represents a novel and unique tool for the study of cholinergic neurons in the peripheral nervous systems. This fact, in combination with the ectopic expression of ChAT in non-neuronal tissue (Ibáñez et al., 1991) and the puzzling difficulties in visualizing cholinergic neurons with ChAT antibodies, particularly in the peripheral nervous system, has limited the use of ChAT as an unambiguous marker for cholinergic neurons (Wu and Hersh, 1994).

Strong AChE activity in a few large ganglion cells and weak reactivity in chromaffin cells was observed in the current study. Moreover, no AChE- positive cells were found in either the capsule or the cortex and AChE- positive nerve fibers were rarely found in the capsule. In parallel, Watanabe et al. (1990) and Iwasa et al. (1991) reported thick and thin bundles of AChE- positive nerve fibers that ran directly through the cortex into the medulla of the mouse adrenal gland. Moreover, groups of AChE-positive chromaffin cells and intramedullary ganglion cells were reported also in the rat (Tomlinson and Coupland, 1990; Dagerlind et al., 1994) and bovine (De Diego, 2010) adrenal medulla. The presence of AChE in the ganglionic cells and some chromaffin cells may be related to the hydrolysis of a large amount of ACh used in controlling the chromaffin secretory activity (Iwasa et al., 1991).

A dense meshwork of cChAT-IR nerve fibers interlaced the entire adrenal medulla through the cortex, but no cChAT-IR cells could be seen anywhere. In partial agreement, ChAT-IR nerve fibers formed a dense meshwork and some of the intramedullary nerve cell bodies were ChAT-IR in rat (Kato et al., 2014), guinea pig and mouse (Colombo-Benkmann et al., 1995; Oomori et al., 2013).

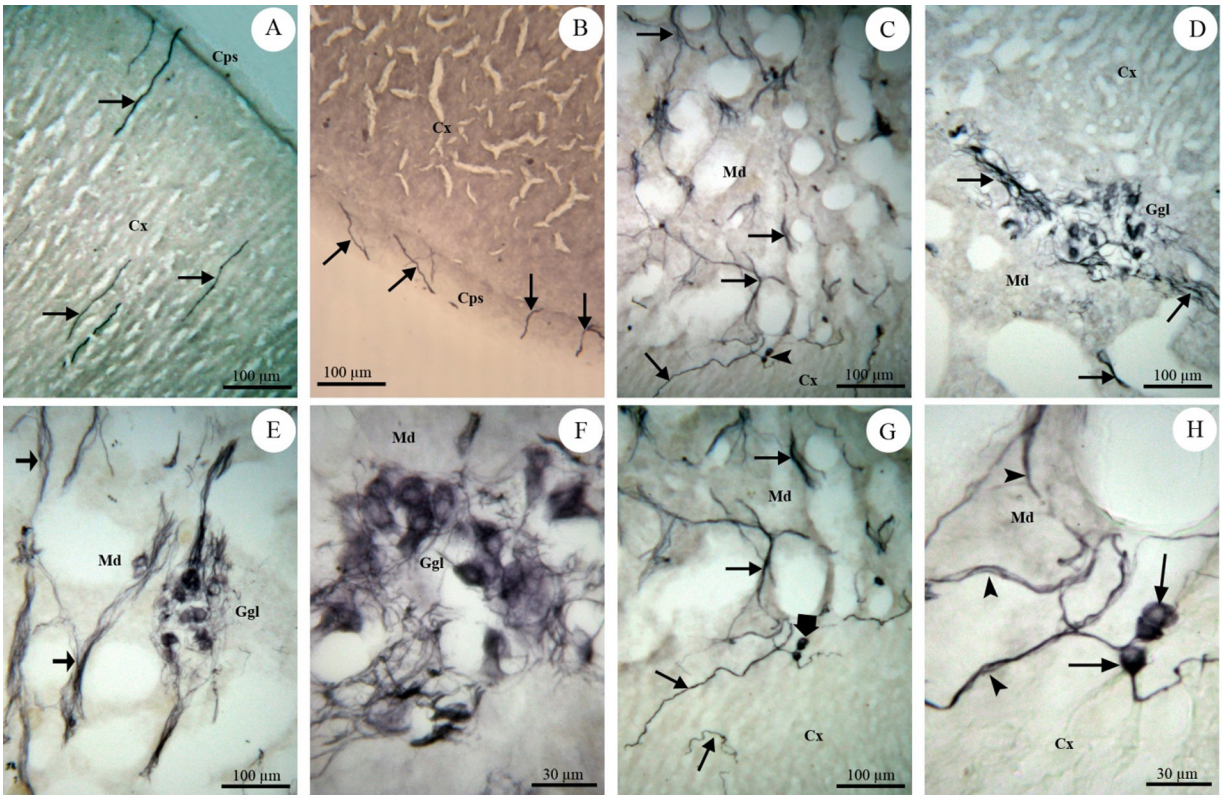

Fig. 4. Light micrograph of pChAT immunoreactivity in the rat adrenal gland. (A), (B) and (C) show pChAT-IR nerve fibers (arrows) in the capsule (Cps), cortex region (Cx) and in the adrenal medulla (Md). Strongly labeled sporadic ganglionic neurons (arrow head) also appear in the medulla. In the capsule, pChAT-IR nerve bundles and fibers were seen to traverse radially through the cortex into the medulla. (D), (E) and (F) show adrenal medulla (Md) with strong pChAT-IR nerve fibers and bundles (arrows) as well as ganglionic neurons (Ggl). (G) shows pChAT-IR fibers (arrows) of some sporadic neurons (broad arrow) leave the adrenal medulla (Md) and enter the cortex. (H) is a higher magnification of (G) showing adrenal medulla (Md) with strong pChAT-IR nerve bundles and fibers (arrow heads) and axons of the pChAT-IR neurons (arrows) at the periphery of adrenal medulla entering the cortex (Cx).

Although the presence of neurons in the adrenal medulla has been recognized in many laboratory and domestic animals (Coupland, 1965a; Tomlinson and Coupland, 1990), no immunohistochemical studies of these elements against acceptable cholinergic markers have been published. In the current study a dense plexus of pChAT-IR fibers innervating the entire adrenal medulla and pChAT-IR ganglionic cells has been demonstrated for the first time. A few pChAT-IR fiber bundles traversing the adrenal cortex into the

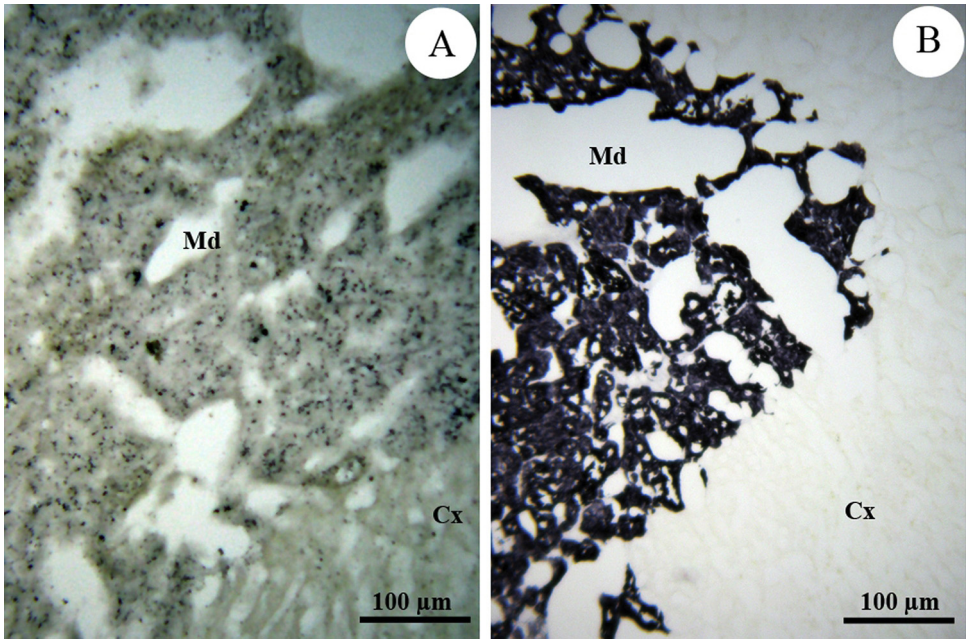

Fig. 5. Light micrographs. (A) shows VAChT immunoreactivity as small VAChT-IR puncta in the nerve fibers only in the medulla (Md) and no VAChT reactivity is found in the cortex (Cx). (B) shows strong TH immunoreactivity only in the rat adrenal medulla (Md) whereas no reactivity is seen in the cortex (CX).

medulla were also observed. Groups of medullary neurons often showed a close packing of somata and some of them had fine axons extending into the adrenal cortex. Intramedullary pChAT-IR neurons, supplying adrenal cortex and medulla, belong to the postganglionic autonomic system and may therefore be both the source of sympathetic and parasympathetic postganglionic fibers, as well as providing one of the targets of the extrinsic adrenal innervation. In agreement, Bellier and Kimura (2011), based on previous studies, suggested that ACh could be released directly from the centripetal and centrifugal pChAT-containing nerves implying that pChAT may function within axons or presynaptic terminals.

The VAChT immunoreactivity in the adrenal medulla of the rat appeared as nerve terminals in the form of puncta around the medullary cells. Some VAChT-IR nerve fibers were also noticed crossing the cortex into the medulla. In parallel, Weihe et al. (1996), Arvidsson et al. (1997), Schafer et al. (1997), Tischler et al. (1999), Hamelink et al. (2002) and Murabayashi et al. (2009) found that VAChT immunoreactivity completely delineate the preganglionic sympathetic terminals in the adrenal medulla. In reasonable accord with this fact, a dense plexus of VAChT-IR nerve terminals in the adrenal medulla and few VAChT-IR fiber bundles traversing the adrenal cortex to the medulla were also observed (Arvidsson et al., 1997; Tischler et al., 1999). Since adrenal chromaffin cells are innervated by terminals from the splanchnic nerve, this indicates that ACh present in the splanchnic nerve is innervating the rat adrenal medulla. Preganglionic fibers of the splanchnic nerve enter the clusters with presynaptic boutons on each glandular cell. A single fiber may of course provide more than one bouton to a particular cell and at least some of the chromaffin cells are polyneuronally innervated (Kajiwara et al., 1997). They control the secretion of adrenal medullary catecholamines (Tomlinson and Coupland, 1990; Martin, 2005) and they are thought to be cholinergic (Kesse et al., 1988). No evidence of an adrenergic innervation of chromaffin tissue of the adrenal medulla has been obtained in rat (Tomlinson and Coupland, 1990). Our result confirmed this fact since we only observed strong TH-IR in the adrenal medulla without any TH-IR nerve fibers in rat adrenal gland as a whole. In contrast, the presence of adrenergic fibers and terminals with *bouton en passage* configuration adjacent to noradrenergic cells in the adrenal medulla of the cat has been reported (Prentice and Wood, 1974, 1975).

According to our results, components of the cholinergic nervous system in the peripheral tissues, whose existence has been controversial, have been confirmed through the existence of new components of the cholinergic nervous system, pChAT. Our study provides for the first time, detailed data about pChAT expression, as a novel and reliable cholinergic marker, in the rat adrenal gland. The significance and physiological role of pChAT in adrenal gland need further study to be elucidated.

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