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Cholinergic Profiles in the Goettingen miniature pig *(Sus scrofa domesticus)* brain

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Abstract

Central cholinergic structures within the brain of the even-toed hoofed Goettingen miniature domestic pig (*Sus scrofa domesticus*) were evaluated by immunohistochemical visualization of choline acetyltransferase (ChAT) and the low-affinity neurotrophin receptor, p75^{NTR}. ChAT immunoreactive (-ir) perikarya were seen in the olfactory tubercle, striatum, medial septal nucleus, vertical and horizontal limbs of the diagonal band of Broca and the nucleus basalis of Meynert, medial habenular nucleus, zona incerta, neurosecretory arcuate nucleus, cranial motor nuclei III and IV, Edinger-Westphal nucleus, parabigeminal nucleus, pedunculopontine nucleus and laterodorsal tegmental nucleus. Cholinergic ChAT-ir neurons were also found within transitional cortical areas (insular, cingulate, and piriform cortices) and hippocampus proper. ChAT-ir fibers were seen throughout the dentate gyrus and hippocampus, in the mediodorsal, laterodorsal, anteroventral and parateanial thalamic nuclei, the fasciculus retroflexus of Meynert, basolateral and basomedial amygdaloid nuclei, anterior pretectal and interpeduncular nuclei, as well as select laminae of the superior colliculus. Double immunofluorescence demonstrated that virtually all ChAT-ir basal forebrain neurons were also p75^{NTR} positive. The present findings indicate that the central cholinergic system in the miniature pig is similar to other mammalian species. Therefore, the miniature pig may be an appropriate animal model for preclinical studies of neurodegenerative diseases where the cholinergic system is compromised.

Keywords

acetylcholine; choline acetyltransferase; p75 low-affinity neurotrophin receptor; cholinergic basal forebrain; miniature pig; RRID: AB_2079751; RRID: AB_310649; RRID: AB_2314520; RRID:

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Conflict of Interest
The authors report no conflict of interest.

Role of Authors
All authors had full access to all the data in the study and take responsibility for the integrity of the data. Study concept and design: E.J. Mufson, S.E. Perez and L. Mahady. Acquisition of data: L. Mahady, S.E. Perez, and E.J. Mufson. Analysis and interpretation of data: L. Mahady, S.E. Perez and E.J. Mufson. Drafting of the article: L. Mahady, S.E. Perez, and E.J. Mufson. Critical revision of the article for important intellectual content: L. Mahady, S.E. Perez, E.J. Mufson, D.F. Emerich, and L.U. Wahlberg.

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Introduction

The distribution of cholinergic neurons in the central nervous system has been examined in numerous mammalian species (Armstrong et al., 1983; Mesulam et al., 1983a; Vincent and Reiner, 1987; Everitt et al., 1988; Maley et al., 1988; Mufson and Cunningham, 1988; Kordower et al., 1989; Alonso and Amaral, 1995; Ichikawa et al., 1997; Varga et al., 2003; Maseko and Manger, 2007; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010, 2012; Pieters et al., 2010; Calvey et al., 2013; Maseko et al., 2013; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c). These neurons play a key role in complex functions including learning, memory, attention, and sleep (Jouvet, 1972; Kodama and Honda, 1996; Hasselmo and McGaughy, 2004; Atri et al., 2004; Green et al., 2005; Klinkenberg et al., 2011). Cholinergic neurons are characterized by the presence of choline acetyltransferase (ChAT), the synthetic enzyme for the neurotransmitter acetylcholine (ACh), while its hydrolytic enzyme, acetylcholinesterase (AChE), is also present in cholinoceptive cells (Emson et al., 1979; Mesulam et al., 1984, Mufson et al., 1989; Woolf, 1991). Cholinergic neurons in the mammalian basal forebrain are found within the medial septum (MS) and vertical limb of diagonal band of Broca (VDB), the horizontal limb of diagonal band of Broca (HDB) and the magnocellular nucleus basalis of Meynert/substantia innominata complex (NBM/SI), which correspond to the Ch1, Ch2, Ch3 and Ch4 subregions, respectively (Mesulam et al., 1983b). The Ch4 complex has been subdivided into anteriomedial (Ch4am), intermediate dorsal (Ch4id) and ventral (Ch4iv), and posterior (Ch4p) subfields (Mesulam et al., 1983b; Liu et al., 2015). Ch1-2 neurons innervate the hippocampus, Ch3 the olfactory bulb and visual cortex (Mesulam et al., 1983b; Rye et al., 1984), while Ch4 neurons provide the major cholinergic innervation to the cortical mantle and amygdala (Armstrong et al., 1983; Mesulam et al., 1983b; Saper, 1984; Kasa, 1986; Eckenstein et al., 1988; Brückner et al., 1992; Semba, 2000). Within the epithalamus cholinergic neurons are found within the medial habenula (Ch7) (Mesulam et al., 1986; Mufson and Cunningham, 1988), which innervates the interpeduncular nucleus (Woolf and Butcher, 1985; Woolf et al., 1990; Woolf, 1991). More caudally, cholinergic neurons are found in the pedunculopontine (Ch5), laterodorsal tegmental (Ch6) nuclei and parabigeminal nucleus (Ch8), which project to the thalamus and superior colliculus, respectively (Mesulam et al., 1986; Mufson et al., 1986). Cholinergic basal forebrain neurons also express the low affinity pan-neurotrophin $p75^{NTR}$ receptor for nerve growth factor (Mufson et al., 1989) and depending upon the mammalian species examined, the ACh inhibitor, galanin (GAL) (Fisone et al., 1987; Kordower and Mufson, 1990; Benzing et al., 1993a; Perez et al., 2001).

Despite the large number of studies of the cholinergic system in mammals, to our knowledge, no study has examined the central cholinergic system in the Goettingen domestic miniature pig, a member of the species *Sus scrofa domesticus*, and order Artiodactyla. However, cholinergic profiles have been evaluated in other members of this order including even-toed ungulates such as sheep (Ferreira et al., 2001), giraffe (Bux et al., 2010), river hippopotamus (Dell et al., 2016a), and cetaceans including the harbor porpoise (Dell et al., 2016b) and minke whale (Dell et al., 2016c). The Goettingen miniature pig is the result of crossbreeding the Minnesota miniature pig, the Vietnamese potbelly pig, and the German Landrace pig (Köhn et al., 2007; Simianer and Köhn, 2010). The miniature pig has a well-defined genetic background, and its organ physiology is similar to humans (Dolezalova et al., 2014). The pig brain is gyrencephalic with gray/white matter proportions closer to humans than the rodent (Howells et al., 2010). Additionally, swine are more cost-effective than non-human primates and have a shorter gestation period (Dolezalova et al., 2014) making the Goettingen miniature pig well-suited for human disease-related translational research including myocardial infarction and stroke (Schuleri et al., 2008; Dolezalova et al., 2014), Alzheimer's (Kragh et al., 2009; Fjord-Larsen et al., 2009; Søndergaard et al., 2012; Jakobsen et al., 2013) and Parkinson's (Mikkelsen et al., 1999; Glud et al., 2011) disease. The latter disorders are associated with cholinergic basal forebrain (CBF) neuronal degeneration (Mufson et al., 1991; Gilmor et al., 1999). The aim of the present study was to immunohistochemically define the distribution of cholinergic profiles within the brain of the Goettingen miniature pig, a potential animal model for studies of the cholinergic system during aging and disease.

Materials and Methods

Tissue Processing—In the current study, six sexually mature female Goettingen miniature pigs (4-month-old, 10kg mean body mass, 67g mean brain mass) were anesthetized with isoflurane (1–2%) prior to intubation, and maintained on the drug until the administration of an intravenous lethal injection of Beuthanasia-D (Merck Animal Health, Madison, NJ). Pigs were perfused under anesthesia via the carotid artery with 0.9% cold saline followed by Zamboni's solution (consisting of picric acid and 4% paraformaldehyde) and brains were removed from the calvarium and post-fixed for 48 hours in the same fixative. Brains were cryoprotected in 30% sucrose, and sectioned in either the coronal or horizontal plane at 40 microns on a sliding freezing microtome. Tissue provided for this study extended from the frontal cortex to the level of the brain stem pontomesencephalic nuclei. The lower brain stem and medulla were not available for analysis. Sections were stored in a cryoprotectant solution (30% glycerol, 30% ethylene glycol, 40% phosphate buffer) at −20° C until processing for immunohistochemistry. The use of these animals and the indicated anesthesia/perfusion protocol was approved by the Animal Care and Use Committees of the Department of Neuroscience at Rush University, Chicago, IL and NsGene Inc, Providence, RI.

Antibody Characterization—Table 1 describes the characteristics of the primary antibodies used in the present study, including: an anti-choline acetyltransferase (ChAT) goat IgG antibody (1:1,000 dilution, Millipore, MA, AB144P, RRID: AB_2079751) raised against human placental ChAT (Hersh et al., 1978; Raghanti et al., 2008). According to the manufacturer western blot analysis of this antibody yields a single band at 70 kDa, and specifically stains cholinergic neurons (Mufson et al., 1989). Additionally, we used an anti-low affinity neurotrophin receptor p75^{NTR} rabbit IgG antibody raised against a glutathione S-transferase (GST) fusion protein corresponding to the intracellular domain (residues 274–

425) of rat p75^{NTR} (1:3,000 dilution, Millipore, 07-476, RRID: AB_310649) (Matusica et al., 2013); and a rabbit polyclonal anti-galanin (GAL) antibody (1:1,000 dilution, gift from Dr. E. Theodorsson, Sweden, RRID: AB_2314520), whose specificity has been reported previously (Theodorsson and Rugarn, 2000; Perez et al., 2001; Diez et al., 2003; Kelley et al., 2011) and reacts with the central and C-terminal part of GAL but does not recognize the N-terminus in a radioimmunoassay (Theodorsson and Rugarn, 2000). Each of these antibodies have been used extensively in immunocytochemical investigations of the mammalian brain (Mufson et al., 1989; Obata et al., 1999; Theodorsson et al., 2000; Diez et al., 2003; McKeon-O'Malley et al., 2003; Ikonomovic et al., 2007; Motts and Schofield, 2009; Fu et al., 2010; Kelley et al., 2011).

ChAT, p75^{NTR} and GAL Immunohistochemistry—Immunohistochemistry was performed as described previously (Mufson and Cuningham, 1988; Mufson et al., 1989; Benzing et al., 1993b; Perez et al., 2000). Briefly, sections were washed in phosphate buffer (3×10 minutes) and tris buffered saline (TBS, 3×10 minutes), to remove excess cryoprotectant, before a 20-minute incubation in 0.1 mol/L sodium metaperiodate (Sigma, St Louis, IL) in TBS to inactivate endogenous peroxidase activity. Tissue was then washed in a TBS solution containing 0.25% Triton X (ThermoFisher, Waltham, MA) (3×10 minutes), and placed in the same solution using 3% horse serum for 1 hour. Sections were then incubated with the appropriate primary antibodies (see Table 1) overnight in a 0.25% Triton X-100 and 1% horse serum solution. All washes and incubations were performed at room temperature on a shaker table. Sections were subsequently washed in TBS and 1% horse serum before incubation (3×10 minutes) with the appropriate biotinylated secondary antibody, horse anti-goat IgG, (1:200, Vector Laboratories, CA) or horse anti-rabbit IgG, (1:200, Vector), for one hour. After TBS washes, ChAT immunoreactivity was amplified by incubating the tissue using the Vectastain ABC kit (Vector) for 1 hour; rinsed in 0.2 mol/L sodium acetate, 1.0 mol/L imidazole buffer, pH 7.4; and developed in acetate-imidazole buffer containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, IL) and 0.0015% H_2O_2. Sections only immunostained for p75^{NTR} or GAL were visualized with DAB and 1% nickel (II) ammonium sulfate hexahydrate and 0.0015% H_2O_2 resulting in a black reaction product. The histochemical reaction was terminated in acetate-imidazole buffer, tissue mounted on slides, dehydrated through graded alcohols (70%–95%–100%), cleared in xylene, and cover-slipped using DPX mounting medium (Biochemica Fluka, Buchs, Switzerland). The specificity of the secondary antibodies was determined by absence of immunocytochemical reaction in sections processed without the primary antibody. Additional sections were counterstained with cresyl violet to aid in cytoarchitectonic analysis. For each antibody, staining of all sections was performed at the same time to reduce batch-to-batch variability. A stereotaxic atlas of the pig brain was used for regional delineation (Félix et al., 1999), and abbreviations were taken from the atlas by Paxinos and colleagues (1999).

Immunofluorescence—Immunofluorescence was conducted on additional forebrain sections using a modification of a previously reported protocol (Oh et al., 2010). Briefly, sections were double labeled for ChAT (1:100) and p75^{NTR} (1:300) overnight at room temperature, and then incubated for 1 hour using Cy2-conjugated donkey anti-rabbit (1:200, Jackson ImmunoResearch, RRID: AB_2340612) and Cy3-conjugated donkey anti-goat IgG (1:200, Jackson ImmunoResearch, RRID: AB_2307351) as secondary antibodies. Cy2 and Cy3 fluorescence was detected using FITC (excitation light= 490 nm) and TRITC (excitation light= 550 nm) filters, respectively.

Charting the distribution of cholinergic neurons in the pig brain

Schematic drawings of the distribution of ChAT-ir profiles were outlined manually under 1× and 10× lenses (numerical aperture (NA) 0.04 and 0.25, respectively) with the aid of a Nikon Optiphot-2 microscope using the Neurolucida Neuron tracing program (MBF Bioscience, VT, RRID: SCR_001775). Chartings were adjusted and edited using the CorelDraw Graphics Suite X7 software (Corel Corporation, Ottawa, ON, Canada, RRID: SCR_014235). Cholinergic cell diameter was determined by manually outlining twenty ChAT-ir neurons from 4 separate sections containing each cholinergic subgroup using the Nikon NIS-element software (Nikon, Melville, NY, RRID: SCR_014329). Measurements are presented as mean diameter ± standard deviation. Immunohistochemistry and immunofluorescence were visualized with the aid of a Nikon Eclipse 80i microscope.

Results

Telencephalon

Cerebral cortex and Olfactory Tubercle—ChAT-ir fibers were observed throughout the cortical areas examined, including the cingulate, insular and piriform cortices (mean diameter: 16 ± 1.73 μm) (Fig. 1A–D). In the cingulate cortex small multipolar and bipolar ChAT positive neurons were found mainly in layer VI (Fig. 1A), whereas in the insular cortex cholinergic neurons were observed scattered within layers III and V (Fig. 1B) and within layer III of the piriform cortex (Fig. 1C). These cortical areas also displayed a few small, bipolar $p75^{NTR}$-ir neurons distributed in a pattern similar to that seen for ChAT-ir neurons (Fig. 1E). Small, spindle shaped ChAT- and $p75^{NTR}$-ir neurons were scattered throughout the more rostral aspect olfactory tubercle (Fig. 1F).

Striatum and Basal Forebrain—ChAT-ir perikarya were identified within the striatum (i.e., caudate and putamen) and in each CBF subfield (Fig. 2). The striatum displayed large multipolar ChAT-ir neurons and fibers (mean diameter: 32 ± 3.21 μm) (Fig. 3A, B), which were $p75^{NTR}$ immunonegative (Fig. 4A). Within the CBF, ChAT-ir neurons were found in the medial (MS, Ch1), VDB (Ch2)/HDB (Ch3), and the NBM (Ch4) (Figs. 2, 3A, C–E, F–H). The lateral septum (LS) also displayed small spindle and multipolar ChAT-ir neurons (mean diameter: 24 ± 2.21 μm) in its lateroventral aspect (Figs. 2C, D, 3A, C). A few scattered ChAT-ir neurons were observed in the nucleus accumbens (Fig. 2A, B) and bed nucleus of the stria terminalis (Fig. 3F). Ch1 contained small bipolar and multipolar ChAT-ir neurons with processes directed dorsally and ventrally, forming an elongated cluster situated along the midline (mean diameter: 28 ± 1.56 μm) (Fig. 3A, D, F). Ch2 displayed medium-sized, round or spindle-shaped ChAT-ir neurons (mean diameter: 30 ± 2.16 μm) (Figs. 2C, D, 3A, E), with larger neurons found more rostrally (Fig. 3E). Ch3 medium-sized cholinergic neurons (mean diameter: 31 ± 2.33 μm) were located ventral to the anterior commissure and lateral to Ch2 (Figs. 2C, D, 3A, F, G). More caudally, ChAT-ir somata were seen within the Ch4 subfield ventral to the internal capsule and globus pallidus, which extended to the level of the anterior amygdala (Figs. 2C, D, 3F, H). At the level of the decussation of the anterior commissure ChAT-ir neurons within Ch4 (NBM/SI) appeared multipolar (mean diameter: 35 ± 2.10 µm) (Fig. 3H), with processes that curved ventrally around the globus pallidus, extending into the internal capsule (Fig. 3F). Although GAL-ir neurons were not observed in any of the CBF subgroups, numerous scattered GAL-ir fibers were seen surrounding neurons in the septum (Fig. 3I) and Ch4 (Fig. 3J). Dual immunofluorescence revealed that virtually all ChAT-ir neurons within the Ch subfields were p75^{NTR} immunopositive (Fig. 4B–E).

Hippocampal Formation and Amygdala—The hippocampal formation contained ChAT-ir neurons and fibers (Fig. 5A–E). Scattered, small bipolar ChAT-ir neurons were found dorsal to the CA1 pyramidal cell layer, mainly in the caudal aspect of the hippocampus (Fig. 5B, inset), but not in the dentate gyrus. Although numerous fine, beaded ChAT-ir fibers were observed throughout the hippocampus and dentate gyrus (Fig. 5A–C), the densest collection of fibers was observed within the polymorphic and molecular layers of the dentate gyrus, stratum oriens, and CA1-3 pyramidal cell layers (Fig. 5B–E).

Although ChAT-ir neurons were not observed in the amygdala, ChAT-ir fibers were found throughout the anterior part of the basolateral, basomedial, and dorsolateral nuclei (Fig. 6A), with the heaviest concentration observed within the anterior aspects of the basolateral amygdaloid nucleus (Fig. 6A).

Diencephalon

Epithalamus—A group of small densely packed, oval-shaped ChAT-ir neurons were seen in the medial habenular nucleus (Ch7) (mean diameter: 15 ± 0.81 µm) (Fig. 6C, D). In contrast, only ChAT-ir fibers were observed in the lateral nucleus of the habenula, which coursed within the fasciculus retroflexus (Fig. 6C, open arrow), presumably projecting to the interpeduncular nucleus, which displayed a dense cholinergic innervation (Fig. 7C).

Thalamus—Several thalamic nuclei displayed ChAT-ir fibers but not positive neurons (Fig. 6A, B). Rostrally, the medial and lateral portions of the anteroventral, and parateanial thalamic nuclei displayed intense ChAT-ir, while the central, periventricular and reticular thalamic nuclei displayed weak staining (Fig. 6B). The thalamic mediodorsal and laterodorsal nuclei were strongly ChAT-ir, followed by the ventrolateral nucleus (Fig. 6A). More caudally, ChAT-ir fibers were observed in rostral aspects of the anterior pretectal nucleus (Fig. 6G), but to a lesser degree more caudally at the level of the superior colliculus. ChAT-ir fibers were also observed in the lateral geniculate nucleus (Fig. 5A). The zona incerta contained small, spindle-shaped neurons. Ventromedial to the dorsolateral geniculate at the level of the medial habenula, a small group of faintly stained ChAT-ir neurons and fibers were observed in reticular thalamic territories.

Hypothalamus—Within the hypothalamus, weakly-stained small, oval shaped ChAT-ir neurons (mean diameter: 14 ± 1.13 µm) (Fig. 6E) were observed within the dorsal portion of the arcuate nucleus. A densely packed group of ChAT-ir neurons of similar shape and size with extensive processes were seen within the medial arcuate nucleus (mean diameter: 12 ± 0.72 µm) (Fig. 6F). ChAT-ir fibers were also seen coursing within the external aspect of the median eminence.

Mesencephalon

Small spindle-shaped ChAT-ir perikarya and fibers were seen in the Edinger-Westphal nucleus (mean diameter: 21 ± 1.84 µm) (Fig. 7A, B), forming a dense cluster running parallel to the midline, ending caudal to the superior colliculus. Laterally, large multipolar ChAT-ir somata were found in the oculomotor nucleus (mean diameter: 35 ± 2.87 µm) (Fig. 7A, B), giving rise to the III cranial nerve roots exiting at the level of the interpeduncular nucleus. ChAT-ir fibers, but not cells, were observed within the superficial layers of the superior colliculus in the intermediate gray, and intermediate optic layers (Fig. 7D).

Several ChAT-ir cell groups were observed within the tegmentum of the pontomesencephalon. ChAT-ir neurons in the pedunculopontine (Ch5) and laterodorsal tegmental (Ch6) nuclei were largely heteromorphic, ranging in appearance from pyramidal to fusiform to multipolar (Fig. 8A–C). ChAT positive neurons within Ch5 were slightly larger (mean diameter: 30 ± 2.70 µm) (Fig. 8B) and more spindle-shaped compared to Ch6 (mean diameter: 27 ± 1.90 µm) (Fig. 8C). A cluster of small oval shaped ChAT-ir perikarya were observed within the parabigeminal nucleus (Ch8) (mean diameter: 18 ± 1.35 µm) (Fig. 8A, D). A dense plexus of ChAT-ir fibers were found in close proximity to the parabigeminal nucleus (Fig. 8A, black arrows). Large ChAT-ir neurons (mean diameter: 38 ± 3.32 µm) and fibers were also observed in the trochlear nucleus (Fig. 7B).

Discussion

The present findings are the first detailed description of cholinergic profiles within the brain of the female Goettingen miniature pig, *Sus scrofa domesticus*. ChAT-ir neurons and fibers were observed within the telencephalon, diencephalon, and mesencephalon similar to other mammalian species (see Table 2).

Telencephalon

Cerebral Cortex—ACh is the major neuromodulatory neurotransmitter in the cortex and is thought to play an important role in learning, memory and attention (Callahan et al., 1993; Andrews et al., 1994; Muir et al., 1994; Hasselmo, 2006). The cortex of the Goettingen miniature pig displayed numerous ChAT-ir fibers similar to other mammals (e.g., humans, Mufson et al., 1989; Mesulam et al., 1992; nonhuman primates, Mrzljak and Goldman-Rakic, 1993; Mesulam, 2004; Raghanti et al., 2008; rodents, Ichikawa and Hirata, 1986; Lysakowski et al., 1986; Mechawar et al., 2000). Small ChAT positive neurons were seen within the cingulate, insular and piriform cortices as described in some rodent species (Eckenstein and Baughman, 1984; Mufson and Cunningham, 1988; Mechawar et al., 2000; Bhagwandin et al., 2006; Consonni et al., 2009) including murid rodents (Kruger et al., 2012) and the Hottentot golden mole (Calvey et al., 2013), but not in the BALB/c ByJ mouse (Kitt et al., 1994), guinea pig (Maley et al., 1988), monotremes (Manger et al., 2002) and most nonhuman primates (Mesulam et al., 1984; Everitt et al., 1988; Kordower et al., 1989). ChAT positive neurons in supragranular cortical layers (II–IV) have been reported in some rodent species (Eckenstein and Baughman, 1984; Mufson and Cunningham, 1988; Mechawar et al., 2000; Bhagwandin et al., 2006; Consonni et al., 2009), rabbit (Varga et al., 2003), feline (Avendaño et al., 1996), fetal *Macaca mulatta* (Hendry et al., 1987) and to a lesser degree in humans (Kasashima et al., 1999). Unlike the miniature pig, ChAT–ir neurons were found within the somatosensory cortex and to a lesser extent in transitional and limbic cortices in murine species and rabbits (Varga et al., 2003; Consonni et al., 2009). These cholinergic perikarya have been classified as interneurons, which contain calretinin and vasoactive intestinal peptide (VIP) and are involved in local cortical modulatory functions (von Endelhardt et al., 2007). Whether intrinsic cortical neurons in the miniature pig express calretinin and VIP, as well as their functional role remain unknown. In contrast to the rodent and pig cortex, a few studies have reported ChAT positive pyramidal neurons in layers III and V in the human motor and secondary sensory cortex (Kasashima et al., 1999; Benagiano et al., 2003). This species difference in the type of cortical neurons requires further investigation to verify the existence of cholinergic neurons in the human cortex. On the other hand, the pig cingulate, insular, and piriform cortices contained $p75^{NTR}$-ir fibers similar to that seen in the rodent (Jaffar et al., 2001), monkey, and human (Mrzljak and Goldman-Rakic, 1993) cortex. Perikarya containing $p75^{NTR}$ have also been identified in somatosensory and motor cortices in the adult macaque (Miller, 2000) and in medial temporal cortex and amygdala in the aged human and Alzheimer diseased brain (Mufson and Kordower, 1992) but not in the rat (Lee et al., 1998) or mouse (Perez et al., 2007) suggesting species differences in the cortical $p75^{NTR}$ cellular phenotype.

Olfactory tubercle, Striatum and Basal Forebrain—The olfactory tubercle of the miniature pig contained ChAT-ir neurons and fibers as described in other mammals (McGeer et al., 1982; Armstrong et al., 1983; Wahle and Meyer, 1986; Woolf, 1991; Varga et al., 2003; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010; Pieters et al., 2010; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c). More caudally, large multipolar ChAT-ir neurons were located in the striatum of the miniature pig similar to other mammals (Sofroniew et al., 1982; Armstrong et al., 1983; Mesulam et al., 1984; Satoh and Fibiger, 1985; Mufson and Cunningham, 1988; Mufson et al., 1989; Woolf, 1991; Geula et al., 1993; Varga et al., 2003; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010; Pieters et al., 2010; Bajo et al., 2014; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c). The major source of cholinergic striatal innervation is the large aspiny interneurons that comprise 1% of striatal neurons (Woolf and Butcher, 1981; Graveland and DiFiglia, 1985), which regulate intrastriatal local circuit activity (Galarraga et al., 1999; Koós and Tepper, 2002). These cholinergic neurons are not $p75^{NTR}$-ir (Sobreviela et al., 1994; Kordower et al., 1994, present study) and are related to the regulation of motoric function (Macintosh, 1941; Hebb and Silver, 1961; Woolf et al., 1984).

ChAT-ir neurons were observed in the MS/VDB (Ch1/Ch2) and HDB (Ch3). The MS/VDB complex projects to the hippocampus, entorhinal, cingulate and retrosplenial cortices in many mammals (Mesulam et al., 1983a; Irle and Markowitsch, 1984; Woolf et al., 1984;

Amaral and Kurz, 1985), while the HDB projects to the olfactory bulb and visual cortex (Mesulam et al., 1983a; Woolf et al., 1984). The human HDB consists of a few cholinergic neurons (Mesulam et al., 1983a) suggesting a diminished functional role compared to other mammals. We also observed lightly immunoreactive cholinergic neurons within the lateral septum of the miniature pig, as reported in the rat (Kimura et al., 1990), raccoon (Brauer et al., 1999) and monkey (Kimura et al., 1990).

More caudally, Ch4 (NBM/SI) displayed ChAT-ir neurons extending from the crossing of the anterior commissure to the level of the lateral geniculate nucleus as well as a few scattered somata embedded within the internal capsule (termed interstitial cholinergic neurons), globus pallidus, and anterior regions of the amygdala; (Mesulam et al., 1983). These neurons share the magnocellular hyperchromic morphology observed in other mammals (Mesulam et al., 1983a, 1983b; Woolf, 1991; Varga et al., 2003). In rodents, and monotremes (the oldest mammalian group) a homologous NBM/SI cell group is found adjacent to the globus pallidus (Mesulam et al., 1983b; Manger et al., 2002) similar to other mammalian species (Woolf, 1991; Varga et al., 2003; Descarries et al., 2004; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010; Pieters et al., 2010; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c; see Table 2). Interestingly, the NBM/SI displays the greatest degree of subfield differentiation in monkeys, great apes (Benzing et al., 1993a) and humans (Gorry, 1963; Mesulam et al., 1983a; Saper and Chelimsky, 1984; Mesulam and Geula, 1988), compared to other mammals (Mesulam et al., 1983b) including the miniature pig (present findings). However, in sheep, the complexity of this region is intermediate between rodents and humans, four subsectors are found including the anterior, intermedioventral, intermediodorsal and posterior subfields but not an anterolateral or medial subfield (Ferreira et al., 2001). Thus Ch4 (NBM/SI) intricacy among species may be associated with a gain in neocortical chemoanatomical and functional complexity (Howells et al., 2010; Lui et al., 2011). In addition to innervating neocortical areas (Mesulam et al., 1983a), cholinergic Ch4 neurons also send a dense projection to the basal nucleus of the amygdala (Woolf, 1991). In this regard, extensive cholinergic innervation was seen in the basolateral and basomedial amygdaloid nuclei in the miniature pig similar to that observed in the rat, golden mole (Calvey et al., 2013), monkey (Woolf and Butcher, 1982; Amaral and Bassett, 1989) and human (Mufson et al., 1989; Benzing et al., 1993b). Although, only ChAT-ir fibers are found in the amygdala of most mammals including the minipig, a recent report revealed ChAT-ir neurons in the amygdala of the golden mole (Calvey et al., 2013).

We observed that the majority of neurons within the CBF of the miniature pig co-express the p75^{NTR} similar to other mammals (Hefti et al., 1986; Mesulam et al., 1989; Mufson et al., 1989; Kordower et al., 1989; Higgins and Mufson, 1989; Hefti and Mash, 1989; Ferreira et al., 2001). The p75^{NTR} receptor plays a key role in regulating the trophic status of CBF neurons (Kramer et al., 1999). Of interest was our observation that CBF neurons in the miniature pig were GAL immunonegative, while in rodents (Melander et al., 1985; Perez et al., 2001) and monkeys (Melander and Staines, 1986; Walker et al., 1989; Kordower and Mufson, 1990; Kordower et al., 1992) these neurons were GAL positive. However, GAL-ir fibers in the miniature pig were seen in close proximity to cholinergic neurons more similar to humans (Chan-Palay, 1988; Kordower and Mufson, 1990; Mufson et al., 1993) and great apes (Benzing et al., 1993a). We have suggested that the separation from intraneuronal GAL within cholinergic neurons to extra-neuronal innervation may relate to a greater control over cholinergic cellular activity in humans and great apes (Mufson et al., 2005; Counts et al., 2010).

Epithalamus, Thalamus, and Hypothalamus—In the epithalamus of the Goettingen miniature pig, ChAT-ir profiles were found in the medial habenular nucleus, fasciculus retroflexus and interpeduncular nucleus as described in other mammals (Woolf and Butcher, 1985; Mesulam et al., 1986; Mufson and Cunningham, 1988; Butcher et al., 1992; Oh et al., 1992; Manger et al., 2002; Kruger et al., 2012; Maseko et al., 2013). Tract tracing studies in mammals have shown that the medial habenula provides the main source of cholinergic innervation via the fasciculus retroflexus to the interpeduncular nucleus (Woolf and Butcher, 1985; Woolf et al., 1990; Woolf, 1991). The functional role of this dense cholinergic innervation remains to be investigated.

ChAT-ir neurons were virtually absent from the thalamic nuclei of the miniature pig (see Table 2), although they have been reported in the centromedian nucleus of the dorsal thalamus in the river hippopotamus (Dell et al., 2016a), an animal related to the pig, and in the paracentral thalamic nucleus of the macaque monkey (Rico and Cavada, 1998). Numerous cholinergic fibers were observed in the anteroventral, parateanial, mediodorsal, and laterodorsal thalamic nuclei as well as in the reticular nucleus in the miniature pig (present study) as reported previously in mammals (Hallanger et al., 1987; Levey et al., 1987; Schafer et al., 1998). In addition, ChAT positive fibers were found in the anterior pretectal nucleus, lateral geniculate nucleus, as well as in the subthalamic nucleus as previously described in the Goettingen minipig (Larsen et al., 2004). Neuroanatomical tract-tracing studies in the rat have demonstrated that the cholinergic neurons located in pedunculopontine tegmental nucleus innervate the thalamic anterior, laterodorsal, central medial, and mediodorsal thalamic nuclei as well as the lateral geniculate nucleus (Mufson et al., 1986; Hallanger et al., 1987; Fitzpatrick et al., 1988; Smith et al., 1988). Retrograde tracing studies have revealed that the cholinergic laterodorsal tegmental nucleus also projects to several thalamic nuclei and the pretectum (Satoh and Fibiger et al., 1986). Whether similar projections exist in the pig remains to be determined. The functional significance of these observations in the pig are not clear, but cholinergic pedunculopontine neurons have been suggested to play a role in regulating wakefulness and sleep-wake transitions (Brown et al., 2012), perhaps via its thalamic innervation.

Unlike the rat (Rao et al., 1987) and other vertebrates (Mason et al., 1983; Ekstrom, 1987; Tago et al., 1987), the hypothalamus of the miniature pig contained ChAT positive neurons only within the neurosecretory arcuate nucleus. Hypothalamic ChAT-ir fibers were observed coursing in the external portion of the median eminence in the minipig. Several mammals related to the miniature pig including the river hippopotamus (Dell et al., 2016a), the harbour porpoise (Dell et al., 2016b), and the minke whale (Dell et al., 2016c) display three separate ChAT positive hypothalamic cell groups located lateral, dorsal and ventral to the third ventricle. These cholinergic neuronal groups could be homologous to the arcuate nuclei in other mammals including the minipig (Table 2). Since arcuate neurons release hypophysiotropic hormones from terminals located within the median eminence via the hypophysial portal system into the anterior portion of the pituitary gland, it is likely that the cholinergic arcuate system regulates pituitary hormone release (Scott and Pepe, 1987). Although the physiological function of ACh in the neurosecretory system is unclear, perhaps it plays a role in the liberation of hormones acting as an autocrine signal (Wessler and Kirkpatrick, 2008).

Mesencephalon—ChAT-ir neurons were found within the Edinger-Westphal nucleus, the motor nuclei of the oculomotor (IIIn) and trochlear (IVn) cranial nerves. Cholinergic neurons of cranial nerves III, IV, and VI innervate the extraocular eye musculature and the levator palpebrae muscle (Warwick, 1953), while the Edinger-Westphal nucleus is considered the source of preganglionic input to the ciliary ganglion (Strassman et al., 1987). Cranial nerve motor neurons are some of the largest cholinergic cells in the brain and exhibit multiple thick processes in the rodent, feline, and primate brain (Strassman et al., 1987; Woolf, 1991), a pattern mirrored in the miniature pig. Cholinergic neurons in the motor cranial nerve nuclei are a persistent trait in mammalian vertebrates (Armstrong et al., 1983; Mesulam et al., 1983a; Vincent and Reiner, 1987; Everitt et al., 1988; Maley et al., 1988; Mufson and Cunningham, 1988; Kordower et al., 1989; Alonso and Amaral, 1995; Ichikawa et al., 1997; Varga et al., 2003; Maseko and Manger, 2007; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010, 2012; Pieters et al., 2010; Calvey et al., 2013; Maseko et al., 2013; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c) suggesting similar biological actions.

Similar to other mammalian-vertebrates (Armstrong et al., 1983; Mesulam et al., 1983b, 1984; Mufson et al., 1986; Mufson et al., 1988; Mesulam et al., 1989; Ichikawa and Hirata, 1990; Woolf et al., 1990; Lavoie and Parent, 1994; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010; Kruger et al., 2010; Motts and Schofield, 2010; Pieters et al., 2010; Kruger et al., 2012; Maseko et al., 2013; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c), ChAT positive neurons were seen in the pedunculopontine (Ch5), and laterodorsal tegmental (Ch6) nuclei in the miniature pig, which provide cholinergic innervation to the thalamus (Hallanger et al., 1987; Woolf et al., 1990; Motts and Schofield, 2010). Like other mammals, porcine ChAT-ir perikarya within Ch5 and Ch6 are morphologically heterogeneous and relatively large, second only to neurons of the Ch4 complex (Mesulam et al., 1989). Cholinergic immunoreactive neurons within the pontomesencephalon subfields of other members of Artiodactyla display morphological differences compared to the miniature pig. For example, in the male giraffe, cholinergic neurons within the lateral tegmental nucleus (Ch6) are larger than those found in the pedunculopontine nucleus (Ch5) (Bux et al., 2010), which is opposite to what we observed for the minipig. In the river hippopotamus, Ch6 is separated into magnocellular and parvocellular subdivisions (Dell et al., 2016). Whereas, Ch5 and Ch6 are divided into parvocellular and magnocellular subdivisions in the rock hyrax (Gravett et al., 2009). By contrast, the cholinergic parabigeminal nucleus consists of small and oval-shaped perikarya (Mufson et al., 1986; Mufson et al., 1988; Mufson and Cunningham, 1988; Henderson, 1989; Woolf et al., 1990; Maseko and Manger, 2007; Maseko et al., 2007; Bhagwandin et al., 2008; Limacher et al., 2008; Gravett et al., 2009; Bux et al., 2010;

Kruger et al., 2010; Pieters et al., 2010; Patzke et al., 2014; Calvey et al., 2015a, 2015b, 2016; Dell et al., 2010, 2016a, 2016b, 2016c), which project to the superficial layers of the superior colliculus (Beninato and Spencer, 1986; Mufson et al., 1986; Henderson, 1989). No cholinergic cells were observed in the superior colliculus of the miniature pig similar to other mammalian species (Woolf, 1991; Manger et al., 2002).

Conclusions

The present study provides the first chemoanatomical distribution of cholinergic neurons and fibers in select regions of the Goettingen miniature pig brain. Cholinergic neurons were found in the cortex, hippocampus, striatum, MS, LS, VDB/HDB, NBM, medial habenula, pedunculopontine and laterodorsal tegmental nuclei, parabigeminal nucleus, and cranial nerve nuclei. In addition, cholinergic fibers displayed a laminar distribution in the cortex and hippocampus and were seen in the thalamus. The present observations provide detailed information showing that the organization of central cholinergic systems within the miniature pig brain are comparable to most other mammalian species (Table 2), suggesting that the miniature pig may be an appropriate model for the study of the central cholinergic system in various neurological disorders.

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Abbreviations

3V	third ventricle
aca	anterior part of the anterior commissure
Acb	accumbens nucleus
APT	anterior pretectal nucleus
ArcD	arcuate nucleus, dorsal part
ArcM	arcuate nucleus, medial part
AVL	lateral part of the anteroventral nucleus of the thalamus
AVM	medial part of the anteroventral nucleus of the thalamus
BLA	basolateral amygdaloid nucleus, anterior part
BMA	basomedial amygdaloid nucleus, anterior part
BST	bed nucleus of the stria terminalis
CA1	field CA1 of hippocampus

CA3	field CA3 of hippocampus
cc	corpus callosum
Cd	caudate
IIIn	oculomotor nucleus (OCN)
IIIr	third cranial nerve root
IVn	trochlear nucleus
IVr	fourth cranial nerve root
cp	cerebral peduncle
DG	dentate gyrus
DLG	dorsal lateral geniculate nucleus
DS	dorsal septum
eml	external medullary lamina
EW	Edinger-Westphal nucleus
f	fornix
fi	fimbria hippocampus
fr	fasciculus retroflexus
gcc	genu of the corpus callosum
GP	globus pallidus
GrDG	granular layer of the dentate gyrus
HDB	horizontal limb of the diagonal band of Broca
ic	internal capsule
InG	intermediate gray layer of the superior colliculus
InWh	intermediate white layer of the superior colliculus
IP	interpeduncular nucleus
LaDL	lateral amygdaloid nucleus, dorsolateral part
lcj	islands of Calleja
LD	laterodorsal thalamic nucleus
LDT	laterodorsal tegmental nucleus
LHb	lateral habenular nucleus

LMol	lacunosum moleculare layer of the hippocampus
lo	lateral olfactory tract
LSD	dorsal part of the lateral septal nucleus
LSV	ventral part of the lateral septal nucleus
LV	lateral ventricle
MD	mediodorsal thalamic nucleus
MHb	medial habenular nucleus
mlf	medial longitudinal fasciculus
Mol	molecular layer of the dentate gyrus
MS	medial septal nucleus
mt	mammillothalamic tract
NBM	nucleus basalis of Meynert
Op	optic layer of the superior colliculus
Or	oriens layer of the hippocampus
ot	optic tract
PB	parabigeminal nucleus
Pi	piriform cortex
Po	posterior thalamic nuclear group
PoDG	polymorphic layer of the dentate gyrus
PPN	pedunculopontine tegmental nucleus
PT	parateanial thalamic nucleus
Pu	putamen
PVA	paraventricular thalamic nucleus
Py	pyramidal cell layer of the hippocampus
Rad	stratum radiatum of the hippocampus
Re	reuniens thalamic nucleus
Rt	reticular thalamic nucleus
SC	superior colliculus
SI	substantia innominata

sm	stria medullaris of the thalamus
Str	striatum
SuG	superficial gray layer of the superior colliculus
Tu	olfactory tubercle
VDB	vertical limb of the diagonal band of Broca
VL	ventrolateral thalamic nucleus
VLG	ventral lateral geniculate
VM	ventral posteromedial thalamic nucleus
VP	ventral posterolateral thalamic nucleus
ZI	zona incerta
Zo	zonal layer of the superior colliculus

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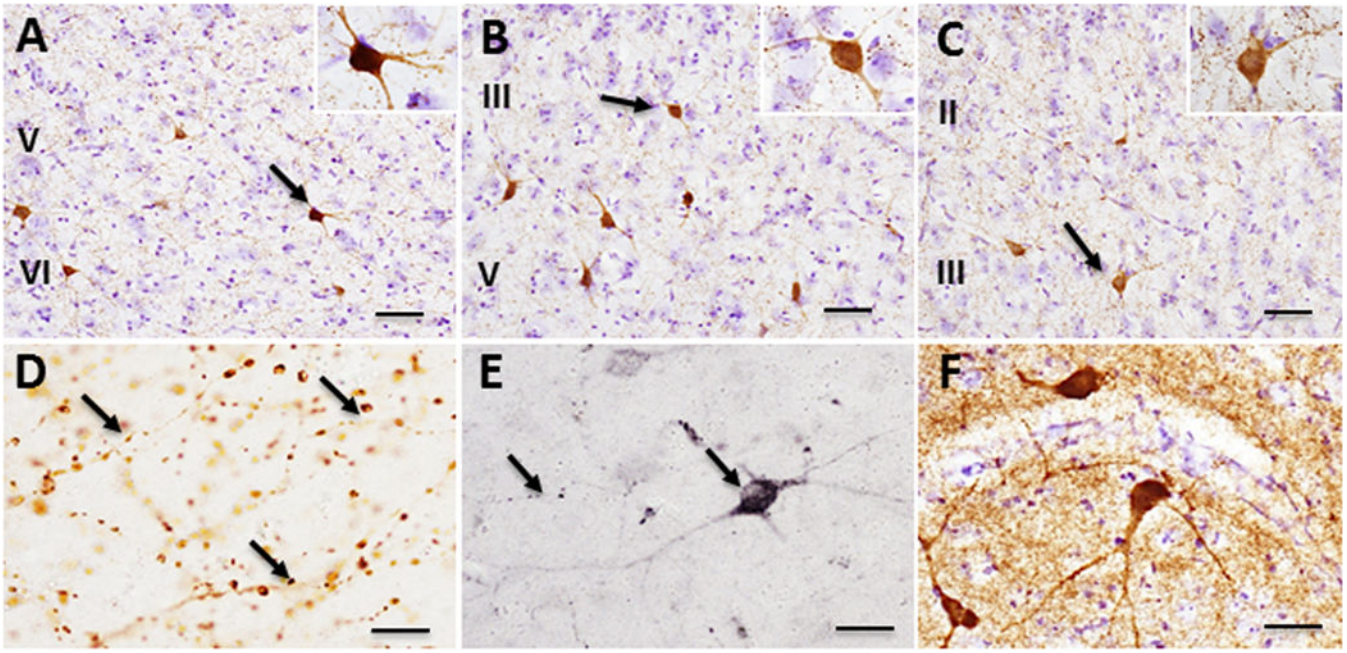

Figure 1.

ChAT-ir profiles in the cortex and olfactory tubercle of *Sus scrofa domesticus*: ChAT-ir multipolar neurons (black arrows) within layers V and VI of the cingulate cortex (**A**), layers III and V of insular cortex (**B**), and layer III of the piriform cortex (**C**). Varicose ChAT-ir fibers within piriform cortex (black arrows) (**D**). P75^{NTR}-ir neuron and fibers (black arrows) in the cingulate cortex (**E**). ChAT-ir neurons and neuropil within the olfactory tubercle (**F**). Tissue in panels A-C and F were counterstained with cresyl violet. Scale bar: 100 μm in A, B, and C; 25 μm in D, E, F. Insets: 40x A, B, C.

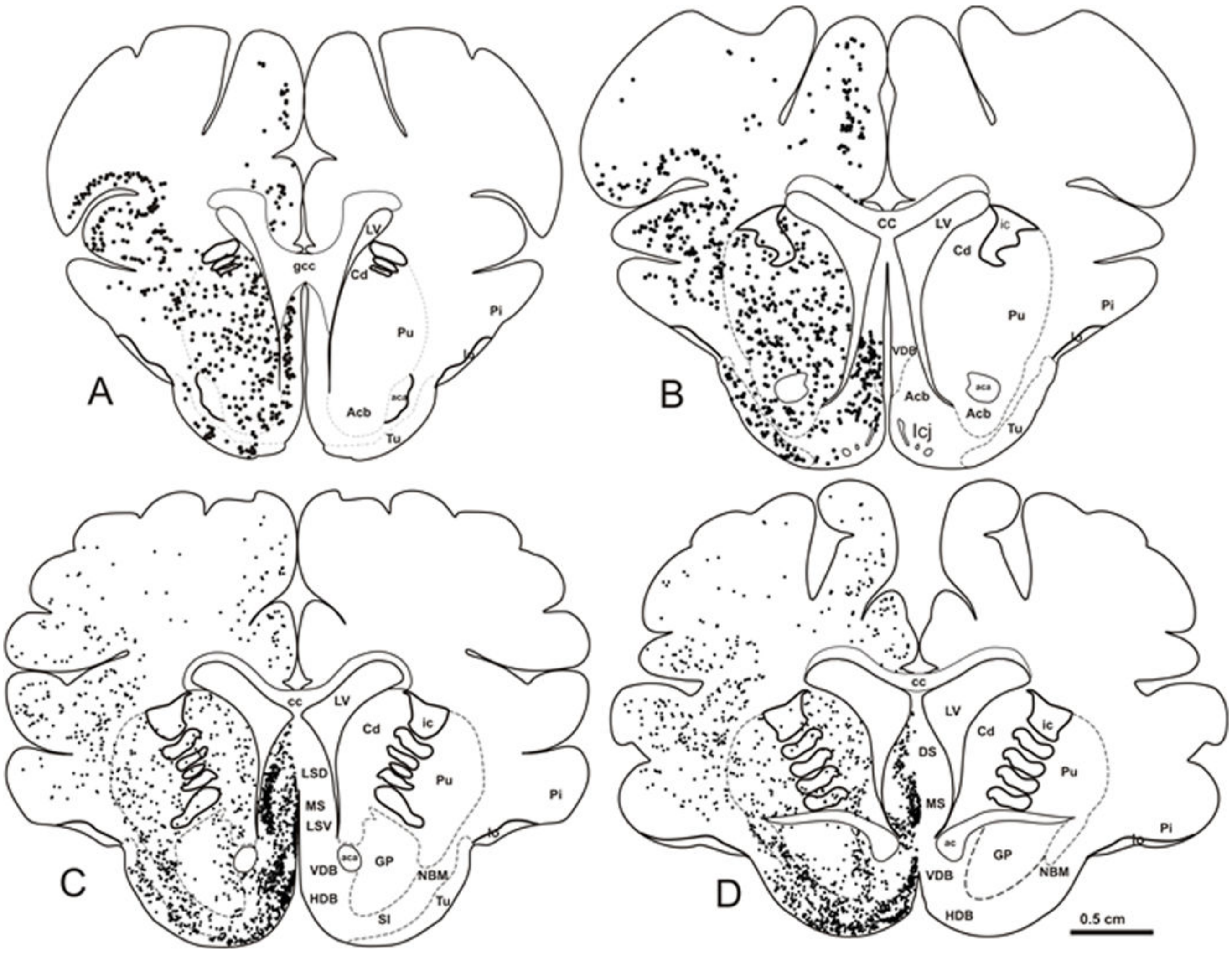

Figure 2.

Schematic drawings of coronal sections (**A-D**) of the telencephalon of *Sus scrofa domesticus* depicting the distribution of ChAT-ir perikarya (black dots). Scale: 0.5 cm A, B, C, D.

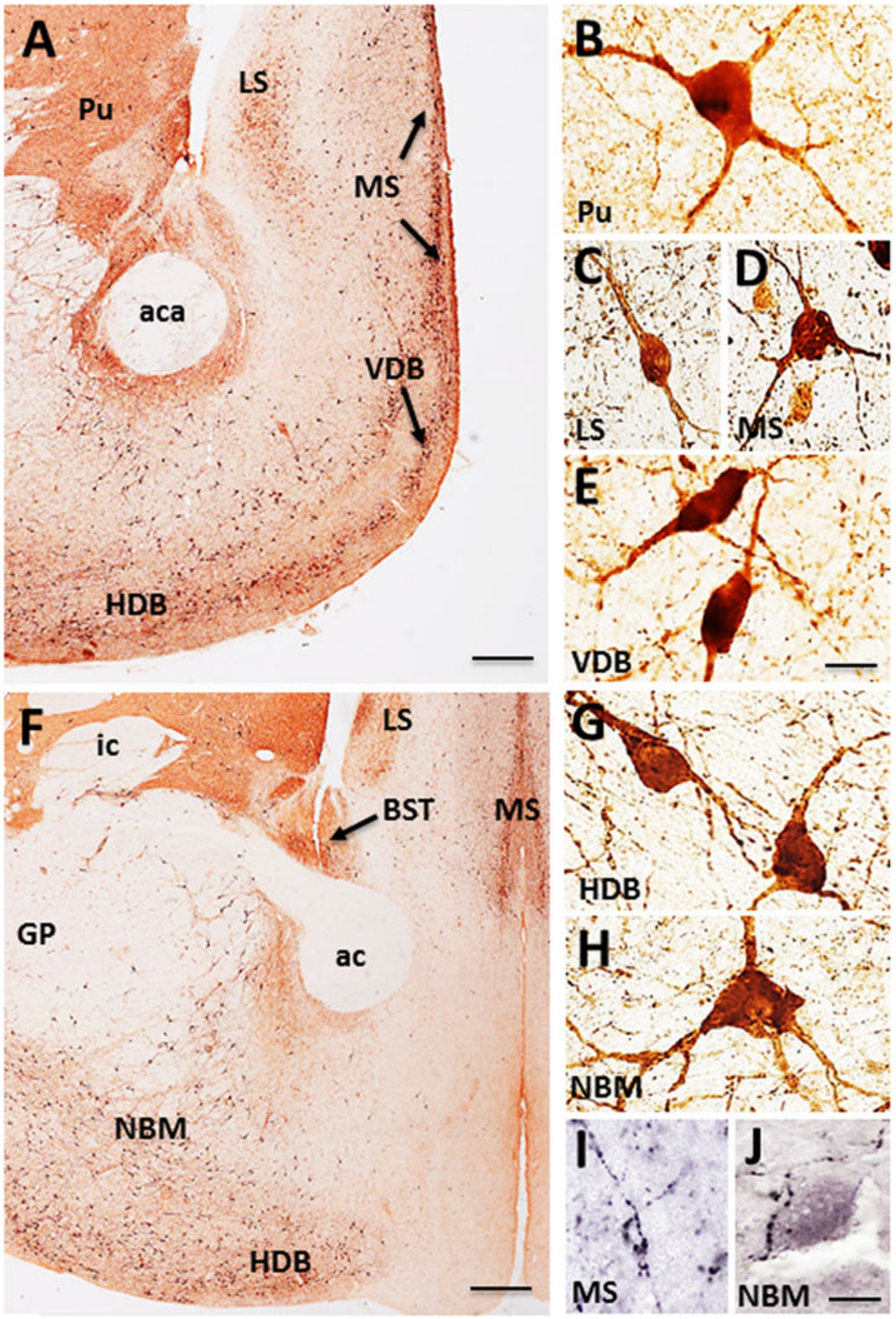

Figure 3.

A–B. ChAT-ir perikarya and neuropil within the striatum and basal forebrain of *Sus scrofa domesticus*. ChAT-ir neurons (black arrows) and neuropil within the putamen (Pu), medial septum (MS), vertical and horizontal diagonal band (VDB, HDB), and lateral septum (LS) (**A**). High-power photomicrographs showing multipolar ChAT-ir neurons in the Pu (**B**), LS (**C**), MS (**D**), and bipolar VDB (**E**) neurons. Caudal section showing ChAT-ir profiles in the LS, MS, bed nucleus of the stria terminalis (BST, black arrow), and the nucleus basalis of Meynert (NBM) (**F**). High-power photomicrographs of multipolar HDB (**G**) and NBM (**H**) neurons and GAL-ir fibers surrounding a neuron within the MS (**I**) and in the NBM (**J**). Scale: 1000 µm A, F; 25 µm B-E, G-J.

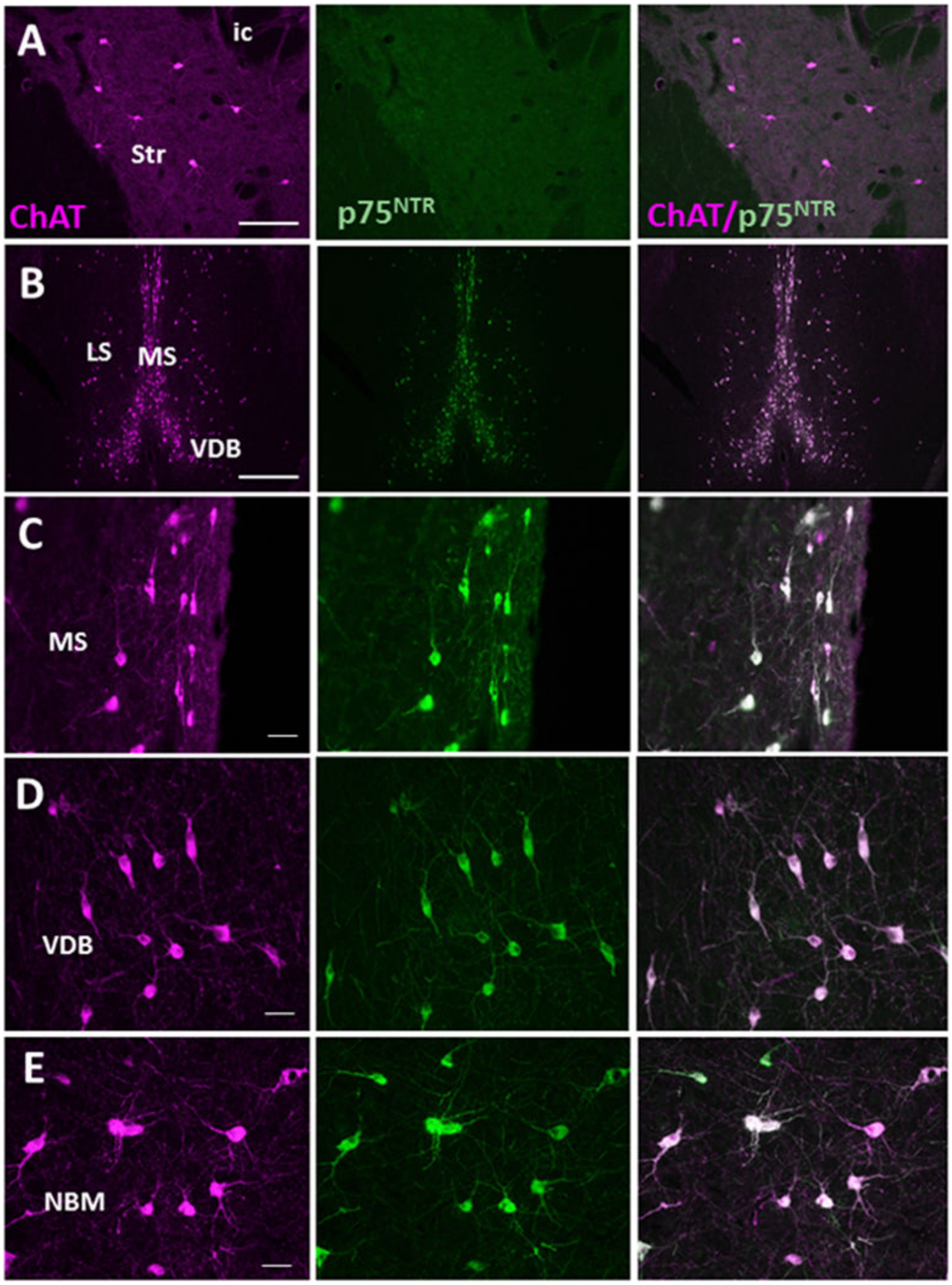

Figure 4.

Immunofluorescent photomicrographs of ChAT (magenta), p75^{NTR} (green) and merged (white) neurons and fibers in the striatum (**A**), MS (**B**, **C**), VDB (**D**), and NBM (**E**) of *Sus scrofa domesticus*. Note that virtually all ChAT-ir neurons and fibers are p75^{NTR} positive in all basal forebrain cell groups, while striatal neurons were only ChAT immunopositive. Scale: 250 μm A, B; 25 μm C, D, E.

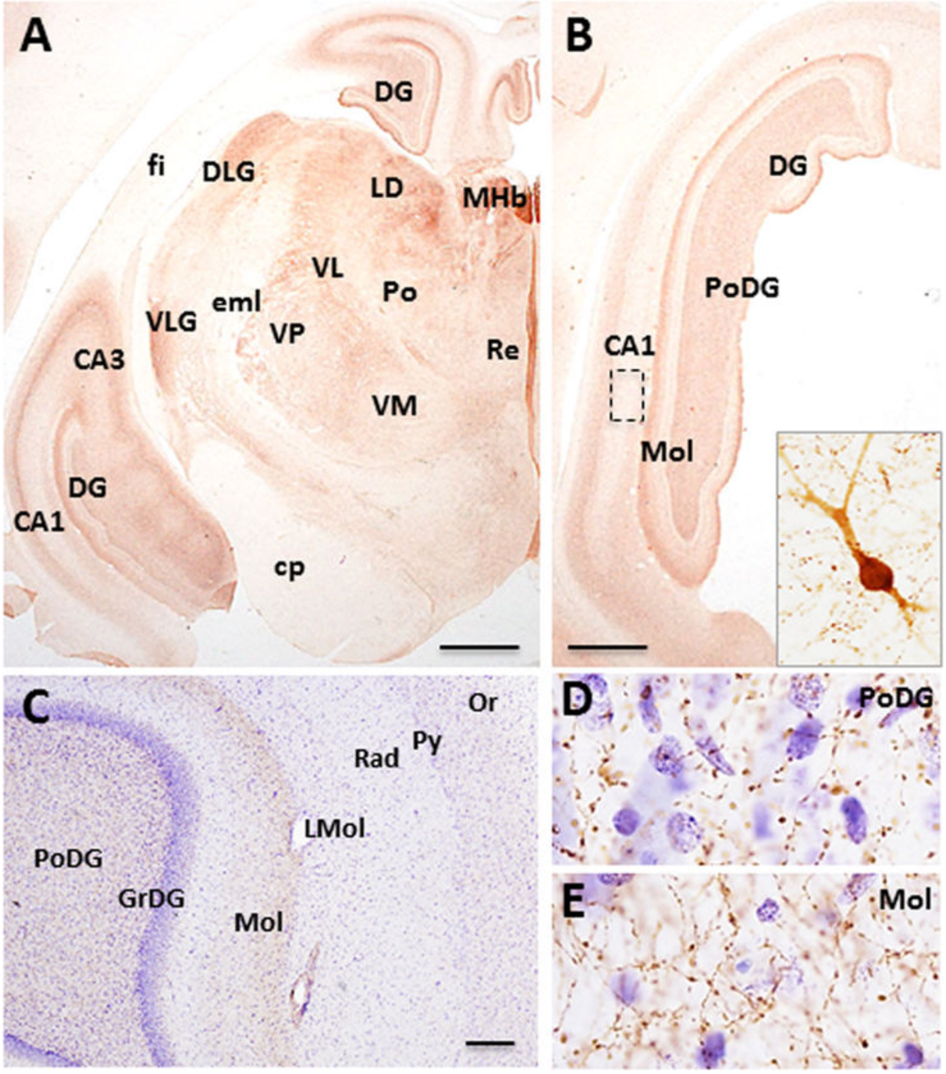

Figure 5.

Photomicrographs showing the distribution of ChAT-ir profiles in the hippocampus and thalamus of *Sus scrofa domesticus*. Coronal sections (**A**, **B**) showing ChAT-ir labeling in the dorsal and temporal aspects of the hippocampal formation and dentate gyrus (DG) and thalamic nuclei. Insert in **B** shows a cholinergic bipolar hippocampal neuron. Low (**C**) and high power (**D**, **E**) photomicrographs of a Nissl counterstained section showing ChAT-ir fibers within the polymorphic (**C**, **D**) and molecular layer (**C**, **E**) of the dentate gyrus. Scale 1000 μm A, B; 100 μm C.

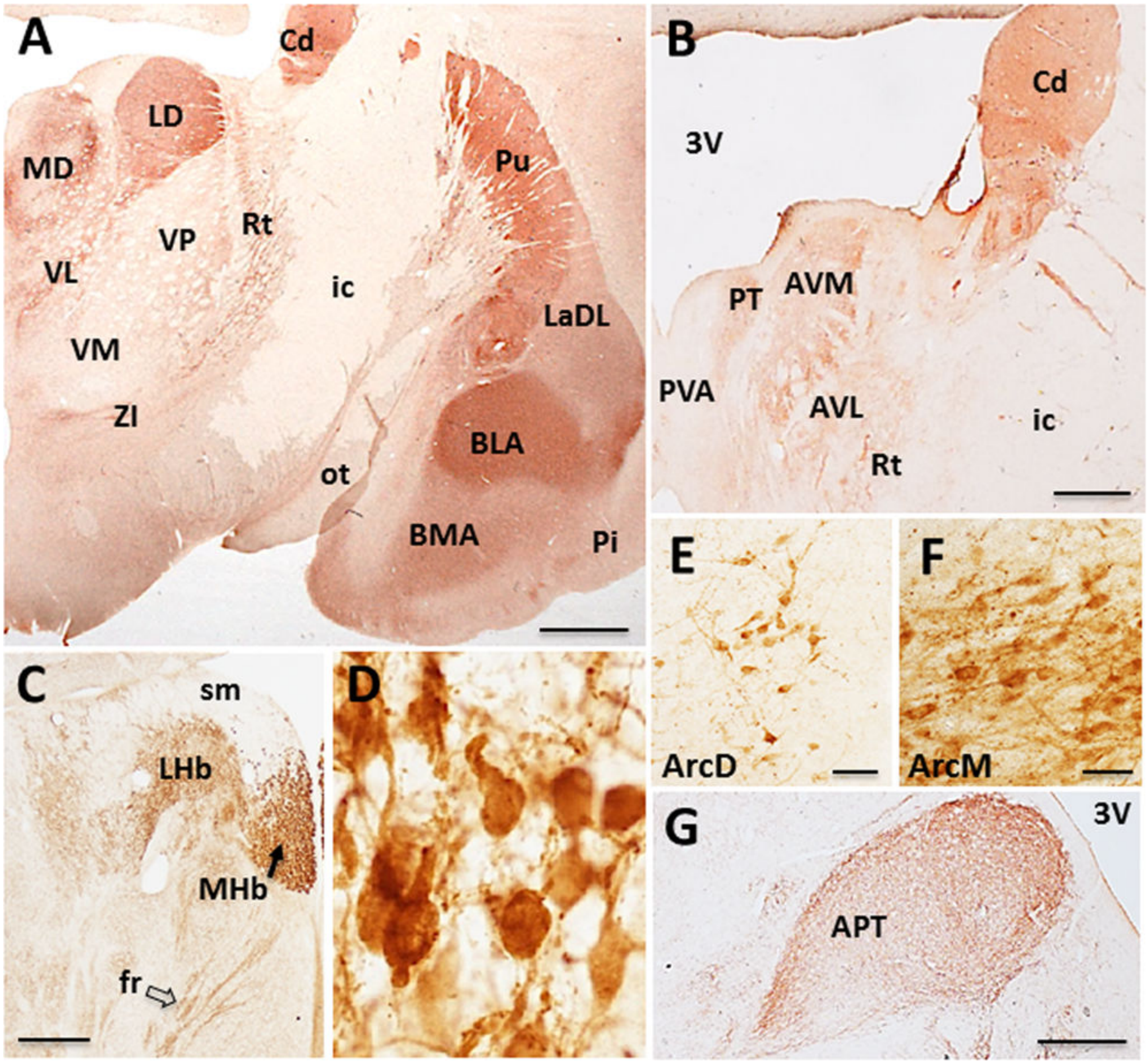

Figure 6.

Photomicrographs showing ChAT-ir profiles in the epithalamus, thalamus, hypothalamus and amygdala of *Sus scrofa domesticus*. ChAT-ir fibers within the caudal (**A**) and rostral thalamus and amygdala (**B**; see text for detailed description). ChAT-ir profiles within the lateral (LHb) and medial (MHb) (black arrow) habenular nuclei and fasciculus retroflexus (fr; open arrow) (**C**) and cholinergic neurons within the MHb nucleus (**D**). ChAT-ir neurons and fibers within the dorsal (**E**) and medial (**F**) arcuate nuclei and neuropil staining within the anterior pretectal nucleus (APT) (**G**). Scale: 500 μm A; 1000 μm C, D, G; 25 μm E, F.

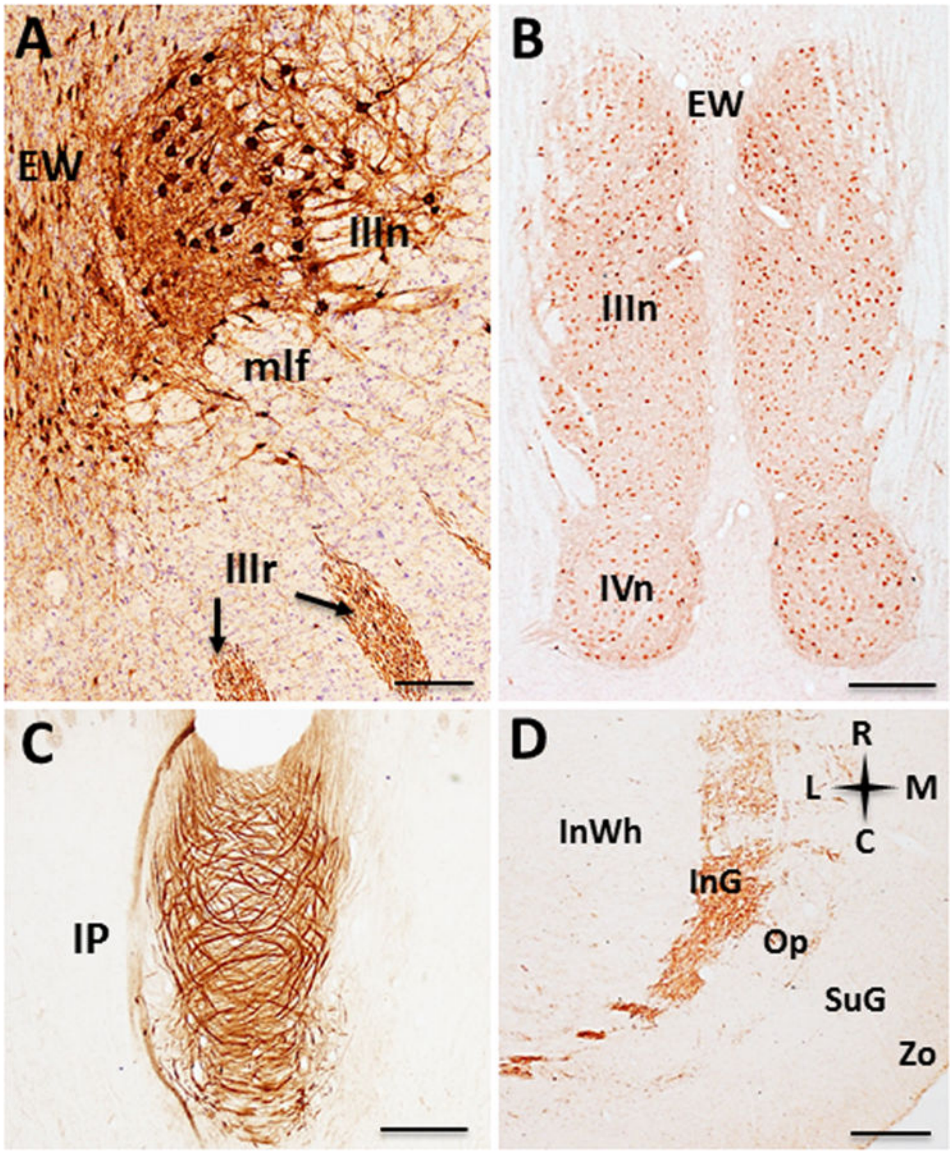

Figure 7.

Photomicrographs of ChAT-ir profiles in the mesencephalon. Coronal section showing ChAT-ir neurons within the Edinger-Westphal (EW) and oculomotor nuclei and third cranial nerve rootlets (black arrows) (**A**). Low-power photomicrograph of a horizontal section showing the Edinger-Westphal, third (IIIn) and fourth (IVn) cranial nerve nuclei (**B**). Horizontal section showing swirling ChAT-ir fibers in the central portion of the interpeduncular nucleus (IP, **C**). Low-power photomicrograph (**D**) of ChAT-ir fibers within the superficial laminae of the superior colliculus. Scale: 250 µm A; 1000 µm B; 500 µm C, D. Anatomic orientation: R= rostral, C= caudal, L= lateral, M= medial.

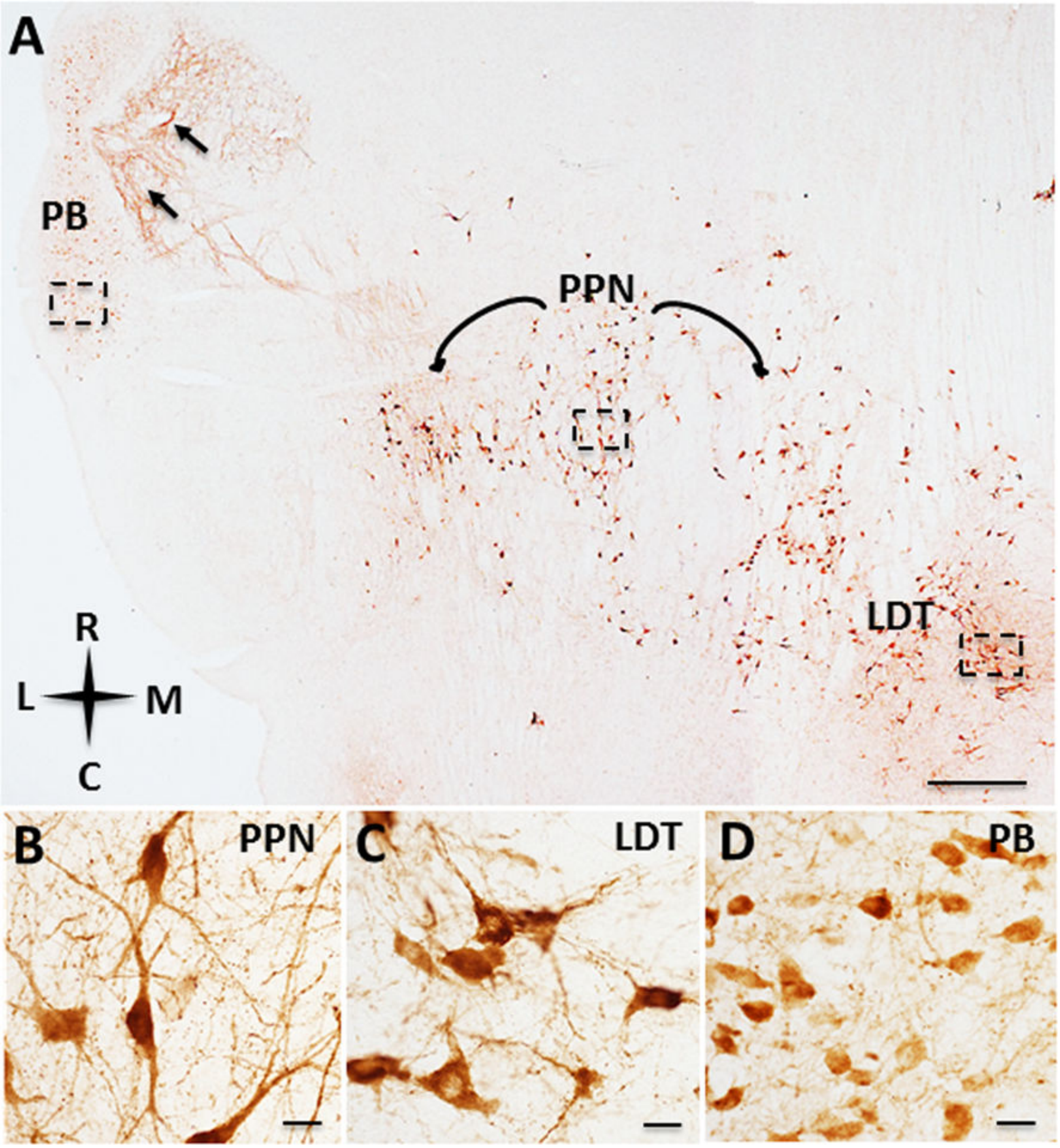

Figure 8.
Photomicrographs of the pontomesencephalic cholinergic cell groups of *Sus scrofa domesticus*. ChAT-ir neurons and fibers (**A**, black arrows) of the parabigeminal (PB), pedunculopontine nucleus (PPN), and the laterodorsal tegmental nucleus (LDT). ChAT-ir neurons and fibers within the PPN (**B**), LDT (**C**) and PB (**D**). Scale: 1000 μm A; 25 μm B, C, D. Anatomic orientation: R= rostral, C= caudal, L= lateral, M= medial.

Table 1

Summary of Antibodies Used

Antigen	Description of Immunogen	Source, Host Species, Cat. #, RRID	Dilution
Choline acetyltransferase (ChAT)	Human placental Choline acetyltransferase	Millipore, goat polyclonal, Cat# AB144P, RRID:AB_2079751	Immunohistochemistry: 1:1,000 Immunofluorescence: 1:100
Low affinity neurotrophin receptor ($p75^{NTR}$)	GST fusion protein corresponding to the intracellular domain (residues 274–425) of rat p75 neurotrophin receptor	Millipore, rabbit polyclonal, Cat# 07-476, RRID:AB_310649	Immunohistochemistry: 1:3,000 Immunofluorescence: 1:300
Galanin (GAL)	Synthetic peptide: GWTLNSAGYLLGPHAIDNHRSFSDKHGLT-amide (rat Galanin 1–29) coupled to BSA via carbodiimide	Gift from E. Theodorsson, rabbit polyclonal, RatGal4, RRID:AB_2314520	Immunohistochemistry : 1:1,000

Table 2

Summary of ChAT positive profiles across species

	Artiodactyla			Cetacea			Carnivora		Lagomorpha	Rodentia			Primates					
	Goettingen pig	**Giraffe**[1]	**Sheep**[2]	**River hippopotamus**[3]	**Harbour porpoise**[4]	**Minke whale**[5]	**Cat**[6–13]	**Dog**[14–16]	**Rabbit**[17–19]	**CD-1 mouse**[20,21]	**Rat**[22–40]	**Guinea pig**[22,41,42]	**Strepsirrhines**[43]	**Macaque**[44–53]	**Marmoset**[54–56]	**Baboon**[57]	**Gorilla**[58–60]	**Human**[61–71]
	S. s. domesticus	***G. camelopardalis***	***O. aries***	***H. amphibius***	***P. phocoena***	***B. acutorostrata***	***F. cattus***	***C. familiaris***	***O. cuniculus***	***M. musculus***	***R. norvegicus***	***C. porcellus***	***G. demidoff*** ***P. potto*** ***L. catta***	***M. mullata*** ***M. nemestrina*** ***M. fascicularis*** ***M. fuscata***	***C. jacchus***	***P. papio***	***G. gorilla*** ***G. g. gorilla*** ***G. b. beringei***	***H. sapiens***
Cortex	+	N/A	−	N/A	N/A	N/A	−	N/A	+	+	−	−	−	−	−	−	−	−/+
Tu	+	N/A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N/A	+
Str	+	+	+	+	+	N/A	+	+	+	+	+	+	+	+	+	+	N/A	+
LS	+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+	N/A	N/A	+	N/A	N/A	N/A	−
MS (Ch1)	+	N/A	+	+	N/A	N/A	−/+	+	+	+	+	+	+	+	+	+	N/A	+
VDB (Ch2)	+	N/A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HDB (Ch3)	+	N/A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	N/A	+
NBM (Ch4)	+	+	+	+	+	+	+	+	+	+	+	+*	+	+	+	+	+	+
Hippocampus	+	N/A	N/A	N/A	N/A	N/A	−	N/A	+	+	+	N/A	−	−	N/A	−	N/A	+
Amygdala	−	N/A	N/A	N/A	N/A	N/A	+	N/A	−	N/A	−	N/A	−	−	N/A	N/A	N/A	−
MHb (Ch7)	+	+	N/A	N/A	+	N/A	+	N/A	N/A	+	+	+	+	+	+	−	N/A	+
LHb	−	N/A	N/A	N/A	N/A	N/A	+	N/A	−	N/A	−	N/A	N/A	N/A	N/A	N/A	N/A	−
Thalamus	−	N/A	N/A	+†	N/A	N/A	−	N/A	−	N/A	−	N/A	N/A	+**	N/A	N/A	N/A	−
ZI	+	N/A	N/A	N/A	N/A	N/A	+	N/A	−	N/A	−	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ArcD	+	+****	N/A	+****	+****	+****	N/A	N/A	N/A	N/A	−/+	N/A	−	−***	N/A	N/A	N/A	−***
ArcM	+	+****	N/A	+****	+****	+****	N/A	N/A	N/A	N/A	−/+	N/A	−	−***	N/A	N/A	N/A	−***
APT	−	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+	−	N/A	N/A	N/A	N/A	N/A	N/A
EW	+	+	N/A	+	+	+	+	N/A	+	N/A	+	+	+	+	N/A	+	N/A	+
IIIn	+	+	N/A	+	+	+	+	N/A	+	+	+	+	+	+	N/A	+	N/A	+
IVn	+	+	N/A	+	+	+	+	N/A	N/A	N/A	+	+	+	+	+	+	N/A	+
IP	−	−	N/A	N/A	N/A	N/A	−	N/A	−	−	−	−	N/A	−	N/A	N/A	N/A	N/A
SC	−	N/A	N/A	N/A	N/A	N/A	+	N/A	N/A	−	− /+	+	−	N/A	N/A	N/A	N/A	N/A
PPT (Ch5)	+	+	N/A	+	+	+	+	+	N/A	+	+	+	+	+	+	+	N/A	+
LDT (Ch6)	+	+	N/A	+	+	+	+	+	N/A	+	+	+	+	+	+	+	N/A	+
PBN (Ch8)	+	+	N/A	+	+	+	+	N/A	N/A	+	+	+	+	N/A	N/A	N/A	N/A	+

N/A= data not reported in cited references, + presence of ChAT-ir neurons, – absence of ChAT-ir neurons and −/+ = conflicting data.

* guinea pig NBM/Ch4 region referred to as substantia innominata,

[**] paracentral nucleus only thalamic subfield to contain ChAT-ir neurons,

[***] arcuate nuclei referred to as infundibular nuclei[33],

[****] ChAT-ir neurons reported in the tuberal nuclei of the ventral hypothalamus which may correspond to arcuate nuclei[1,3–5],

[†] ChAT-ir neurons reported in intralaminar thalamic nuclei[3]. Cholinergic subfield nomenclature (Ch1-8) according to Mesulam and colleagues[23]. Tabular data was derived from the present and previous reports indicated by superscripted numbers:

[1] Bux et al., 2010;

[2] Ferreira et al., 2001;

[3] Dell et al., 2016a;

[4] Dell et al., 2016b;

[5] Dell et al., 2016c;

[6] Kimura et al., 1981;

[7] Wahle and Meyer, 1986;

[8] Vincent and Reiner, 1987;

[9] Jones and Beaudet, 1987;

[10] Strassman et al., 1987;

[11] Shiromani et al., 1988;

[12] Hashikawa, 1989;

[13] Schnurr et al., 1992;

[14] Isaacson and Tanaka, 1986;

[15] St-Jacques et al., 1996;

[16] Tafti et al., 1997;

[17] Chao et al., 1982;

[18] Balaban, 2003;

[19] Varga et al., 2003;

[20] Mufson and Cunningham, 1988;

[21] Mufson et al., 1986;

[22] Kimura et al., 1980;

[23] Mesulam et al., 1983;

[24] Armstrong et al., 1983;

[25] Eckenstein and Sofroniew, 1983;

[26] Frotscher et al., 1986;

[27] Ichikawa and Hirata, 1986;

[28] Contestabile et al., 1987;

[29] Levey et al., 1987;

[30] Rao et al., 1987;

[31] Rye et al., 1987;

[32] Tan and Harvey, 1989;

[33] Tago et al., 1989;

[34] Tinner et al., 1989;

[35] Jones, 1990;

[36] Kimura et al., 1990;

[37] Woolf, 1991;

[38] Oh et al., 1992;

[39] Schnurr et al., 1992;

[40] Schafer et al., 1998;

[41] Maley et al., 1988;

[42] Motts et al., 2008;

[43] Calvey et al., 2015a;

[44] Mesulam et al., 1984;

[45] Tago et al., 1987;

[46] Amaral and Bassett, 1989;

[47] Kimura et al., 1990;

[48] Mrzljak and Goldman-Rakic, 1993;

[49] Alonso and Amaral, 1995;

[50] Ichikawa and Shimizu, 1998;

[51] Rico and Cavada, 1998;

[52] May et al., 2008;

[53] Raghanti et al., 2011;

[54] Everitt et al., 1988;

[55] Maclean et al., 1997;

[56] Wu et al., 2000;

[57] Satoh and Fibinger, 1985;

[58] Benzing et al., 1993a;

[59] Perez et al., 2013;

[60] Perez et al., 2016

[61] Mizukawa et al., 1986;

[62] Tago et al., 1987;

[63] Mesulam and Geula, 1988;

[64] Mufson et al., 1988;

[65] Mesulam et al., 1989;

[66] Mufson et al., 1989;

[67] Ransmayr et al., 1989;

[68] Heckers et al., 1992;

[69] Kasashima et al., 1999;

[70] Manaye et al., 1999;

[71] Benagiano et al., 2003.