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The Distribution of Cholinergic Neurons, and their Co-localization with FMRFamide in Central and Peripheral Neurons of the Spider *Cupiennius salei*

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Abstract

The spider *Cupiennius salei* is a well-established model for investigating information processing in arthropod sensory systems. Immunohistochemistry has shown that several neurotransmitters exist in the *C. salei* nervous system, including GABA, glutamate, histamine, octopamine and FMRFamide, while electrophysiology has found functional roles for some of these transmitters. There is also evidence that acetylcholine (ACh) is present in some *C. salei* neurons, but information about the distribution of cholinergic neurons in spider nervous systems is limited. Here, we identified *C. salei* genes that encode enzymes essential for cholinergic transmission: choline ACh transferase (ChAT) and vesicular ACh transporter (VAChT). We then used *in-situ* hybridization with an mRNA probe for *C. salei ChAT* gene to locate somata of cholinergic neurons in the central nervous system (CNS), and immunohistochemistry with antisera against ChAT and VAChT to locate these proteins in cholinergic neurons. All three markers labeled similar, mostly small neurons, plus a few mid-sized neurons, in most ganglia. In the subesophageal ganglia, labeled neurons are putative efferent, motor or interneurons, but the largest motor and interneurons were unlabeled. Groups of anti-ChAT labeled small neurons also connect the optic neuropils in the spider protocerebrum. Differences in individual cell labeling intensities were common, suggesting a range of ACh expression levels. Double labeling found a subpopulation of anti-VAChT labeled central and mechanosensory neurons that were also immunoreactive to antiserum against FMRFamide-like peptides. Our findings suggest that ACh is an important neurotransmitter in the *C. salei* central and peripheral nervous systems.

Keywords

Choline acetyltransferase; vesicular acetylcholine transporter; sensory neurons; *in-situ* hybridization; central nervous system

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Introduction

Due to an exquisite array of mechanosensory organs, the tropical wandering spider *Cupiennius salei* has served as a model system for mechanotransduction for decades (Barth 2002; 2004; French et al. 2002). The architecture of the *C. salei* central nervous system (CNS) has also been extensively investigated, with many neurons involved in motor control and processing of mechanical and visual information identified (Babu and Barth 1984; 1989; Babu et al. 1985; Strausfeld et al. 1993; Strausfeld and Barth 1993). *C. salei* legs contain numerous touch sensitive hairs and delicate trichobothria hairs that detect air movement, as well as slit sensilla that monitor strains and substrate vibrations (French et al. 2002; Barth 2002; 2004). Together these and other sense organs allow the spider to accurately monitor its environment and the positions and movements of its body parts. All spider mechanosensilla receive extensive efferent innervation via GABAergic, glutamatergic and octopaminergic nerve fibers (Fabian-Fine et al. 1999a, 2000; Widmer et al. 2005) and they respond to each transmitter by diverse changes in sensitivity to mechanical stimuli (Panek et al. 2002, Pfeiffer et al. 2009; Torkkeli et al. 2011; 2012). This presumably allows the spider to select and discriminate amongst behaviorally relevant mechanical stimuli.

Ultrastructural investigations have identified a variety of synaptic contacts between the efferents and mechanosensory neurons that innervate VS-3 slit sensilla in the spider patella (Fabian-Fine et al. 2000). Some nerve fibers containing GABA or glutamate have electron lucent synaptic vesicles, but there are also efferents that have large electron dense vesicles or mixed vesicle populations whose transmitters have not been identified. An additional small molecule transmitter that is often found in clear synaptic vesicles is acetylcholine (ACh), and some efferents probably contain neuropeptides that are known to reside in electron dense vesicles (Brownlee et al. 2000; Salio et al. 2006). It is also likely that some efferent fibers contain more than one neurotransmitter, as in many neurons across all animal phyla (Trudeau 2004; Salio et al. 2006). Recently, the spider CNS was shown to contain neurons immunoreactive to both GABA and glutamate as well as their transporters (Fabian-Fine et al. 2015). Some of these neurons are probably efferents innervating the sensory neurons.

ACh is widely distributed in arthropod central and peripheral nervous systems, including mechanosensory and visual pathways (Yasayuma and Salvaterra 1999; Clark et al. 2005; Fusca et al. 2013) and there is significant evidence that ACh is also an important transmitter in spiders. The *C. salei* mechanosensory neurons and some efferents were immunoreactive to an antiserum against the enzyme that catalyzes ACh biosynthesis, choline acetyltransferase (ChAT), as well as a monoclonal antibody against muscarinic ACh receptors (Fabian and Seyfarth 1997; Fabian-Fine et al. 2002; Widmer et al. 2006). These neurons also contained acetylcholine esterase (AChE; Fabian and Seyfarth 1997), and four different genes that produce this ACh degrading enzyme have been found in the *C. salei* transcriptomes as well as in genomes of other spider species (French et al. 2014; Meng et al. 2016).

It is not known which neuropeptides inhabit the electron dense vesicles in *C. salei* efferents, but likely candidates are members of the large family of FMRFamide related peptides that are widely distributed among every invertebrate phylum and often co-expressed with other

neurotransmitters (Mercier et al. 2003; Nässel and Homberg 2006; Iwano and Kanzai 2006; Berg et al. 2009). Immunolabeling using antisera against FMRFamide-like peptides, which is likely to detect several members of this family, produced extensive labeling in *C. salei* protocerebrum (Becherer and Schmid 1999) and subesophageal ganglia (summarized in Barth 2002).

Here, we identified *C. salei ChAT* and *VAChT* (Vesicular ACh Transporter) genes and used three markers to identify cholinergic neurons in spider CNS: (1) We prepared an RNA probe for the *CsChAT* gene and used *in-situ* hybridization to locate this gene in the somata of cholinergic neurons. We selected commercial antisera that matched the spider sequences to detect (2) ChAT and (3) VAChT proteins that are synthesized in the somata and transported to the synaptic terminals (Ichikawa et al. 1997; Gilmor et al. 1996; Prado et al. 2013). We also used an antiserum against FMRFamide-like peptides to investigate its distribution in peripheral nerves and to test whether it is co-expressed in cholinergic neurons. This is the first investigation that uses genetic information to identify specific neurons in spider CNS and links this information to immunohistochemistry. This study (i) establishes markers for cholinergic neurons in spider nervous tissue, and (ii) provides a firm background for further studies to investigate the effects of environmental stresses on sensory organs and associated central pathways including changes in the expressions of neurotransmitters and their receptors as well as associated metabolic proteins.

Materials and Methods

Experimental Animals

Central American wandering spiders, *Cupiennius salei*, were raised under laboratory conditions. All experiments conducted in this study were in accordance with IACUC, Animal Welfare regulations and approved by the Dalhousie University Committee on Laboratory Animals. The spiders were housed individually at 22°−26°C. All animals were fed regularly with fruit flies throughout the hatchling stage and crickets or cockroaches in the juvenile and adult stages. All experimental animals used in this study were adult females (10 months or older).

Tissue dissection for immunohistochemistry

CNS dissection: Spiders were deeply anesthetized with CO2 and perfused with ice cold 6% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS) at pH 7.4 for approximately 2 minutes. Prior to perfusion the tarsal leg segments were removed to allow adequate flow of fixative through the body and prevent tissue damage. Fixative was perfused directly into the heart using a hypodermic needle and syringe. After removal of all legs, pedipalps and opisthosoma, the prosoma was kept in fixative overnight at 6°C. The sternum plate was then removed and the CNS extracted from the prosoma using fine micro scissors. After removal of surrounding muscle tissue and the esophagus, the extracted CNS was embedded in 4% agarose (Invitrogen 1652–050 or Sigma-Aldrich #A9539) and sectioned using a vibrating microtome (Leica Biosystems VT1000S). Horizontal serial brain sections were cut at 60–100 μm thickness, collected with a fine paintbrush and transferred to 16-well dishes filled with PBS.

Sensory neuron dissection: The legs of animals perfused with 6% PFA in PBS were cut below the coxa and patella joints, so that only the femur and patella segments remained. These segments were kept in fixative overnight at 6°C. To expose the sensory neurons, leg segments were split longitudinally, from dorsal to ventral using a fine micro scalpel, so that the anterior and posterior parts of the legs containing the VS-3 and HS-3 mechanosensilla remained intact. After removal of muscle tissue, the underlying hypodermis containing sensory neurons attached to the cuticle was gently pulled away from the cuticle, with part of the hypodermis left attached to the cuticle to prevent damage to the delicate hypodermis during processing. After the immunolabeling procedure was completed, the hypodermis was detached from the cuticle and mounted on glass slides in Mowiol medium, prepared as described previously (Fabian-Fine et al. 2015). The preparations were left to set overnight at 6°C.

Immunohistochemistry

Anti-FMRFamide labeling: Brain sections and hypodermis preparations were washed in PBS for 4×5 min prior to exposure to incubation and blocking medium (IBM; 0.25% bovine serum albumin (Sigma A4503), 10% normal goat serum (Sigma G9023), 0.05% Triton-X 100 in PBS) for 20 min. The polyclonal antiserum against FMRFamide-like peptides (rabbit anti-FMRFamide, Millipore AB15348) was diluted 1:1000 in IBM overnight at 6°C. After subsequent washing in PBS (4 × 5 min followed by 2×1 h), samples were preincubated in IBM for 20 min prior to incubation in the secondary Cy3 conjugated goat-anti-rabbit antibody (1:600, overnight 6°C; Jackson ImmunoResearch Laboratories 111–165-003). After washing with PBS (3×5 min), samples were incubated with Hoechst stain solution (Sigma H6024) in PBS at a dilution of 1:3000 for 20 min for nuclear labeling. After washing (2×1 h) the tissue samples were mounted on glass slides using Mowiol medium and left to set overnight at 6°C.

Anti-ChAT labeling: The anti-ChAT labeling was performed as described above for FMRFamide except for the primary antiserum. The polyclonal antiserum against ChAT (rabbit anti-ChAT, Millipore AB143) was diluted 1:1000 in IBM.

Anti-ChAT and anti-vesicular acetylcholine transporter (anti-VAChT-N) double labeling: The CNS was fixed with 6% PFA in PBS and immunolabeling for the primary rabbit ChAT antiserum was carried out as described above with the exception that normal goat serum was replaced with horse serum (Sigma H0146) in the IBM since the antiserum against the N-terminus of the VAChT was raised in goat (dilution 1:100 in IBM; Santa Cruz Biotechnology sc-7717). Alexa Fluor® 488 donkey-anti-goat (Jackson ImmunoResearch Laboratories 705-545-147) was used as secondary antibody to detect anti-VAChT-N labeling and Cy3 donkey-anti-rabbit (Jackson ImmunoResearch Laboratories 711-165-152) was used at a dilution of 1:500.

Anti-VAChT-N and anti-FMRFamide double labeling: Immunolabeling was carried out as described for Anti-VAChT-N and anti-ChAT double labeling with the exception that the ChAT antiserum was replaced with primary rabbit FMRFamide antiserum (see above) at a dilution of 1:1000.

Image acquisition and analysis

Examination and digital image acquisition were carried out using a LSM 510 meta or LSM 710 laser-scanning confocal microscope equipped with x10/0.45, x20/0.8, x40/1.3 and x63/1.4 Plan Neofluar objectives (Carl Zeiss, Oberkochen, Germany). We used an argon–krypton laser for Alexa Fluor 488 (488 nm) and a helium–neon laser for CY-3 (543 nm) witht eh LSM 510. The LSM 710 microscope uses prisms and barriers to direct the specific wavelength to the detector. For image processing and analysis, the Zeiss ZEN light laser scanning microscope software was used. Single confocal optical sections, 1–4 μm thick are shown in images of the CNS and maximum intensity projections for the mechanosensory neurons. Final images were processed with Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

RNA probe construction and in-situ hybridization

Two genes homologous to mammalian and arthropod choline acetyltransferase gene (*ChAT*) were identified from the *C. salei* transcriptome by searching the databases at low stringency against amino acid sequences of published arthropod genes using the transcriptome walking method described previously in detail (French 2012; Torkkeli et al. 2015). Custom designed primers (Table 1) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Digoxigenin-labeled antisense and sense (control) RNA probes were transcribed *in-vitro* from spider hypodermis cDNA using T7 polymerase (Roche Diagnostics, Laval, Quebec, Canada; Liu et al. 2016) following protocols recommended by the manufacturer. Probe quality was confirmed by agarose gel electrophoresis. The probes were then stored at −80°C until use.

All steps of the *in-situ* hybridization were performed under RNase free conditions and all chemicals were purchased from Sigma-Aldrich Oakville, Ontario, unless otherwise indicated. Adult spiders were anesthetized with CO_2 then perfused with ice cold 4% PFA through the heart and their brains removed and fixed overnight in 4% PFA. The brains were then dehydrated in graded methanol in 0.1% Triton X-100 in PBS (PBST), left in 100% methanol −20°C for a minimum of 24 h, then rehydrated through a series of methanol solutions. The brains were embedded in a mixture of bovine serum albumin and gelatin type A with 6.47% formaldehyde and 0.075% glutaraldehyde added just before use (Levin 2004; von Trotha et al. 2014). The blocks were hardened at 4°C for 5–7 days and then sectioned using a Leica VT-1000S vibratome. 20 μm thick serial sections were collected on Netwell mesh bottom inserts (mesh size 74 μm, diameter 15 mm) (Electron Microscopy Sciences, Hatfield, PA.) in 12-well tissue culture plates each well filled with 3 ml of PBS. Sections were then processed using the same protocol as described previously for *in-situ* hybridization of *C. salei* whole-mount preparations (Liu et al. 2016). Finally, the anti-Digoxigenin-Alkaline-Phosphatase, Fab-fragment (Roche) antibody (dilution 1:8,000) reaction was visualized using 0.5% Nitroblue Tetrazolium Chloride (NBT, Roche) and 0.375% 5-Bromo-4-Chloro-3-Indolyl-Phosphate, 4-Toluidine salt (BCIP, Roche). The blue color was clearly visible within 3–4 hours at room temperature. All samples were washed in PBS with 0.1% Tween-20. Sections were collected on glass slides and mounted in Vectamount aqueous mounting media (Vector labs Burlingame, CA). Samples were

inspected and photographed using an Axiovert 100 microscope fitted with an Axiocam camera (Zeiss); the images were processed with Adobe Photoshop CS5.

Western blot analysis

Spider brain tissue was placed in a 1 ml glass tissue grinder containing ice-cold spider saline with protease inhibitor (Sigma S-8830) at a dilution of 1 part protease inhibitor to 10 parts spider saline. The brain was finely ground and the homogenate centrifuged for 20 min at 16,000 x g in a refrigerated centrifuge at 4°C. The supernatant was transferred into a centrifuge tube and an equal volume of 2x Laemmli sample buffer was added. The proteins were denatured at 95°C for 5 min. Protein separation followed using a Mini-PROTEAN TGX precast polyacrylamide gel (456–1083, BIO-RAD, Hercules, CA, USA). For each antiserum, two wells containing 3 and 6 μl were loaded. A molecular weight marker standard ranging from 10–180 kDa was used (Invitrogen #26616). The gel was washed in distilled water 3×5 min and incubated in blotting solution (BIO-RAD) for 15 min. Blotting of proteins was performed according to the manufacturer's instructions. The nitrocellulose membrane was immersed in blocking buffer (Tris buffered saline; TBS containing 1% Tween 20, 5% bovine serum albumin and 5% normal goat serum) for 1 h followed by overnight incubation with the VAChT antiserum (1:1000) in blocking buffer diluted 1:10 at 4°C. After subsequent washing in TBS (3×10 min) the secondary antibody (peroxidase conjugated goat anti-rabbit; Jackson 111–035-0003) was applied at a dilution of 1:10,000 and agitated for 1 h. After renewed washing 3×10 min in TBS, immunoreactive protein bands were visualized using a chemiluminescence kit (BioRad 170–5070), according to the manufacturer's instructions.

Results

Anatomy of the spider nervous system

CNS: The spider CNS consists of a fused complex located in the cephalothorax, also known as the prosoma. It consists of two neural masses and is penetrated from anterior to posterior by the esophagus (Fig. 1a). The mass located dorsally to the esophagus is referred to as the supraesophageal complex. It contains the optic mass with optic tracts, three successive optic neuropils (ON1-ON3) for one pair of principal and three pairs of secondary eyes. The dorsal protocerebral cell layer (DCL) contains the ON3 of secondary eyes and is called the mushroom body while the ON3 of principal eyes is located on the rear of the brain in the central body (CB, also called arcuate body) that also serves probably as a motor and association center. The most posterior cell layer (PCL) consists of cell bodies. The left and right cheliceral ganglia (CG) rest above the esophagus (Fig. 1b). The mass ventral to the esophagus is the subesophageal complex. Each hemisphere contains one opisthosomal ganglion (OG) on the posterior side, one pedipalpal ganglion (PPG) on the anterior side and four walking leg ganglia (LG) in between (Fig. 1b) (Babu and Barth 1984: Strausfeld and Barth 1993; Strausfeld et al. 1993; Loesel et al. 2011).

Mechanosensory neurons

The spider lyriform slit sensilla are located on the anterior (German: Vorderseite, VS) and posterior sides (German: Hinterseite, HS) of the eight walking legs. The slit sensilla are

numbered VS-1 to VS-5 on the anterior side and HS-1 to HS10 on the posterior side (from proximal to distal) (Barth and Libera 1984). Each mechanosensory organ consists of numerous slits, each innervated by a pair of large bipolar neurons (soma diameter 20–110 μm). Dendrites, somata and parts of the axons are embedded in the hypodermis adjacent to the exoskeleton and can be dissected and used as whole-mount preparations. Axons of these and other mechanosensory neurons terminate in the subesophageal ganglia (Milde and Seyfarth 1988; Babu and Barth 1989: Anton and Barth 1993).

Identification of the C. salei ChAT gene

Searches of *C. salei* brain and leg hypodermis transcriptome libraries found two genes that were homologous to mammalian and arthropod *ChAT* genes. Based on GenBank searches these were identified as putative *C. salei* choline acetyltransferase (*CsChAT* Accession number KX892709) and carnitine O-acetyltransferase (*CsCrAT* Accession number KX892707). For the *CsChAT* gene we found only 292 residues from the carboxyterminal end. This region has 42% similarity with human and 40% similarity with *Drosophila ChAT* genes. The *CsCrAT* gene coding DNA sequence (CDS) is complete, it encodes a protein with a molecular weight of 72 kDa and 40% similarity with the human and 33% similarity with the *Drosophila ChAT* genes. Compared to actin, the relative abundances were: *CsCrAT* 1.62×10^{-2} in the brain and 7.05×10^{-3} in the hypodermis, and *CsChAT* 2.69×10^{-5} in the brain and 4.08×10^{-5} in the hypodermis. Probes for *in-situ* hybridization were constructed for each gene based on the primer sequences shown in Table 1. The *CsChAT* antisense probe produced specific labeling in sections from all brain regions as shown in Figs. 2–5 while the control sense probe did not produce any labeling (Fig. 3d). The *CsCrAT* antisense or sense probes did not produce signals in any areas of the brain, but a small level of labeling was seen in the muscle tissue that surrounds the brain (data not shown). The spider brain tissue that was used to create the transcriptome also included some of this surrounding muscle tissue and it is likely that the *CsCrAT* gene RNA originated from this muscle.

Expression of ChAT in the spider CNS

To examine the distribution of ChAT in the spider brain, we performed immunohistochemistry and *in-situ* hybridization. The polyclonal ChAT antiserum used here has previously been shown to specifically label *C. salei* mechanosensory neurons, while Western blots revealed a clear band at 65 kDa in spider CNS and hypodermis tissues (Fabian and Seyfarth 1997). Seven CNS preparations were immunolabeled with the ChAT antiserum and four brains processed with the *CsChAT* antisense and sense probes. In some areas of the brain, almost all neurons were labeled by ChAT antiserum and the *CsChAT* probe but other areas, even within the same ganglion, were devoid of labeling. Because of this, and the fact that neurons were densely packed within the labeled areas, it is not possible to give accurate numbers of labeled versus unlabeled neurons. However, we estimate that about 10–20% of the neurons were labeled. Their distributions in different parts of the CNS are shown in schematic Figs. 2a–d and actual data in Figs. 3–5 and the results are described below.

Leg ganglia (Figs 2a and 3): Immunoreactive somata were observed throughout each walking leg ganglia. Most labeled neurons were small with diameters between 5–20 μm (Fig. 3a) located in the ventral cell layer. However, some labeled neurons located in the

medial and lateral areas of the ganglia were larger with somata between 30 and 40 μm in diameter. Confocal microscopy revealed that the labeling intensities within individual neurons varied (Figs. 3a and b). Hoechst Blue nuclear staining revealed that many neurons in the leg ganglia were not immunoreactive to the ChAT antiserum.

The same areas of leg ganglia that displayed anti-ChAT labeling also had strong signals with the antisense *CsChAT* probe (Figs. 3c and f), while the sense (control) probe produced no signal (Fig. 3d). As in immunolabelled preparations, strongly and weakly labeled neurons were observed (Fig 3c). Most of the labeled neurons had small diameter (5–20 μm) while a few neurons with a soma diameter of 30–40 μm were also labeled. However, the largest neurons (diameter 50–100 μm) were not labeled in any sections through the leg ganglia (Figs. 2a, 3c). These large neurons were mostly found in the mid-ventral region of the subesophageal ganglia and are likely motor and interneurons (Milde and Seyfarth 1988; Babu et al. 1989). Characteristic neuron clusters on the anterolateral part of each leg ganglion (anterolateral neuron clusters; ANC) consisting mainly of small neurons (5–10 μm soma diameter) also displayed anti-ChAT labeling and expressed the *CsChAT* gene (Figs. 2a, b and 3e, f). A total of 56% (± 9%, $n = 16$) of the neurons within ANCs were labeled by the *CsChAT* antisense probe.

Opisthosomal ganglia (Figs. 2a, b and 4 a–c): Many neurons in the opisthosomal ganglia were labeled either strongly or faintly by the ChAT antiserum (Figs. 4a and b) and a similar labeling pattern was also seen in sections treated with the antisense *CsChAT* probe (Fig. 4c). Labeled neurons were 5–15 μm by diameter and often in clusters. Small numbers of larger neurons (20–30 μm diameter) were also labeled. However, many opisthosomal neurons were not labeled and the largest neurons (40–100 μm in diameter) were not labeled in any section.

Pedipalpal ganglia (Figs. 2a and 4 d–f): *CsChAT* antisense probe indicated gene expression in lateral and medial areas of the pedipalpal ganglia (Fig. 4d). Almost all labeled neurons in the pedipalpal ganglia were small (soma diameter 5–15 μm) with only a very small number of medium sized neurons (soma diameter 20–30 μm) found in some sections and the largest neurons (40–100 μm in diameter) were not labeled. Similar labeling patterns were observed in sections immunolabeled by ChAT antiserum (Figs. 4e and f). Hoechst blue nuclear stain revealed that only a subpopulation of neurons in the lateral areas of the pedipalpal ganglia was ChAT immunoreactive (Fig. 4f inset).

Cheliceral ganglia (Figs. 2b, c and 4g, h): Many neurons in the cheliceral ganglia also displayed anti-ChAT labeling in both anterior medial and lateral areas (Fig. 4g). *In-situ* hybridization with the antisense *CsChAT* probe produced clear signal in the same areas (Fig. 4h). Neurons that were labeled in cheliceral ganglia had predominantly soma diameters of 10–15 μm while many similar size neurons and all larger neurons (40–110μM diameter) were unlabeled.

Supraesophageal ganglia (Figs. 2d and 5a–f): Photoreceptors from the spider eight eyes synapse with second order neurons in first optic neuropils (ON1) that, in turn, supply two successive optic neuropils, ON2 and ON3 (Fig. 2d). L-cells (Lamina cells) pass

information from secondary eye ON1 to ON2 and T-cells (Tangential cells or globuli cells) further from ON2 to ON3 (Fig. 2d inset) (Strausfeld and Barth 1993; Strausfeld et al. 1993). Many cell bodies at the approximate locations of L- and T-cell somata were labeled by both the ChAT antiserum and the *CsChAT* probe (Figs. 2d and 5 a–e). Similarly to the subesophageal ganglia, there were clear differences in labeling intensities in different neurons with both the *CsChAT* probe and the ChAT antiserum. Some somata within the neuron clusters remained unlabeled (Figs. 5 a, b and d). Strongly labeled neurons were visible especially in the lateral areas close to the brain midline (Figs. 5a and c). Adjacent to the ChAT immunoreactive cell bodies, strong labeling was visible in nerve fibers within the optic neuropils where synaptic contacts are located (Figs. 5d and e). Strongly labeled somata were also found in the mushroom body area (Figs. 2d and 5d). We also observed a small number of labeled neurons in the posterior cell layer, particularly in the lateral areas, but most neurons in this layer were not labeled (Fig. 5 f).

Double labeling of spider CNS and mechanosensory VS-3 neurons by anti-ChAT and anti-VAChT

To further confirm that ChAT expressing neurons are cholinergic, we performed double labeling using the same ChAT antiserum as above and an antiserum that detects another typical epitope for cholinergic transmission: the vesicular acetylcholine transporter (VAChT; Fig. 6). Since the latter antiserum was made in goat, it could also be used for double labeling with the FMRFamide antiserum (*below*). Western blot analysis for anti-VAChT showed distinct bands at molecular weights of about 70 and 85 kDa for the spider brain homogenate (Fig. 6b inset). We found one gene in the *C. salei* transcriptome that codes a putative vesicular ACh transporter, *CsVAChT* (Accession number KX966397) and a partial homologous gene (KY074556). The antiserum was raised against a peptide sequence in the amino terminal region of human VAChT that is highly homologous to the same regions in the two spider genes (proprietary information provided by Santa Cruz Biotechnologies). The *CsVAChT* was only found in the hypodermis transcriptome, where its abundance is 1.33×10^{-4} compared to the abundance of actin and it codes a protein with a molecular weight of 65 kDa. The partial gene was present in both the brain and hypodermis transcriptomes with abundances of 3.21×10^{-4} and 3.59×10^{-6} relative to actin, respectively.

Neurons that were immunoreactive to ChAT antiserum in the leg ganglia were also labeled by anti-VAChT. However, the VAChT antiserum also labeled some areas that were not labeled by anti-ChAT (Figs. 6 a–d). Since the images are single confocal sections, it is possible that the two types of antisera label different subcellular areas of the same neuron. Both antisera also labeled the somata of VS-3 neurons in the hypodermis of leg patella (Figs. 6 e–h, see Fig. 1c for schematic diagram). The glial cells that surround the VS-3 neurons, which have elongated ellipsoid nuclei (Liu et al. 2017) were not stained by either antiserum.

FMRFamide-like peptides in spider CNS and mechanosensilla and their co-localization in cholinergic neurons

We investigated the distribution of FMRFamide-like peptides in the spider CNS and mechanosensilla using an antiserum that recognizes these peptides. The spider transcriptomes have at least two FMRFamide-like peptides (Accession numbers KY074554

and KY07455) and the antiserum may also detect additional related peptides. Anti-FMRFamide labeling was previously shown to be very strong and widespread in the *C. salei* protocerebrum (Becherer and Schmid 1999) and our findings were similar (data not shown). As in previous studies (Barth 2002), we found anti-FMRFamide labeled somata in all subesophageal ganglia. However, we also found strongly labeled nerve fibers projecting into each leg ganglion (Figs. 7a, b) that were not described in the previous investigation. Interestingly, mechanosensory neurons in the VS-3 organ that were previously found immunoreactive to ChAT (Fabian and Seyfarth 1997) were also labeled by anti-FMRFamide. However, there were differences in the intensity of labeling among the VS-3 neurons (Fig. 7c). In addition, some of the putative efferent fibers that innervate mechanosensory neurons were labeled by anti-FMRFamide as seen in Fig. 7d where fine labeled fibers branch around the three neurons of a hair sensillum in the patella.

To test if cholinergic neurons in the CNS also contain FMRFamide-like peptides we performed double labeling with the FMRFamide antiserum and the VAChT antiserum. As shown in Fig. 8 for the opisthosomal ganglion, we found four groups of neurons: Some were immunoreactive for both anti-VAChT and anti-FMRFamide (Figs. 8a–c), others were labeled only by anti-VAChT (Figs. 8 d–f), the third group was only labeled by anti-FMRFamide (Figs. 8 g–i) and the fourth group remained unlabeled by either antiserum (Figs. 8 g–i). The labeling intensity for anti-VAChT was generally weaker in neurons that were also labeled by anti-FMRFamide when compared to neurons that were not labeled by anti-FMRFamide (Figs. 8 b and e). In contrast, the labeling intensity for anti-FMRFamide was similarly strong whether it was co-localized with anti-VAChT or not (Fig. 8 a and g).

Discussion

In this study, we mapped the distribution of putative cholinergic neurons in the central nervous system of the spider *C. salei.* We first identified genes encoding two proteins that are essential for cholinergic transmissions, *ChAT* and *VAChT* (Ichikawa et al. 1997), and then used this genetic information to produce probes for *in-situ* hybridization and to identify suitable antisera for immunolabeling. An RNA probe for *C. salei* ChAT (*CsChAT*) and antisera against ChAT and VAChT labeled similar distinct clusters of neurons in all parts of the spider CNS as well as some individual neurons. The abundances of putative *ChAT* and *VAChT* genes were relatively low in the spider CNS transcriptome and the ratio of labeled versus unlabeled neurons varied greatly throughout the CNS. Together with previous reports of cholinergic markers, such as ChAT, AChE and muscarinic ACh receptors in the spider mechanosensory and efferent neurons (Fabian and Seyfarth 1997; Fabian-Fine et al. 2002; Widmer et al. 2006) our findings establish the importance of cholinergic neurons for the functions of spider central and peripheral nervous systems. Furthermore, we have extended previous research on the distribution of FMRFamide related peptides in the spider nervous system (Becherer and Schmid 1999; Barth 2002) and demonstrated that they are co-localized with some anti-VAChT labeled neurons in the CNS.

C. salei ChAT and VAChT genes and specificities of antisera

Our searches of the *C. salei* brain and hypodermis transcriptomes found a partial putative *ChAT* gene sequence and another homologous gene, carnitine acetyltransferase (*CsCrAT*). The ChAT antiserum used in this study was raised against the complete human placental ChAT enzyme. It shares high amino acid similarity with the CsChAT and CsCrAT, and would be likely to bind both epitopes. The antisense RNA probe for *CsChAT* produced labeling in the somata of specific neurons in the spider CNS, but the probe for *CsCrAT* only labeled some areas of muscle tissue. The *CsCrAT* gene may code a protein with a similar role in fatty acid transport through muscle cell mitochondrial membranes as CrAT proteins in other arthropods (Haunerland 1997). The binding specificity of the same ChAT antiserum has previously been determined by Western blot on spider brain tissue (Fabian and Seyfarth 1997) and is further supported by the fact that it labeled the somata of similar sized neurons in the same areas of the brain as the *CsChAT* RNA probe.

For VAChT detection we used an antiserum that was raised against an amino terminal segment of human VAChT. This region contains a 30-amino acid long highly conserved region that is identical in two genes found in *C. salei* transcriptomes. One of these was only found in the hypodermis transcriptome while the other is a fragment found in both the brain and hypodermis transcriptomes. Our Western blot analysis produced two clear bands at 70 and 85 kDa, suggesting that the antiserum binds two spider proteins, or it may detect two different posttranslationally modified forms of the same protein. The full length VAChT that was found only in spider leg hypodermis transcriptome has a molecular weight of 65 kDa and may also be expressed in the brain, but its abundance there is too low to find in the transcriptome. Labeling with the VAChT antiserum was clearly detected in neurons that were also labeled with anti-ChAT. However, some labeling was also seen in areas that did not have anti-ChAT labeling. Since VAChT is concentrated on synaptic vesicles at axon terminals and VAChT antibodies have been shown to label these locations in other animals more strongly than ChAT antibodies (Gilmor et al. 1996) it is possible that some of the anti-VAChT labeling in spider ganglia was in the nerve terminals that were not as strongly labeled by anti-ChAT. These may be the terminals of sensory neurons that terminate in the subesophageal ganglia (Babu and Barth 1989; Anton and Barth 1993).

Cholinergic neurons in the subesophageal ganglia

Babu and Barth (1984) classified the neurons of *C. salei* CNS based on the size of their somata and nuclei. Their smallest Type A neurons were the globuli cells that were only found in the protocerebrum (*see below*) while Type B cells (12–20 μm by diameter) were present in all ganglia. We found many 5–20 μm neurons in the cheliceral and all subesophageal ganglia expressing ChAT. A large portion of these neurons were smaller than the Type B cells and were probably not visible with the staining method used by Babu and Barth (1984). We also found a small number of ChAT expressing neurons that were 30–40 μm by diameter, the same size as the smallest of the Type D neuron group that consists of motor and interneurons (Babu et al. 1985; Milde and Seyfarth 1988). None of the larger Type D neurons (40 μm - 120 μm) or the Type C (45 μm) neurosecretory cells were labeled by any of the three cholinergic markers.

We were not able to trace projections of anti-ChAT or anti-VAChT labeled somata through the various ganglia. However, some of the cholinergic somata probably lead to axons of efferent fibers that innervate mechanosensory neurons and other efferents in *C. salei* legs (Fabian-Fine et al. 1999a; 1999b; 2000; 2002). Many of these efferent fibers are very thin and difficult to trace, but we detected punctate anti-ChAT labeling in some of the efferent fibers surrounding VS-3 and other mechanosensory neurons, and similar findings have been reported previously (Fabian-Fine et al. 2002). Further evidence for cholinergic efferent fibers comes from previous findings of the ACh degrading enzyme AChE as well as muscarinic ACh receptors in efferent fibers and mechanosensory neurons (Fabian and Seyfarth 1997; Widmer et al. 2006).

Many ChAT labeled cell bodies were found in similar locations to GABA and/or glutamate immunoreactive neurons in previous studies (Barth 2002; Fabian-Fine et al. 2015). One example is given by the anterolateral neuron clusters (ANC) of the leg ganglia that were shown here to have 56 % of somata expressing ChAT. Over 90% of somata in similar clusters were previously found to be immunoreactive to GABA, and many also to glutamate (Barth 2002; Fabian-Fine et al. 2002). Spider mechanosensory neurons and some efferent neurons have GABA, glutamate and ACh receptors and the neurons respond to these transmitters in electrophysiological experiments (Panek et al. 2002; Panek and Torkkeli 2005; Widmer et al. 2006; Torkkeli et al. 2012; 2015). An attractive hypothesis is that neurons in the ANC may be efferents, containing one or more of these three transmitters, each being released in different behavioral situations.

Large neurons in *C. salei* subesophageal ganglia have been identified as motor neurons or interneurons and, as in other arthropods, spider muscles are innervated by multiple excitatory and inhibitory nerve fibers (Babu and Barth 1984; Babu et al. 1985; Maier et al. 1987; Milde and Seyfarth 1988). The fact that we did not see any cholinergic markers in the largest neurons of the subesophageal ganglia, agrees with the recent discovery that spider leg muscle is devoid of mRNA that codes nicotinic ACh receptors (Liu et al. 2016). Therefore, it is likely that, as in other arthropods, the major fast acting excitatory motor neurons in spiders are not cholinergic, but more likely use glutamate as their primary transmitter (Takeuchi and Takeuchi 1964; Usherwood et al. 1968, Jan and Jan 1976). Further evidence for this is that many large neurons in the subesophageal ganglia were previously shown to be immunoreactive to glutamate and/or GABA, and both transmitters were also present in axon terminals innervating the leg muscle (Fabian-Fine et al. 2015). However, some of the smaller Type D neurons that were also labeled by ChAT may be cholinergic motoneurons that act on muscarinic ACh receptors and modulate muscle excitability either directly or presynaptically via other motor neurons.

Some cholinergic neurons in the subesophageal ganglia may also be interneurons that transmit sensory information arriving from numerous mechanosensory nerve terminals as well as hygro- and thermoreceptors that terminate in the subesophageal ganglia (Milde and Seyfarth 1988; Babu and Barth 1989; Anton and Barth 1993; Anton and Tichy 1994).

Cholinergic fibers connect visual pathways

The visual areas of *C. salei* protocerebrum are rich with cell bodies having morphologically variable projections to successive optic neuropils (Strausfeld and Barth 1993; Strausfeld et al. 1993). The bipolar L-cells connect visual pathways between first and second optic neuropils (ON1 and ON2) and the T-cells (or globuli cells) pass information from the second to third optic neuropil (ON2 and ON3) (Fig. 2d). We found many cell bodies labeled with both the ChAT antiserum and the *CsChAT* probe at the locations of both L- and T-cell bodies. In addition, the nerve fibers within optic neuropils were strongly labeled by the ChAT antiserum, clearly indicating that ACh is used in synaptic transmission between the optic neuropils. Spider ON1 is analogous to insect optic lamina, ON2 to the medulla and ON3 to the lobula plate (Strausfeld and Barth 1993). The corresponding neuropils in *Drosophila* also contain cholinergic neurons (Yasayuma and Salvaterra 1999: Kolodziejczyk et al. 2008). Most of the cell bodies in the visual areas were weakly labeled, but we also found some strongly labeled neurons, especially within the dorsal protocerebral cell layer that is located above the mushroom body (Figs. 1b and 2d). Some of these cell bodies may be descending neurons that have been shown to send processes to other areas of the brain and some neurons may serve other sensory modalities than vision (Strausfeld et al. 1993; Strausfeld and Barth 1993). We found no anti-ChAT labeling in the photoreceptor cells, which agrees with previous suggestion that histamine is the neurotransmitter of *C. salei* photoreceptors (Schmid and Duncker 1993). Previous studies have described immunoreactivity to other neurotransmitters in some of the same visual areas. For example, all *C. salei* optic neuropils were immunoreactive to GABA (Becherer and Schmid 1999) and many globuli cells were labeled by antisera for both GABA and glutamate (Fabian-Fine et al. 2015). Extensive labeling with anti-FMRFamide was also found in all optic neuropils while proctolin and octopamine limmunoractive cells were less common (Seyfarth et al. 1993; Becherer and Schmid 1999: Loesel et al. 2011). These findings, and the fact that there are morphologically different types of L- and T-cells (Strausfeld et al. 1993), suggest that several transmitters mediate inhibitory and excitatory synaptic transmission in the spider visual pathways.

FMRFamide-like peptides in C. salei central and peripheral nervous systems

FMRFamide-like peptides form a large family of related neuropeptides found in all animal phyla (Kobayashi and Muneoka 1989; Osborne 1996; Mercier et al. 2003; Peymen et al. 2014). Commercial FMRFamide antisera recognize multiple members of this family and have shown widespread labeling in arthropod central and peripheral nervous systems (Persson and Nässel 1999: Brownlee et al. 2000; Nichols 2003; Nässel and Homberg 2006). Extensive anti-FMRFamide labeling was previously found in the *C. salei* CNS (Becherer and Schmid 1999; Barth 2002) and our findings here were similar. Interestingly, we also found strong anti-FMRFamide labeling in the peripheral nerves leaving each subesophageal ganglia and in some of the fine efferent nerve fibers surrounding mechanosensory neurons that were not described previously (Barth 2002). The detailed distribution FMRFamide immunoreactive neurons in *C. salei* subesophageal ganglia has not yet been described. We also found anti-FMRFamide labeling in leg mechanosensory neurons that were previously shown to be immunoreactive to antisera against ChAT and histamine and have AChE activity (Fabian and Seyfarth 1997). Similar to the FMRFamide labeling, some of these neurons

were more strongly labeled with AChE and histamine than others. Moreover, this difference correlated with neuronal firing pattern so that the very rapidly adapting neurons were more strongly stained by AChE than neurons that adapt more slowly (Fabian-Fine et al. 1999b).

Our main interest here was to discover if FMRFamide related peptides are co-expressed in cholinergic neurons. FMRFamide related peptides have previously been shown to co-localize with other peptides and with GABA in insect nervous systems (Kobayashi and Muneoka 1989; Persson and Nässel 1999; Berg et al. 2009).

Neurotransmitter Co-expression

Over the past several decades it has been firmly established that most neurons in all animal nervous systems, from invertebrates to human, release two or more neurotransmitters (Whim and Lloyd 1989; Trudeau 2004; Gutierrez 2005; Salio 2006; El Mestikawy et al. 2011; Hnasko and Edwards 2012). It is especially common to find multiple neuropeptides in the same neurons, but classical small molecule neurotransmitters such as ACh, GABA and glutamate, have also frequently been shown to co-localize with one or more neuropeptides or with each other (Brownlee et al. 2000). Our findings here indicate that some *C. salei* central and peripheral neurons co-express ChAT and FMRFamide related peptides. Previously, GABA and glutamate were shown to co-localize in many neurons of the spider CNS (Fabian-Fine et al. 2015) and, based on the locations of ChAT expressing neurons found here, some of them may also co-express GABA and/or glutamate. Some of the neurons that express more than one transmitter are likely to be efferents that innervate peripherally located mechanosensory neurons, and thus candidates for the transmitters inhabiting the different types of synaptic vesicles that were previously found by electron microscopic investigation (Fabian-Fine et al. 2000). The Type 3 synapses in *C. salei* central and peripheral nervous systems were shown to contain mixed vesicle populations with both small and large clear vesicles as well as dense core vesicles (Fabian-Fine et al 2000; 2015). The clear vesicles usually contain classical transmitters such as GABA, glutamate, biogenic amines and ACh while the dense core vesicles contain neuropeptides such as FMRFamide or (Brownlee et al. 2000; Salio et al. 2006).

In spider VS-3 mechanosensory neurons, GABA acts on ionotropic receptors to produce a brief inhibition followed by long lasting excitation, while glutamate usually has a purely inhibitory effect and octopamine an excitatory one (Widmer et al. 2005; Pfeiffer et al. 2009; Torkkeli et al. 2012). This poses the questions that if these transmitters are released from the same synapses why is it done and when? Another question is why some neurons co-release more than one transmitter whereas others do not? One possible answer to the first question is plasticity; some neurons may develop activity dependent protective mechanisms that help to counteract overexcitation and excitotoxicity during stressful situations through the fast release of counteracting neurotransmitters. An example of this mechanism is activity induced upregulation of GABA in mammalian hippocampal glutamatergic granule cells (Sloviter et al. 1996; Gutiérrez 2005). For the second question, one hypothesis is that neurons that express only one transmitter, innervate a larger subset of postsynaptic neurons that control both inhibitory and excitatory pathways, whereas neurons with more than one transmitter may predominantly contact either only excitatory or only inhibitory pathways.

Activity dependent co-release of neuropeptides and classical transmitters has been described in some invertebrate neuromuscular junctions. For example, when an *Aplysia* motor neuron was firing slowly, it produced a purely cholinergic response in the muscle whereas high frequency firing led to mixed cholinergic/peptidergic responses (Whim and Lloyd 1989). In the cockroach neuromuscular junction, where glutamate is the main excitatory transmitter, proctolin was released from some of the same motor neurons during elevated neuronal activity and its release potentiated the glutamate effect (Adams and O'Shea 1983). Neuropeptides act mainly on G-protein coupled receptors that activate or inhibit second messenger pathways and can have long lasting responses such as changes in gene expression (Whim and Lloyd 1989). Research into the roles of FMRFamide related peptides in spider synaptic transmission is only beginning but the abundance of anti-FMRFamide labeling in *C. salei* poses the questions (1) how FMRFamide related peptides modulate synaptic transmission and neuronal responses, and (2) whether FMRFamide related peptides are co-expressed in neurons that contain classical neurotransmitters such as GABA or glutamate. It is also important to investigate if expression of FMRFamide related peptides is associated with activity dependent up- or downregulation of other proteins. Some of these experiments are currently ongoing in our laboratories.

Differences in labeling intensity

Several different antibodies against neurotransmitters have been shown to produce different labeling intensities in *C. salei* neurons (Seyfarth et al. 1993; Becherer and Schmid 1999; Barth 2002; Fabian-Fine et al. 2015). Interestingly, double labeling for GABA and glutamate found that weakly labeled GABAergic neurons often co-localized with glutamate, suggesting that weakly labeled neurons may represent those that co-release two or more neurotransmitters, whereas strongly labeled neurons may only carry one transmitter. This could be the case with weaker labeling intensity in VAChT immunoreactive neurons that co-localize anti-FMRFamide than those that do not have anti-FMRFamide labeling. Another hypothesis is that the activity levels of weakly labeled neurons may be lower than those of strongly labeled ones. Activity dependent, up- and downregulation of neurotransmitters in vertebrate neurons has been documented previously (Ramirez and Gutiérrez, 2001; Borodinski et al. 2004). We are currently conducting experiments to address this hypothesis in the spider nervous system.

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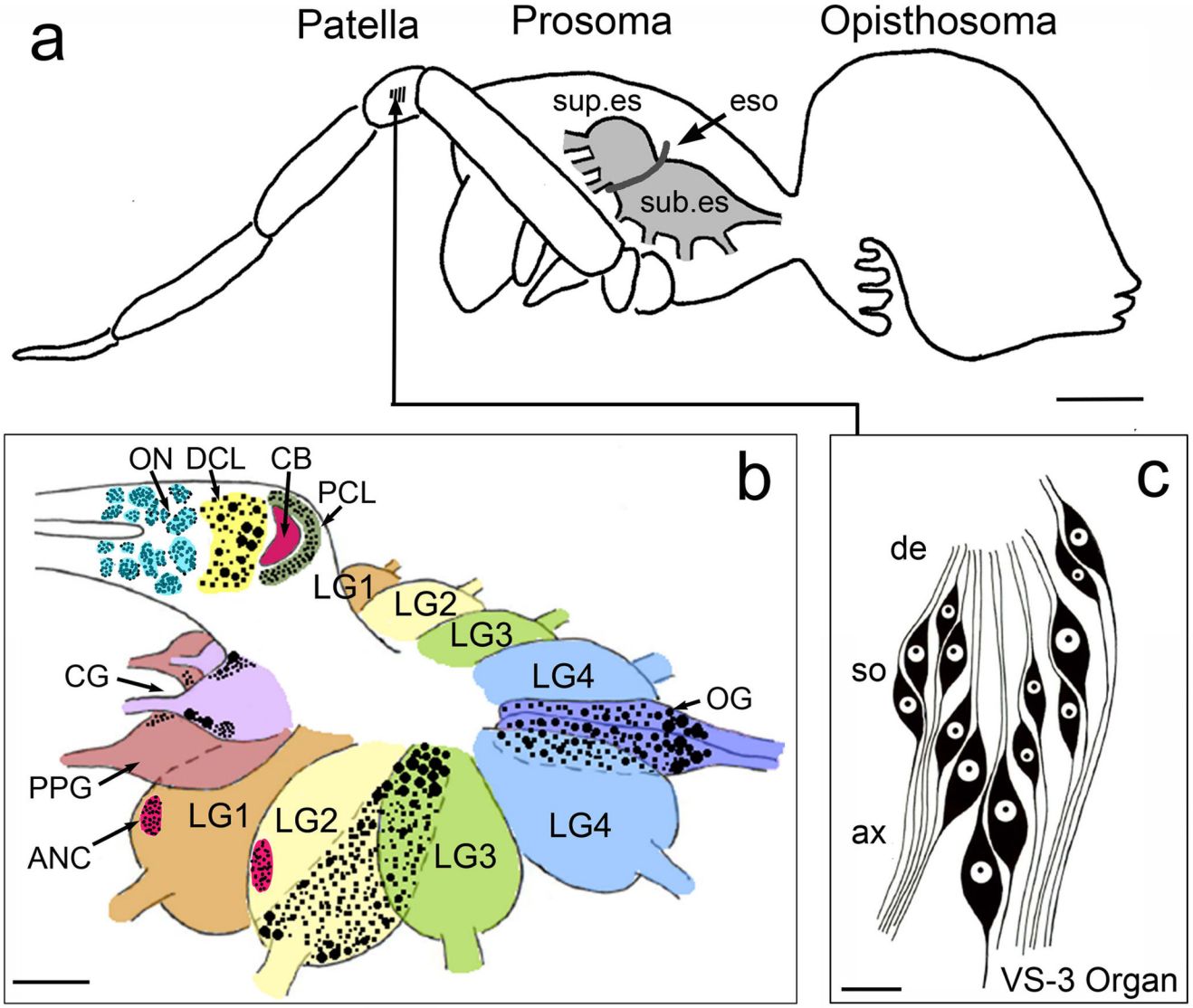

Fig. 1.

Schematic drawing of the *C. salei* CNS and sensory neurons in the VS-3 organ in the leg patella. **(a)** Sagittal section through the spider body; for clarity, only the first walking leg is shown. The VS-3 sensillum is located on the anterior part of the patella. The CNS is in the prosoma and penetrated by the esophagus (*eso*). Dorsal to the esophagus is the supraesophageal ganglion (*sup.es*), the ventral part is the subesophageal ganglion (*sub.es*). **(b)** The esophagus (not shown) penetrates the CNS from anterior to posterior between the pedipalpal (*PPG*) and cheliceral ganglia (*CG*). Each hemisphere of the subesophageal ganglion consists of four walking leg ganglia (*LG1–4*) whose somata are predominantly located in the ventral cell layer as indicated by black dots in the left LG2. Characteristic neuron clusters are located on the anterior part of each leg ganglion (anterior neuron clusters, *ANC*). The *PPG* is in the anterior and the opisthosomal ganglion (*OG*) in the posterior central areas of the subesophageal complex. The supraesophageal ganglion consists of the *CG*, the posterior cell layer (*PCL*), the central body (*CB*), the dorsal

protocerebral cell layer (*DCL*) with the underlying mushroom body and optic neuropils (ON) that are sites for the synaptic contacts between optic nerves. The approximate locations of nerve cell bodies within each ganglia are represented by black dots. For clarity, neurons in the ventral part of the leg ganglia are only shown in LG2 of the left hemisphere. Larger black dots represent the locations of large motor neurons within the ganglia. **(c)** Typical arrangement of mechanosensory neurons in the VS-3 organ. Each slit is innervated by two bipolar neurons whose dendrites (*de*), somata (*so*) and initial axon segments (*ax*) are in the hypodermis adjacent to the exoskeleton. *Scale bars*: a: 5 mm; b: 2 mm; c: 50 μm

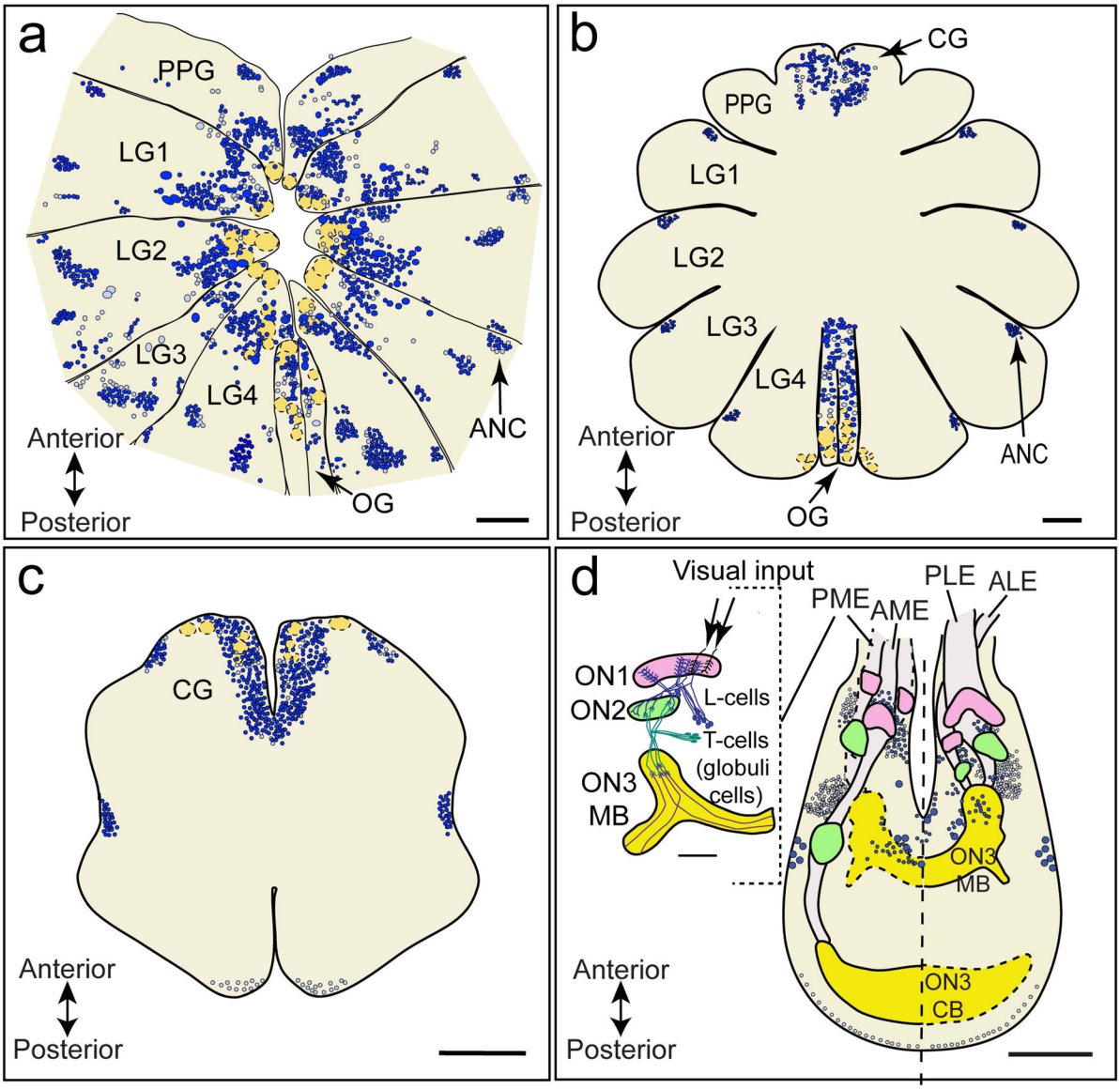

Fig. 2.

Schematic drawing showing the positions of the neuronal somata labeled with the *CsChAT* probe and ChAT antisera in horizontal sections through the spider CNS (Original data in Figs. 3–5). The strongly labeled neurons are indicted as dark blue circles, the lightly labeled neurons as light blue circles and the large neurons that were not labeled are indicated as yellow dashed circles. Note that each ganglion had many unlabeled smaller cells that are not indicated in these figures. **(a)** The ventral region of subesophageal ganglion. Each leg, opisthosomal and pedipalpal ganglia (LG1–4, OG and PPG, respectively) had many strongly or lightly labeled small somata (5–20 μm by diameter). Small numbers of mid-sized (30–40 μm) neurons were also labeled. Many small neurons (5–10 μm) in the anterolateral nerve clusters (ANC) were also strongly labeled. Large neurons (50–100 μm diameter) at the midventral region of each leg ganglion and along the OG were not labeled. **(b)** At the dorsal region of subesophageal ganglion the cheliceral (CG) and opisthosomal ganglia had mainly

small, darkly or lightly labeled neurons. Labeled neurons in the ANC of the leg ganglia were also visible in these sections. Large neurons at the distal region of the OG were not labeled. **(c)** The ventral part of supraesophageal ganglion shows many strongly or lightly labeled 10–15 μm neurons in the CG as well as in distinct regions on the perimeter. Large unlabeled neurons were also present in the CG. **(d)** Visual complex in the dorsal part of the supraesophageal ganglion. The left half shows the dorsal part of the ganglion, with the tracts and optic neuropiles of the anterior and posterior median eyes (AME and PME). The first optic neuropiles (ON1) are indicated in pink, the second optic neuropiles (ON2) in green and the third optic neuropiles (ON3) in yellow. The right half shows the visual pathways of the anterior and posterior lateral eyes (ALE and PLE). Inset on the left shows neural organization between optic neuropils of the PME. The visual nerve fibers terminate in ON1. L-cells pass the visual information from ON1 to ON2, and T-cells (also called globuli cells) further from ON2 to ON3. This diagram only show a small number of L- and T- cells, but there are hundreds of these small cells around the ONs. The ON3 for secondary eyes is also called mushroom body (MB) and the ON3 for principal eyes is central body (CB). Mostly lightly labeled small cell bodies were located close to the optic neuropiles on the expected areas for L- and T- cells. Some larger cell bodies were found close to the brain midline and in the cell body layer dorsal to the mushroom body. The labeled cell bodies were outside the optic neuropiles but the ChAT antiserum also labeled ON1s and ON2s strongly. Each drawing is traced from 3 −5 individual 20 μm sections. For d, the optic nerves and neuropiles have been adapted from Strausfeld and Barth 1993 and Becherer and Schmid 1999. Scale bars: a-d 200 μm, inset in d: 50 μm.

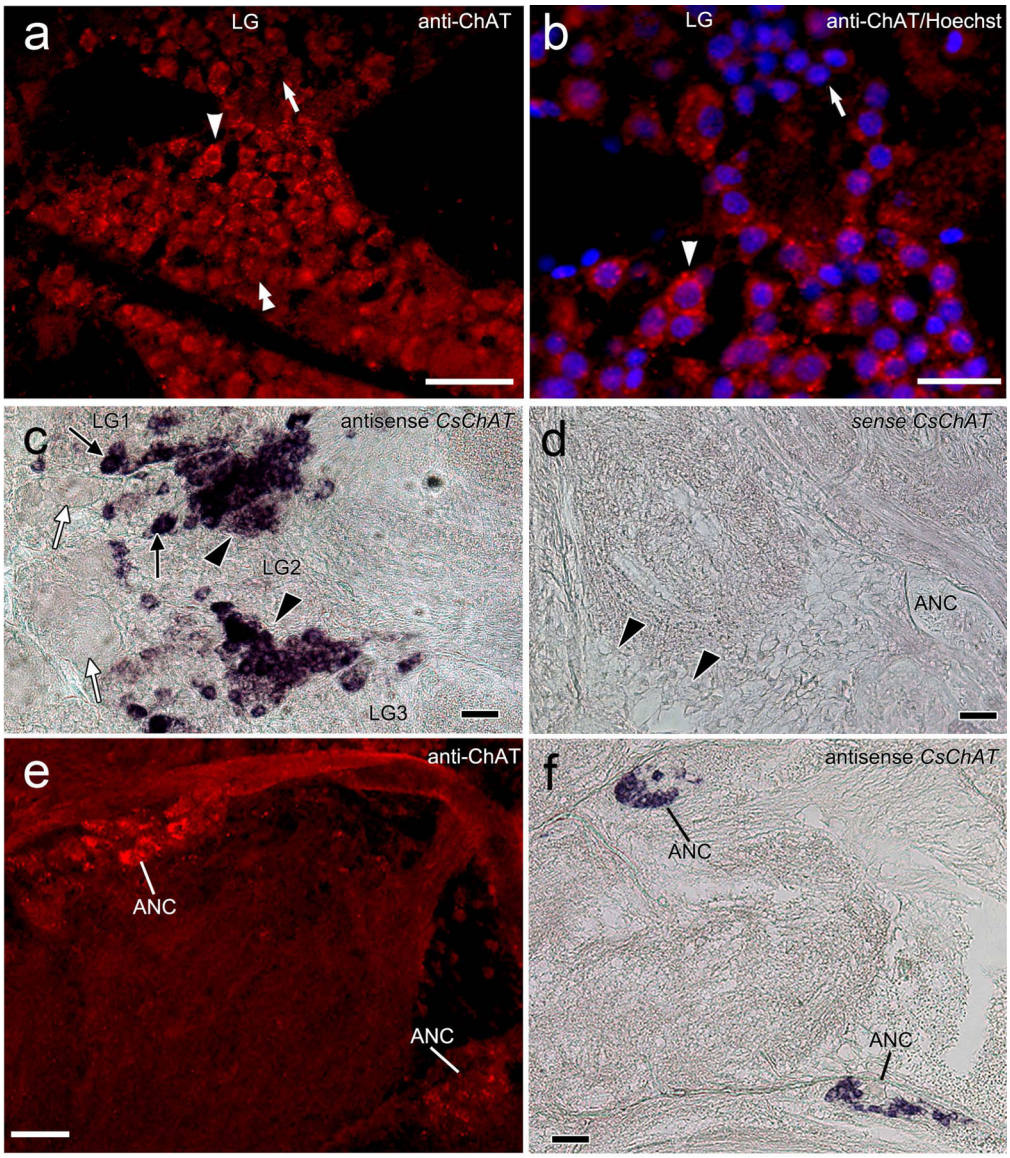

Fig. 3.

ChAT expression in the spider leg ganglia (*LG*). (See Fig. 2a for schematic representation). **(a)** Many neurons in the ventral cell layer were strongly labeled with the ChAT antiserum (*arrowhead*) while others were weakly labeled (*double arrowhead*) and some areas did not have labeling (*arrow*). **(b)** Higher magnification and Hoechst nuclear stain revealed anti-ChAT labeled neurons (*arrowhead*) and neurons that were not labeled by anti-ChAT (*arrow*). **(c)** *In-situ* hybridization using digoxigenin labeled antisense probe for *CsChAT* produced a strong signal in the ventral cell layer of the leg ganglia (*LG1–3*). Clusters of small (5 – 20 μm) neurons (*arrowheads*) and several larger (30–40 μm) diameter neurons (*black arrows*) were labeled. The largest neurons (50–100 μm) at the mid-ventral region of the ganglia (*white arrows*) were not labeled. **(d)** The sense (control) probe for *CsChAT* did not show any signal in the anterior neuron clusters (*ANC*) or any other neuron in the ganglion (*arrowheads*). Clusters of small (5–10 μm) anteriolateral neurons in leg ganglia (*ANC*)

labeled with anti-ChAT **(e)** and with the *CsChAT* antisense probe **(f).** *Scale bars*: a, c, d, f: 50 μm; b, e: 20 μm.

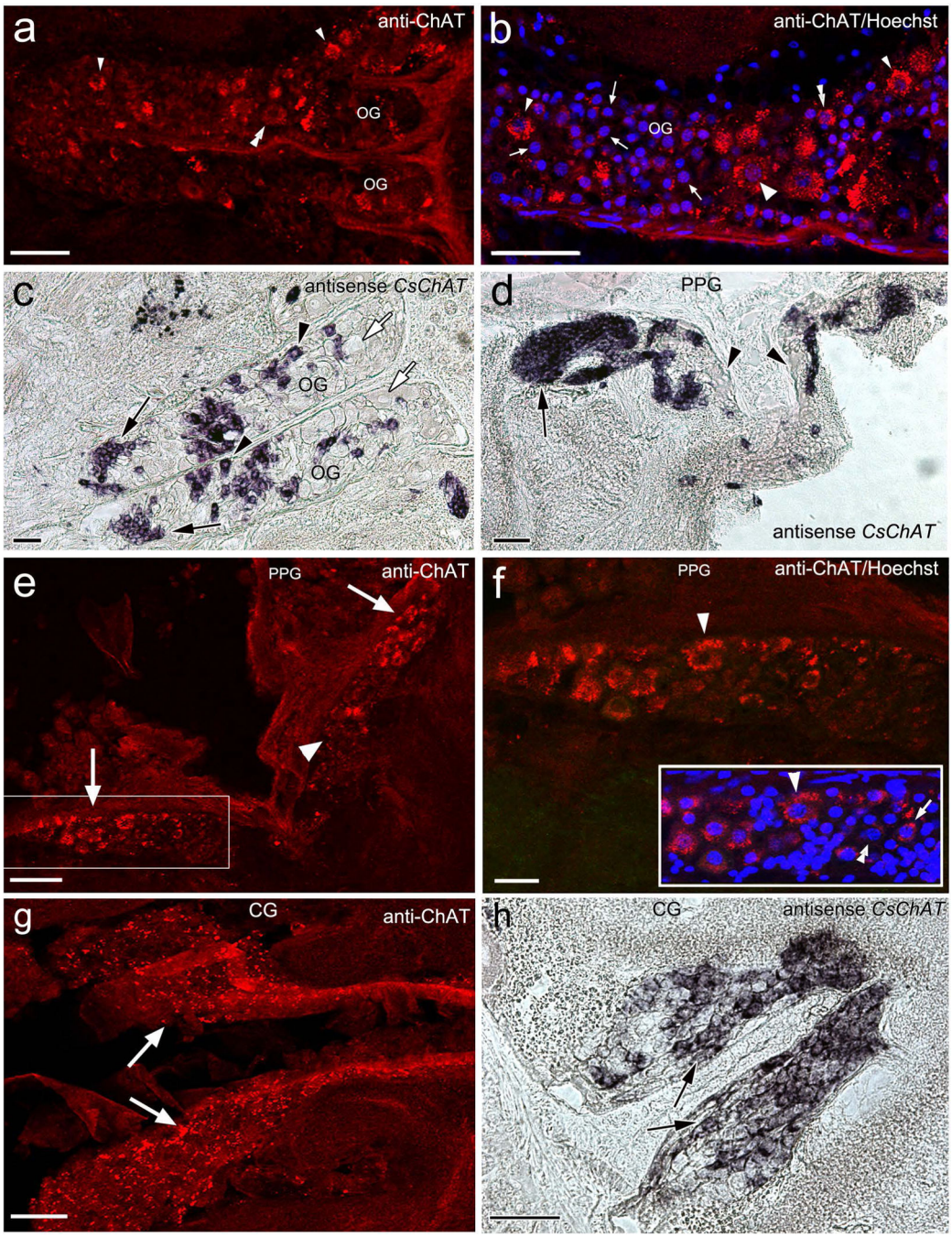

Fig. 4.

Expression of ChAT in opisthosomal (*OG*), pedipalpal (*PPG*) and cheliceral (*CG*) ganglia. (See Figs. 2 a, b and c for schematic representations.) **(a, b)** Numerous neurons in the opisthosomal ganglion were labeled either strongly (*arrowheads*) or weakly (*double arrowhead in a*) by the ChAT antiserum. Hoechst Blue stain revealed the nuclei of many unlabeled neurons (*arrows*). Smallest neurons had nuclei diameters of about 4 μm (*double arrowhead in b*). In the larger neurons, the nuclei were about 10 μm in diameter (*large arrowhead*). **(c)** Antisense probe for *CsChAT* gene revealed a similar staining pattern for the

opisthosomal ganglia (*OG*) as for the ChAT antiserum: Many mid-sized neurons (soma diameter 20–30 μm; *arrowheads*) and small neurons (soma 5–15 μm diameter; *black arrows*) had a strong hybridization signal while none of the largest neurons were labeled (*white arrows*). **(d)** In a section through the pedipalpal ganglia the *CsChAT* hybridization signal was predominantly observed in clusters of small cells (5–15 μm) in lateral parts of the ganglia (*arrow*). The medial areas showed fewer labeled neurons (*arrowhead*). **(e**, **f)** Anti-ChAT labeled section through the pedipalpal ganglia also displayed strong labeling in the lateral areas of the ganglia (*arrows*), whereas fewer neurons in the medial areas were labeled. ***Inset in e:*** Area magnified in **f**. showing the lateral area in a pedipalpal ganglion where several somata displayed relatively strong immunolabeling (*arrowhead*). ***Inset in f:*** Hoechst blue nuclear stain revealed the presence of numerous neurons that were not labeled by anti-ChAT (*double arrowhead*). Most anti-ChAT labeled neurons were small with soma diameters between 5 μm (*arrow*) and 15 μm (*arrowhead*). Neurons in the anterior medial region of the cheliceral ganglion show anti-ChAT labeling **(g)** and *CsChAT* hybridization signal **(h)** (*arrows*). The labeled neurons were 5–15 μm in diameter. *Scale bars*: a – e, g and h: 50 μm; f: 20 μm.

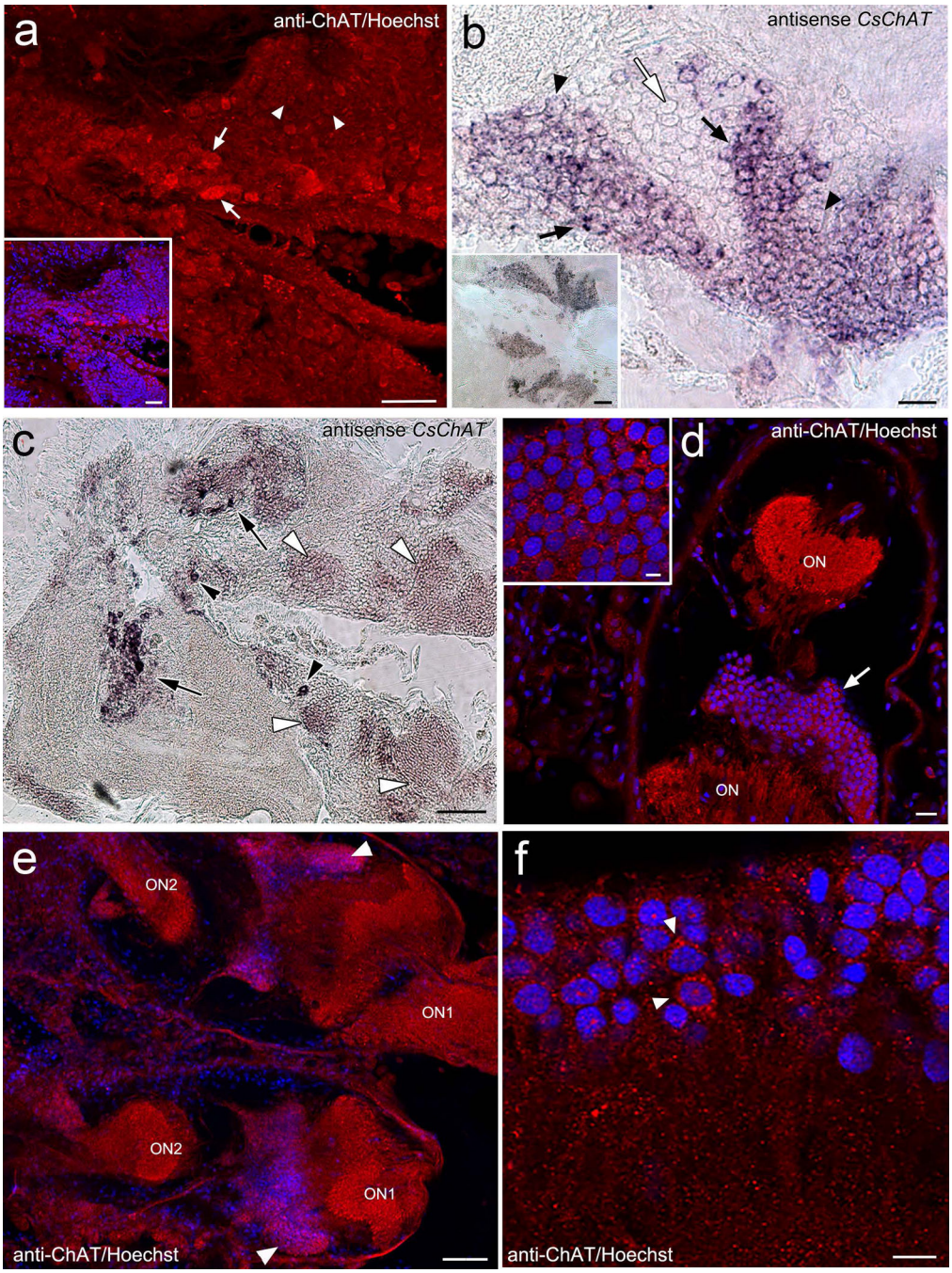

Fig. 5.

Expression of ChAT in visual areas of the protocerebrum. (See Fig. 2d for schematic representation. **(a)** Somata of T-cells (globuli cells) that project to optic neuropils appeared mainly weakly labeled by the ChAT antiserum (*arrowheads*); a small number of cells were more strongly labeled (*arrows*). *Inset:* Lower magnification image and Hoechst blue nuclear stain reveal the nuclei of all cells in the same area. **(b)** *In-situ* hybridization with the antisense probe for *CsChAT* showing relatively weak but clear signal in many L- and T-cells (*arrowheads*) while some cells were more strongly labeled (*black arrows*) and others were

not labeled (*white arrow*). *Inset:* Lower magnification image shows the bilateral arrangement of the optic lobes. **(c)** *CsChAT* antisense labeling in the dorsal cell layer (*black arrows*) was very strong in some cells while others appeared weakly labeled. Somata of many L- and T-cells were weakly labeled by *CsChAT* (*white arrowheads*). A small number of larger, strongly labeled neurons were located close to the midline (*black arrowheads*). **(d)** Anti-ChAT labeling in the somata of globuli cells (*arrow, inset*) that project to two optic neuropils (*ON*), both of which have strongly labeled nerve fibers. **(e)** Strong anti-ChAT labeling was present in nerve fibers in first and second optic neuropils (*ON1* and *ON2*) in both hemispheres. Many somata of the globuli cells were also labeled (a*rrowheads*). **(f)** Only a small number of neuronal somata were labeled by anti-ChAT in the posterior cell layer (*arrowheads*). *Scale bars*: a, inset in a: 50 μm; b, inset in b: 20 μm; c: 100 μm; d: 20 μm, inset in d: 5 μm; e: 100 μm; f: 10 μm.

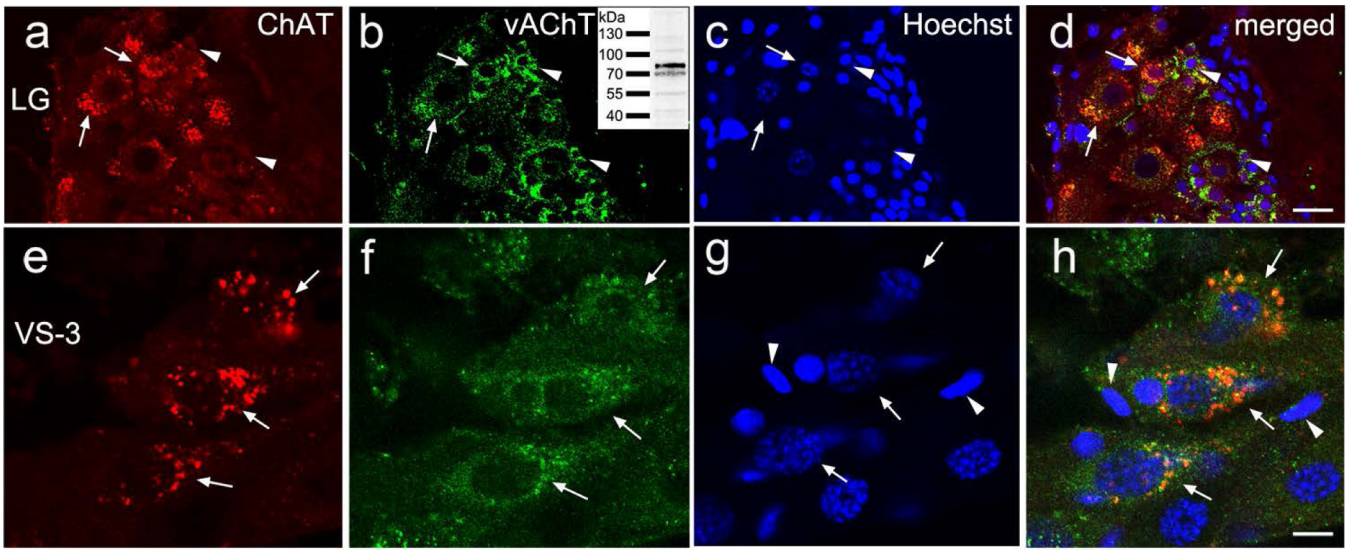

Fig. 6.

Double Immunolabeling of the leg ganglia (*LG*) and neurons of the mechanosensory VS-3 slit sensilla by ChAT and VAChT antisera. **(a - d)** Anti-ChAT and anti-VAChT labeling and Hoechst nuclear staining in the same optical section through a leg ganglion. ChAT and VAChT antisera labeled the same neurons (*arrows*). Anti-VAChT labeling was visible also in some locations where anti-ChAT labeling was not observed (*arrowheads*). The Hoechst Blue staining reveals nuclei of many cells that were not stained by either of the two antisera. Inset in **b** shows Western blot with the VAChT antiserum giving two strong bands at about 70 and 85 kDa. **(e - h)** Maximum intensity projection of confocal images through the mechanosensory neurons of a VS-3 slit sensillum (schematic diagram in Fig. 1c). The sensory neurons were labeled by both the ChAT and VAChT antisera (*arrows*), although the intensity of the latter staining was somewhat weaker. Hoechst blue labeling revealed spherical nuclei (*arrows*) of the VS-3 neurons that were stained by the two antisera. The glial cells surrounding VS-3 neurons have elongated ellipsoid nuclei (*arrowheads*) (Liu et al. 2017) that were not labeled by either antiserum. Scale *bars*: 10 μm.

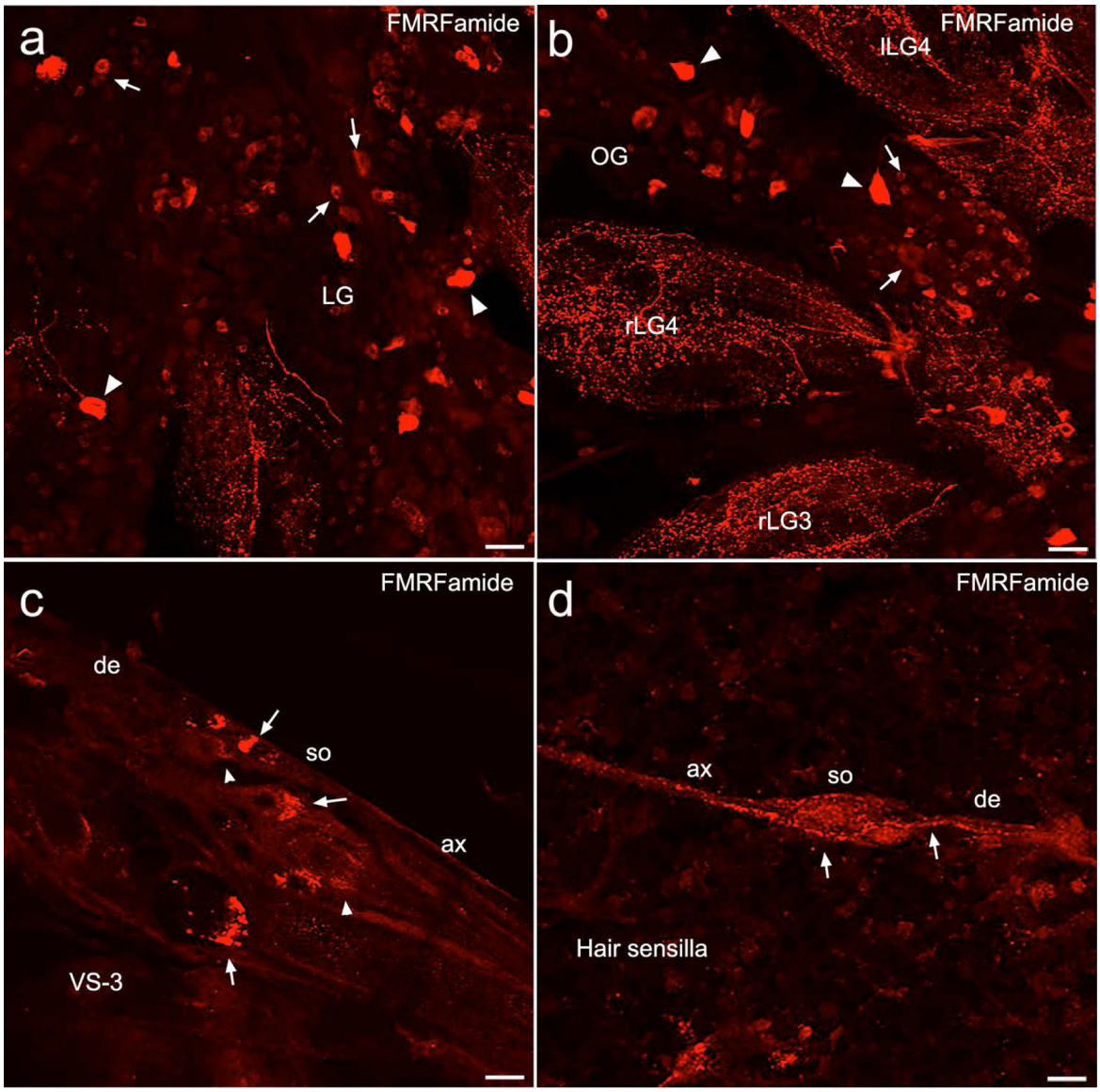

Fig. 7.

Anti-FMRFamide labeling of the subesophageal ganglia and leg mechanosensilla. **(a, b)** The ventral cell body layer of a leg ganglion (*LG in a*) and the opisthosomal ganglion (*OG in b*) had numerous strongly (*arrowheads*) and weakly (*arrows*) labeled neuronal somata. The neuropils of leg ganglia had many anti-FMRFamide labeled fibers (*rLG3* and r*LG4* neuropils of the 3rd and 4th leg ganglia on the right side and *lLG4* the 4th leg ganglion on the left hemisphere). **(c)** In the VS-3 mechanosensilla, some neurons appeared intensely labeled (*arrows*), whereas others showed only weak labeling (*arrowheads*). **(d)** Anti-FMRFamide labeled fine nerve fibers (*arrow*) branching around sensory neurons of a hair sensillum. *So*: somata, *de*: dendrites; *ax*: axons. *Scale bars*: a, b: 10 μm; c: 40 μm; d: 20 μm.

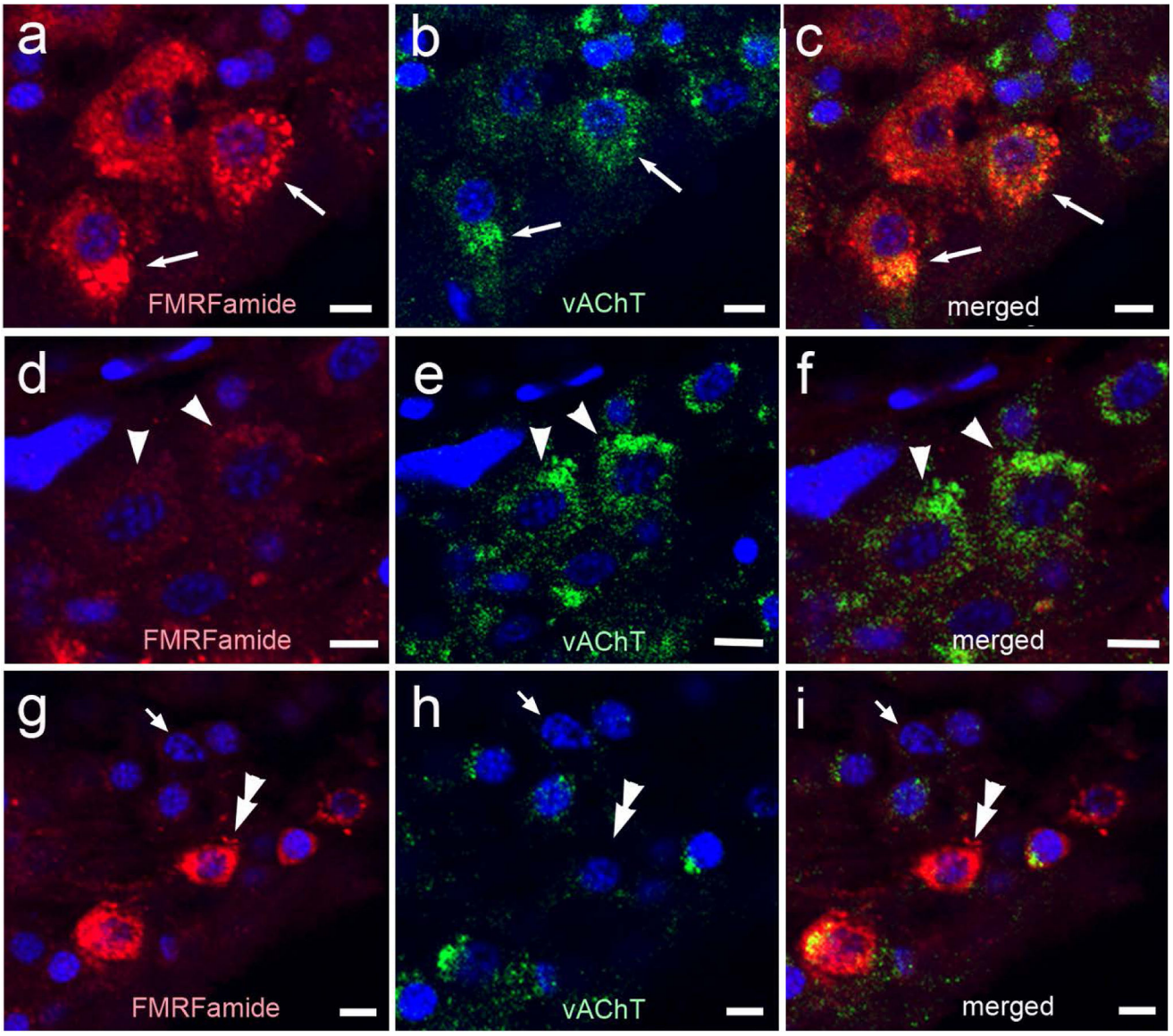

Fig. 8.

Double labeling for anti-FMRFamide and anti-VAChT in neurons of the opisthosomal ganglion. **(a-c)** Some strongly FMRFamide immunoreactive neurons were also weakly labeled by the VAChT antiserum (*long arrows*). **(d-f)** Another population of neurons within the same ganglion was not labeled by anti-FMRFamide but clearly by anti-VAChT (*arrowheads*). **(g-i)** The third population of neurons was strongly labeled by anti-FMRFamide but not immunoreactive to anti-VAChT (*double arrowheads*). A fourth population of neurons remained unlabeled by either antiserum (*short arrows*). *Scale bars*: 10 μm.

Table1.

Primer sequences used for generation of RNA probes for *in-situ* hybridization

Subunit	Accession number	Probe Size (bp)	Primers (T7 promoter sequence in bold)	
CsCh AT	KX892 709	676	AS	GTTGTGCTGAACTGGGAAATAGA
				TAATACGACTCACTATAGGGAGAAGTCCGAACTCGTCTCCTC
			S	**TAATACGACTCACTATAGGG**GTTGTGCTGAACTGGGAAATAGA
				AGAAGTCCGAACTCGTCTCCTC
				GCGACCTCAGACCATTTAACATCG
CsCr AT	KX892 707	777	AS	CAGTCCAGCAGAGGGTCCTC
				TAATACGACTCACTATAGGGGATGAATGTGTTGCAGGACTGC
			S	**TAATACGACTCACTATAGGG**CAGTCCAGCAGAGGGTCCTC
				GATGAATGTGTTGCAGGACTGC

AS=antisense; S=sense. T7 promoter sequence is shown in bold.