Evidence for the cholinergic markers ChAT and vAChT in sensory cells of the developing antennal nervous system of the desert locust Schistocerca gregaria

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Abstract
Sensory and motor systems in insects with hemimetabolous development must be ready to mediate adaptive behavior directly on hatching from the egg. For the desert locust S. gregaria, cholinergic transmission from antennal sensillae to olfactory or mechanosensory centers in the brain requires that choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (vAChT) already be present in sensory cells in the first instar. In this study, we used immunolabeling to demonstrate that ChAT and vAChT are both expressed in sensory cells from identifiable sensilla types in the immature antennal nervous system. We observed ChAT expression in dendrites, neurites and somata of putative basiconic-type sensillae at the first instar stage. We also detected vAChT in the sensory axons of these sensillae in a major antennal nerve tract. We then examined whether evidence for cholinergic transmission is present during embryogenesis. Immunolabeling confirms that vAChT is expressed in somata typical of campaniform sensillae, as well as in small sensory cell clusters typically associated with either a large basiconic or coeloconic sensilla, at 99% of embryogenesis. The vAChT is also expressed in the somata of these sensilla types in multiple antennal regions at 90% of embryogenesis, but not at earlier (70%) embryonic stages. Neuromodulators are known to appear late in embryogenesis in neurons of the locust central complex, and the cholinergic system of the antenna may also only reach maturity shortly before hatching.

Keywords Locust · Antenna · Development · Sensory cells · ChAT · vAChT

Introduction
Acetylcholine is a ubiquitous neuromodulator with extensive roles in insect physiology and behavior (Heinrich et al. 1997; Kunst et al. 2011; Boppana et al. 2017; Deshpande et al. 2020; Showell et al. 2020). In the fly, for example, most chemosensory, olfactory, chordotonal, and auditory primary sensory neurons are cholinergic (Salvaterra and Kitamoto 2001). In the locust, components of cholinergic transmission including acetylcholinesterase, choline acetyltransferase, the high affinity choline transport system, and acetylcholine receptors are expressed throughout the adult CNS (see Homberg 2002 for review). The central projections of antennal afferents in the brain are also cholinergic (Knipper et al. 1989; Rind and Leitinger 2000), although the sensilla types involved remain unidentified.

In insects with a hemimetabolous mode of development, sensory and motor systems must contribute to adaptive behavior directly on hatching from the egg (Stevenson and Kutsch 1986). Cholinergic transmission in the antennal nervous system of the desert locust S. gregaria, for example, must therefore be functional at this first instar stage for olfactory and mechanosensory information to reach regulatory centers in the brain (Gewecke 1972). Despite this, developmental data on cholinergic neurotransmission in this sensory system are lacking.

In this study, we use immunolabeling to demonstrate that two essential components of a functional cholinergic
system—choline acetyltransferase (ChAT) which catalyzes the synthesis of acetylcholine and the vesicular acetylcholine transporter (vAChT) which packs the transmitter into vesicles for synaptic release (see Deshpande et al. 2020) are present in sensory cells of the locust antenna at this first instar stage. We propose identities for the sensilla types involved and also demonstrate that vAChT expression in these cells commences during embryogenesis, but only during the later stages (after 90%), suggesting that the cholinergic system of the antenna may only reach maturity shortly before hatching.

Materials and methods

Eggs from a crowded colony of Schistocerca gregaria were maintained as described in Ehrhardt et al. (2015, 2016) and embryos staged according to Bentley et al. (1979). Prior to immunolabeling, preparations were either embedded and sectioned (14 µm), or treated with ultrasound (sonication) to render the cuticle porous and allow imaging of the intact antenna (for details see Ehrhardt et al. 2015, 2016).

Immunolabeling

For protocols detailing solutions and incubation conditions, see Ehrhardt et al. (2015, 2016).

Primary antibodies

ChAT the polyclonal antibody against mammalian choline acetyltransferase (α-ChAT, rabbit, Millipore AB143, dilution 1:100 in incubation medium) labels neuronal somata, dendrites and axons in the locust (see also Geffard et al. 1985; Lutz and Tyrer 1987).

vAChT the polyclonal antibody against the mammalian vesicular acetylcholine transporter (α-vAChT, guinea pig, Millipore AB 1588, dilution 1:500) labels auditory sensory cells in bushcrickets (Weber et al. 2005).

HRP the antibody against horseradish peroxidase (HRP) recognizes a neuron-specific cell surface epitope in insects (Jan and Jan 1982). In single-labeling experiments, we used: polyclonal α-HRP (Dianova, rabbit, 323-005-021, dilution 1:150). For double-labeling experiments with α-ChAT, we used polyclonal α-HRP (Dianova, goat, 123-005-021, dilution 1:200), and for experiments with α-vAChT, we used polyclonal α-HRP (Dianova, rabbit, 323-005-021, dilution 1:150).

Secondary antibodies

For single-labeling experiments with HRP, we used Alexa® 488 (goat anti-rabbit, Invitrogen A11034) or Cy3 (goat anti-rabbit, Dianova 111-165-003) each diluted 1:200. For double-labeling experiments, the combinations were α-ChAT (Cy3, donkey anti-rabbit, Dianova 711-167-003, dilution 1:200)/α-HRP (Alexa® 488, donkey anti-goat, Dianova 705-547-003, dilution 1:450) and α-vAChT (Alexa® 488, goat anti-guinea pig, Invitrogen A 11073, dilution 1:150)/α-HRP (Cy3, goat anti-rabbit, Dianova 111-165-003, dilution 1:150).

Imaging

Confocal and fluorescence microscopy was performed as previously described (Boyan et al. 2010; Ehrhardt et al. 2015, 2016). ImageJ software (public domain) was used for all image processing and involved adjusting only contrast and resolution, the allocation of false colors, and the application of a convolution algorithm on selected fluorescence photomicrographs.

Results

Our results confirm the presence of the cholinergic markers choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter (vAChT) in sensory cells of the developing antenna of the desert locust S. gregaria.

Sensory cell clusters of the flagellum

On hatching (1st instar stage), the flagellum of the locust comprises 11 segments known as meristal annuli (A1–A11, see Chapman 2002) each delimited by septal-like cuticular restrictions (Fig. 1a). Large numbers of basiconic, coeloconic and campaniform sensillae cover the antennal surfaces (see Slifer et al. 1959; Chapman and Greenwood 1986; Ochieng et al. 1998 for details). Depending on the type, these sensillae are innervated by one or more dendrites originating from HRP-positive sensory cells located at the base, whose axons project via either a ventral (vT) or dorsal (dT) nerve tract to the brain (Fig. 1b).

ChAT in sensory neurons

Double-labeling experiments against neuron-specific horseradish peroxidase (α-HRP) and anti-choline acetyltransferase (α-ChAT) demonstrate the presence of ChAT in HRP-positive sensory neurons in sonicated first instar antennae (Fig. 2a, b). Morphological features such as the bulge in the neurite at the sensilla base (Fig. 2b) are consistent with those of basiconic-type sensillae (c.f. Slifer et al. 1959) and are intact following sonication (see Methods here and Ehrhardt et al. 2015). ChAT also appears to be present in the dendrite (Fig. 2a) and neurites (Fig. 2b) of these sensory units.
Confirmation that neurites contain ChAT was obtained from 3D reconstructions following double-labeling (α-HRP, α-ChAT) in sonicated antennae (Fig. 3). Confocal imaging of a preparation viewed at a level just below the cuticle (Fig. 3a, top view) reveals clusters of HRP-positive neurites in each of which ChAT-labeling is visible. 3D confocal imaging through the antenna and subsequent rotation of the optical stack to either a side (Fig. 3b) or oblique (Fig. 3c) view confirms ChAT labeling to be co-localized to neurites which run from the cuticular surface deeper into the epithelium toward their sensory somata (not in view).

The vAChT in sensory axons

The presence of the vesicular acetylcholine transporter (vAChT) in sensory axons would be consistent with cholinergic synaptic transmission in the antennal nervous system already at the first instar stage. Double-labeling (α-HRP, α-vAChT) after longitudinal sectioning at this first instar stage revealed an HRP-positive sensory cell cluster (white asterisks, Fig. 4a) from which bundled axons project within a nerve tract toward the antennal base (open arrowhead, Fig. 4a). En route these axons appear to form a plexus with a further, unidentified, HRP-positive neuron (open/white arrowhead, Fig. 4b). Examination of the vAChT channel (Fig. 4c, d) confirms the presence of the vAChT, which superposition (Fig. 4e, f) shows, is co-localized to both the sensory axons in the nerve tract (white arrowhead), their terminals in the plexus region (open/white arrowhead) and the putative target neuron (white cross).

The vAChT in the embryonic antenna

If components of a cholinergic neuromodulatory system such as ChAT (Figs. 2 and 3) and the vAChT (Fig. 4) are already present in sensory units of the antenna on hatching, then it is more than likely that these have developed
earlier, during embryogenesis. In the course of this study, we found that the signal strength using α-vAChT was consistently clearer and stronger than with α-ChAT (this may be a methodological problem and needs to be addressed in future studies). As a consequence, we reasoned that to test for, and interpret, any developmentally based changes in expression we would need the strongest signal to begin with, and this was with α-vAChT.

We therefore first double-labeled (α-HRP, α-vAChT) sectioned antennae from preparations just prior to hatching (99%. Fig. 5a, b). Large HRP-positive individual somata typical of campaniform sensillae (Fig. 5a, b; i, iii), as well as small HRP-positive sensory cell clusters (Fig. 5a, b; ii) typically associated with either a large basiconic or ceoloconic sensilla (c.f. Slifer...
et al. 1959), direct axons to the dorsal antennal nerve tract (dT, Fig. 5a). Both neuron types also express vAChT which co-localizes to the sensory somata from sensilla of all three regions examined at this developmental stage (Fig. 5b).

We then investigated younger antennae (90% of embryogenesis) for evidence of the vAChT (Fig. 5c, d). The HRP-positive ventral (vT) and dorsal (dT) antennal nerve tracts are visible in the section, along with clusters of HRP-positive sensory somata whose processes project to these tracts. Somata of putative basiconic or coeloconic sensilla selected from four regions of the antenna are vAChT-positive (Fig. 5d).

We were unable to document the vAChT in the antennal sensory system at a still earlier embryonic stage (70%).

**Discussion**

In this study, we focused on the expression of two components of cholinergic transmission in the developing antennal nervous system of the desert locust *S. gregaria*—choline acetyltransferase (ChAT) which catalyzes the synthesis of acetylcholine, and the vesicular acetylcholine transporter (vAChT) which packs the transmitter into vesicles for synaptic release. Our results cover the developmental stages from 90% of embryogenesis to the 1st postembryonic instar and are summarized schematically in Fig. 6.

**Evolutionary aspects**

Classical transmitters are present in all phyla that have been studied (see Walker and Holden-Dye 1991 for a review). With respect to the cholinergic system, Banzai et al. (2015) show that both choline acetyltransferase (ChAT) and the vesicular acetylcholine transporter...
(vAChT) are conserved across phyla. Their comparisons of ChAT-cDNA reveal about 64% sequence homology between Bombyx mori, Drosophila, and Aedes, while ChAT-cDNA from mouse has a 58% homology to that in Bombyx mori. Further, vAChT-cDNA is apparently even more highly conserved: there is a 78% sequence homology between Bombyx and Drosophila, while mouse vAChT-cDNA has a 70% homology to that in Bombyx.

In adult bushcrickets, Weber et al. (2005) used a mammalian polyclonal antibody to demonstrate that auditory sensory neurons in the forelegs express a vAChT corresponding to the molecular mass of Drosophila vAChT (c.f. Kitamoto et al. 1998), while Leitinger and Simmons (2000) also employed a mammalian polyclonal antibody to localize ChAT in neurons of the locust CNS and used a Western blot to confirm that the mammal ChAT antibody was specifically labeling a protein of the expected molecular weight in the locust. The high affinity obtained with the polyclonal antibodies against mammalian ChAT and vAChT we used for the immunolabeling experiments in this study (see Materials and methods section) are further testimony to the conserved nature of these key cholinergic molecules.

**Acetylcholine in the insect sensory nervous system**

While insect nervous systems express a wide range of neuromodulators (see Homberg 2002; Nässel 2002; Python and Stocker 2002; Deshpande et al. 2020), cholinergic transmission in particular features strongly in their chemosensory and mechanosensory subsystems (Lutz and Tyrer 1987; Rind and Simmons 1998; Leitinger and Simmons 2000; Knipper et al. 1989; Leitch et al. 1993; Hermsen et al. 1998; Python and Stocker 2002; Salvaterra and Kitamoto 2001). In the fly, for example, most chemosensory, olfactory, chordotonal and auditory primary sensory neurons are cholinergic (Salvaterra and Kitamoto 2001). In the adult locust too, central projections from the antennal nerve to the brain are also cholinergic (Knipper et al. 1989; Rind and Leitinger 2000) but the identity of the sensory sensillae involved remains unclear, and a timeline for the development of cholinergic expression in the antennal system is lacking.

**Choline acetyltransferase (ChAT)**

We show that ChAT is present in the somata of sensory cells associated with basiconic-type sensillae on the antenna of the first instar locust (Figs. 2 and 6). The dendrites and neurites from these sensillae are also labeled (Figs. 2, 3 and 6), and this has a precedent in mammalian nervous systems where dendrites from pontomesencephalic neurons of tegmental nuclei in the cat are also known to express ChAT (Jia et al 2003). Our data are also consistent with findings from the fly, where ChAT is expressed in sensory neurons associated with basiconic, trichoid and coeloconic sensillae (Yasayama and Salvaterra 1999). While ChAT is found in chemosensory and proprioceptive sensory neurons of stage-15 fly embryos, it is not present in tactile bristles of the adult (Yasayama and Salvaterra 1999) which express histamine (Buchner et al. 1993), one of several neuromodulators reported for insect (Persson and Nässel 1999; Python and Stocker 2002) and spider (Fabian-Fine et al. 2017) mechanosensory pathways.

**The vesicular acetylcholine transporter (vAChT)**

The vAChT (see Boppana et al. 2017; Deshpande et al. 2020 for reviews) not only facilitates quantal transmitter packing (Song et al. 1997), but in Drosophila also plays a role in glial differentiation (Soustelle et al. 2002), cognitive performance...
and locomotion (Showell et al. 2020). Given the homologous nature of insect nervous systems (see Meier and Reichert 1991; Meier et al. 1991; Boyan and Ball 1993; Casares and Mann 1998), we expected to find the vAChT in the developing locust antennal nervous system as well. The vAChT was present in HRP-positive axons from antennal cell clusters of the first instar locust (Figs. 4 and 6). Further, our data show that en route to the brain these sensory axons form a plexus with at least one other HRP/vAChT-positive cell (Figs. 4b, d, f and 6) which leads us to speculate that presynaptic interactions similar to those reported for the cercal nervous systems of the first instar cockroach (Blagburn and Satelle 1987) and adult locust (Boyan 1988) may also contribute to sensory processing in the locust antennal system.

Developmental aspects

The presence of the vAChT in the antennal nervous system of the first instar locust suggests that cholinergic transmission develops earlier, that is during embryogenesis. We show that the vAChT is immunohistochemically detectable in the somata of antennal sensory cells typical of campaniform, large basiconic or coeloconic antennal sensillae at 99% (Figs. 5a, and 6) and 90% (Figs. 5b, and 6) of embryogenesis. We failed to detect vAChT at still earlier stages (70%), and it is possible that the vAChT is expressed in younger embryos but remains below the detection limits of our protocols. During metamorphosis of the moth antennal system, levels of ChAT, ACh and AChE expression only increase significantly at metamorphosis as more and more neurons differentiate (Sanes and Hildebrand 1976; Sanes et al. 1977). Our data, however, are consistent with other studies in the embryonic locust which report that while receptors for neuromodulators develop quite early (Goodman and Spitzer 1980), the synthesis of the neuromodulators themselves commences considerably later in the central nervous system (Goodman et al. 1979) and follows a programmed timeline unique to each neuromodulator (Thompson and Siegler 1991). In neuronal pathways of the central complex, for example, the expression pattern of neuromodulators only reaches maturity shortly before, or at hatching from the egg (Boyan et al. 2010; Boyan and Liu 2016) as may prove to be the case for the antennal system here.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests. All experiments were performed in accordance with the guidelines for animal welfare as laid down by the Deutsche Forschungsgemeinschaft.

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