Cholinergic axons regulate type I acini in salivary glands of *Ixodes ricinus* and *Ixodes scapularis* ticks

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Regulatory factors controlling tick salivary glands (SGs) are direct upstream neural signaling pathways arising from the tick’s central nervous system. Here we investigated the cholinergic signaling pathway in the SG of two hard tick species. We reconstructed the organization of the cholinergic gene locus, and then used in situ hybridization to localize mRNA encoding choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) in specific neural cells in the *Ixodes* synganglion. Immunohistochemical staining revealed that cholinergic axonal projections exclusively reached type I acini in the SG of both *Ixodes* species. In type I acini, the rich network of cholinergic axons terminate within the basolateral infoldings of the lamellate cells. We also characterized two types (A and B) of muscarinic acetylcholine receptors (mAChRs), which were expressed in *Ixodes* SG. We pharmacologically assessed mAChR-A to monitor intracellular calcium mobilization upon receptor activation. In vivo injection of vesamicol—a VAChT blocker—at the cholinergic synapse, suppressed forced water uptake by desiccated ticks, while injection of atropine, an mAChR-A antagonist, did not show any effect on water volume uptake. This study has uncovered a novel neurotransmitter signaling pathway in *Ixodes* SG, and suggests its role in water uptake by type I acini in desiccated ticks.

The European castor-bean tick *Ixodes ricinus*, and the North American black-legged tick *Ixodes scapularis*, are the principal vectors of spirochetes causing Lyme disease¹,². These allopatic hard tick species have a life cycle spanning three years. *Ixodes* ticks take a blood meal during each of their hematophagous stages, (larva, nymph, and female), to facilitate molting into the next stage or lay eggs³. A tick’s parasitic stage—characterized by active blood uptake from a host—is often recognized as the biological hallmark, however they spend the majority of their life cycle in a non-parasitic fasting state. During both periods, their survival depends on effectively maintaining water homeostasis.

Tick salivary glands (SGs) play crucial osmoregulatory roles during both the on- and off-host stages and their activity directly reflects the biological success of ticks in the environment⁴–⁷. In female hard ticks, grape-like SG clusters are composed of three types of spherical acini (type I, II, and III hereafter). Agranular type I acini are almost exclusively located at the anterior part of the main salivary duct, while the granular type II and III acini are associated with more distally-located secondary and tertiary ducts, respectively⁸–¹⁰. Studies examining tick SG physiology have primarily focused on the on-host tick feeding period, during which the secretion activities of this tissue are the most apparent. In particular, catecholamines dopamine and norepinephrine, and cholinomimetic agent pilocarpine, have been shown to be powerful activators of tick SG secretions¹¹–¹⁵. Subsequently, recent technological developments in the post-genomic era have led to several breakthroughs in understanding

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the molecular physiology of tick SG, providing solid evidence that saliva production is under the control of autocrine/paracrine dopamine and synaptic release of neuropeptides in both granular type I and III acini. Furthermore, it has been clearly documented that these structures are highly innervated by different axons arising from the synganglion, the tick's central nervous system. Generally, there have been fewer investigations into type I acini, that unique morphology and proposed role make them far different from the functionally similar types II and III. Over the last four decades, multiple reports have primarily associated type I acini activity with the fasting period, when ticks often undergo stress due to desiccation. In dry conditions, tick secrets Na+/K+-rich hygroscopic saliva onto their mouthpart surfaces, forming a crystalized matrix. When environmental humidity increases, this salty deposit is deliquesced with water vapor and subsequently ingested. Then, ingested water is absorbed by type I acini, thus keeping the tick hydrated. Moreover, type I acini also appear to have an additional role in sodium ion uptake from the primary saliva generated by types II and III acini during tick feeding.

Mediation of tick SG fluid secretion by cholinomimetic drug pilocarpine, has been considered as a common saliva induction process in many tick species worldwide. Injection of the muscarinic acetylcholine receptor (mAChR) agonist, pilocarpine, into partially-fed hard tick females induces robust long-lasting SG secretion, whereas it fails to induce saliva from isolated SG. Subsequently, it has been suggested that the synganglion is essential for pilocarpine-mediated SG fluid secretion. To date, only indirect evidence indicate the presence of mAChR in ticks. In addition, the molecular characterization of choline acetyltransferase (ChAT), the enzyme involved in acetylcholine (ACh) synthesis, and the vesicular acetylcholine transporter (VACHT), responsible for loading ACh into secretory granules in pre-synaptic cells, remain understudied.

In the present study, we investigated the organization of cholinergic gene loci in two Ixodid tick species. Distribution of cholinergic neurons along their axonal projections reaching acini type I in SG was also examined. Two different types of mAChRs were characterized and functionally tested. Subsequently, an in vivo experiment was conducted in order to determine, whether the cholinergic axons regulate the type I SG acini activities in desiccated ticks.

Materials and methods
Experimental animals and chemicals. We confirm that all experiments were performed in accordance with relevant guidelines and regulations. Ixodes ricinus ticks were obtained from the colonies of UMR-BIPAR, Maisons-Alfort, France or the Institute of Parasitology, Biological Centre of Czech Academy of Sciences, Czech Republic. Unfed adult I. scapularis ticks were sourced from a tick-rearing facility at Oklahoma State University. Approximately 30 ticks, males or females, were each kept individually in a polypropylene tube containing a piece of filter paper (3 × 1 cm). Vials were maintained with a light–dark (12 h/12 h) cycle in a glass desiccator with >97% relative humidity at 22 °C. Ticks were fed on New Zealand rabbits and the protocol was approved by the ComEth Anses/ENVA/UPEC Ethics Committee for Animal Experimentation, (permit No. E 94 046 08).

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Molecular cloning and sequence analyses. BLAST searches of the I. scapularis genome were performed within Vectorbase and NCBI databases. This search retrieved partial sequences of the putative machr-a and -b protein sequences, JQ922421.1 and JX028235.1 respectively. To obtain the full ORFs for both receptors, the primers (Supplementary Table S1) were designed based on the I. ricinus genes.

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The D. melanogaster ChAT and VACHT protein sequences NP_996239.2; NP_477138.1, respectively, were used to query the Ixodes databases. This search retrieved partial sequences of the putative I. scapularis chat and vacht genes. I. ricinus transcript sequence of chat (in-house I. ricinus transcript project, Czech Republic, Biology Centre, České Budejovice) was used to complement the full-length exon–intron structure of the chat I. scapularis gene. Shared exon 1 of chat and vacht and mutually exclusive exons 13 and 14 of chat were experimentally confirmed by reverse transcription polymerase chain reaction (RT-PCR) using cDNA of I. scapularis or I. ricinus syntanglia. The short PCR products of chat/vacht shared exon 1 was commercially sequenced.

The PCR amplicon of chat exons 9–14, vacht exon 2 and both full length ORFs of machr-a and -b were inserted into the pGEM-T Easy vector (Promega) followed by transformation of competent DHα bacteria (prepared using the Mix & Go kit, Zymo Research). Plasmid DNA was purified using the Nucleospin Plasmid kit (Macherey–Nagel). Recombinant plasmids were commercially sequenced. The primers information see Supplementary Table S1.

The exon–intron structure of chat gene was manually analyzed using Vector NTI software (Invitrogen) and graphical images were generated using the Exon–Intron Graphic Marker version 4 (WormWeb.org). Putative translations were aligned using the Clustal program. For phylogenetics, a neighbor-joining tree was constructed using the Clustal program. For phylogenetics, a neighbor-joining tree was constructed using the Clustal program. For phylogenetics, a neighbor-joining tree was constructed using the Clustal program. For phylogenetics, a neighbor-joining tree was constructed using the Clustal program.
Whole-mount immunohistochemistry. We followed validated protocols previously described by Šimo et al.\(^{19,23,43}\), which were successfully used for tick-tissue immunohistochemistry (IHC). Briefly, Ixodes SGs and synganglia were dissected from unfed adults and fixed with Bouin’s solution for 2 h at room temperature (RT), then washed with PBS + 0.3% Triton X-100 (PBST). Tissues were incubated with monoclonal anti-mouse antibody against D. melanogaster ChAT (ChAT481, Developmental Studies Hybridoma Bank of Iowa) diluted 1:500 in PBST for 3 days at 4 °C. After washes in PBST, the specimens were incubated overnight at 4 °C with a goat anti-mouse Alexa 488 conjugated secondary antibody (Life Technologies) diluted at 1:1000. Samples were mounted in Prolong Antifade Diamond Mountant containing DAPI (Life Technologies) and analyzed by inverted confocal microscopy Zeiss LSM 700. Image adjustment was performed in Adobe Photoshop Cs6 (Adobe System Incorporate, 2012). For neuronal cells we used nomenclatures as per Šimo et al.\(^{33}\). The first two letters refer to the position of each neuron in a specific lobe of the synganglion, prothoracic (Pt.), pedal 1–4 (Pd1–4), opisthosomal (Os), preoesophageal (Pe) or postoesophageal (Po), and the letters that follow refer to the anatomical location of the neuron: dorsal (D), ventral (V), anterior (A), posterior (P), medial (M) or lateral (L). Neurons innervating acini of salivary glands were labeled SG.

In situ hybridization. We followed the validated in situ hybridization (ISH) protocol previously developed by Šimo et al.\(^{19}\) for tick synganglia. Briefly, single-stranded digoxigenin-labelled DNA probes for chat and vacht (954 and 505 bp long, respectively), were prepared using asymmetric PCR using the DIG probe synthesis kit (Roche Diagnostic, Germany). To generate antisense probes, only the reverse primer was used. As for the control sense probes (see Supplementary Fig. S1), we only used the forward primer. For the primers information see Supplementary Table S1. Synthetized probes were gel-purified and stored at −20 °C. Synganglia of unfed I. ricinus females were dissected in cold PBS and fixed with 4% paraformaldehyde for 3 h at RT. Cell membrane permeability was enhanced by treating tick synganglia with Proteinase K (New England Bio Labs). Specimens were incubated with single-stranded DNA probes for 27 h at 48 °C, followed by incubation with mouse anti-digoxigenin/AF (Alkaline phosphatase; Roche Diagnostics, Germany) overnight at 4 °C. Hybridized probes were detected following the addition of substrate/chromogen ready-to-use NBT-BCIP tablets (Roche Diagnostics, Germany). Finally, samples were incubated 5 min in 50% glycerol and subsequently mounted into 100% glycerol and observed by light microscopy (Olympus BX53). Images were assembled in Adobe Photoshop Cs6.

Transmission electron microscopy. For immunogold labeling of SG we used methodology successfully used in our recent study by Vancová et al.\(^{24}\). Concisely, SGs intended for ChAT immunolocalization were dissected from unfed I. ricinus females and fixed in a mixture containing 4% formaldehyde and 0.1% glutaraldehyde in 0.1 M HEPES for 1 h at RT. After washing in HEPES buffer with 0.02 M glycine, specimens were cryo-protected in 2.3 M sucrose for 72 h at 4 °C and frozen by plunging into liquid nitrogen. Ultrathin cryosections were cut at −100 °C, picked up with 1.15 M sucrose/1% methylcellulose solution (25 µg. Cpm). Sections were incubated for 30 min at RT in a blocking buffer (BB) composed of 1% fish skin gelatin (Sigma) and 0.05% Tween 20 and labelled for 1 h at RT with anti-mouse antibody against D. melanogaster ChAT diluted 1:30 in BB. After washing in BB, sections were incubated with goat anti-mouse IgG coupled with 5 nm gold nanoparticles (British Biocell International) diluted 1:40 in BB. After 1 h, sections were washed in HEPES, post fixed for 5 min in 2.5% glutaraldehyde, washed in 100% ethanol, then contrasted/embedded using a mixture of 2% methacrylate and 3% aqueous uranyl acetate solution (9:1).

For ultrastructural studies we slightly modified the protocol previously used by Bílý et al.\(^{44}\). SGs isolated from a partially-fed ticks (5 days, guinea pig) were frozen under high pressure (Leica EM PACT2) in the presence of 20% bovine serum albumin. Freeze substitution was performed in 2% OsO4 in 100% acetone (−90 °C, 96 h). Then the temperature was increased to −20 °C (3 °C/h) and after 24 h up to 4 °C (5 °C/h). Samples were washed three times for 15 min in 100% acetone, infiltrated, and embedded in EMBed 812 resin (EMS). Ultrathin sections were stained in ethanolic uranyl acetate for 30 min and lead citrate for 20 min. All samples were observed using a JEOL 1010 transmission electron microscope.

Functional receptor assays. The full-length ORF of mAChR-A was inserted into the expression plasmid pcDNA3/Zeo(+)(Invitrogen), mAChR-A was transiently expressed with the aequorin reporter (human cytoplasmic aequorin\(^{45}\)) in Chinese hamster ovary (CHO-K1, Sigma) cells to monitor intracellular calcium mobilization-triggered bioluminescence upon activation of the receptor.\(^{19,43}\) The assay was performed in opaque 96-well microplates (Nunc) using the Fluostar Omega microplate reader (BMG Labtech). Data obtained were analyzed in Excel (Microsoft Office) and the dose response curves, including the half maximum response values (EC\(_{50}\)) in Excel (Microsoft Office) and the dose response curves, including the half maximum response values (EC\(_{50}\)) were calculated using the GraphPad Prism 5 software package (GraphPad Software, La Jolla California USA).

Cells were simultaneously co-transfected with pcDNA3/Zeo(+)/mAChR, pcDNA3/Zeo(+)/human cytoplasmic aequorin, and pcDNA3.1(+)/wild type human G protein alpha 15 subunit (G\(_{\alpha15(16)}\)) cDNA Resource Center, Bloomsburg University of Pennsylvania) constructs. The use of chimeric G\(_{\alpha15(16)}\) subunit is advocated due to its high efficiency when linking calcium mobilization signaling pathways to transfected G\(_{\alpha15(16)}\) coupled receptors.\(^{46}\) The receptor’s activity was also assessed in the absence of the G\(_{\alpha15(16)}\) subunit. Cells were pre-equilibrated with coelenterazine h (Promega) for 3 h at RT. Various doses of agonist ligands in 50 μL were added into each well followed by injection of a 50 μL cell suspension (containing approximately 15,000 cells). Immediately after the injections, changes in luminescence were monitored for 20 s and their integrated values over time were normalized to the largest positive control response in each plate (10 μM ACh) after background substractions. For the antagonist assay, cells were pre-incubated with different doses of atropine (an mAChR-A agonist) in a 96-well microplate at RT for 5 min and subsequently treated with 10 μM ACh. Emission of luminescence (over 20 s) was.
measured immediately after injecting ACh and time integrated values were normalized to the lowest response (highest dose of atropine) in each plate after subtracting background.

Mock transfections using only the reporter and Gα₁₅(16) were used as negative controls. At least three biological replicates were performed for each assay, with two wells per sample for each given ligand dose. Conditions for handling cell lines and transfection details are provided in Simo et al. Information regarding the mAChR-B functional assay, which monitors cAMP elevations, are provided in the Supplementary Methods.

**Tissue-specific and quantitative real-time reverse transcriptase PCR (qRT-PCR).** Total RNA for tissue-specific PCR was extracted from different tick tissues such as: SGs, synganglia, Malpighian tubules, ovaries, tracheas, and intestines of partially fed (6 days) *I. ricinus* females. In addition, only the dorsal part of the cuticle and carcass (ventral cuticle with legs, muscles, and fat bodies) was also used for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription was performed using Superscript III according to the manufacturer’s protocol (Invitrogen) in presence of oligo(dT) primers, and was followed by classical PCR amplification. For qRT-PCR, the synganglia and SGs of unfed *I. ricinus* females maintained in either 98% or 25% relative humidity (RH) for 30 h were used. The dissected tissues from 10 (first replication) and 20 (second replication) individuals were pooled for RNA extraction using the RNA micro kit (Qiagen). Real-time PCR was performed in a LightCycler 480 II (Roche) using SYBR premix Ex Taq (Roche). The ribosomal protein S4 (GenBank Accession number DQ066214) was used as a reference gene. mRNA level was quantified using the ΔΔCt method, corrected by the amplification efficiency of each target gene, and expressed as a fold difference. Data were analyzed by Microsoft Excel and final graphs were prepared in GraphPad Prism 5 (GraphPad Software, La Jolla California, USA). Statistics for the qRT-PCR values were calculated using a two-tailed *t*-test for minimum of three technical and two experimental replicates.

**Tick fluid ingestion assay.** We slightly modified the methods previously used by Kim et al. Prior to the experiments, ticks were exposed to severe dehydrating conditions of 28 °C and 25% RH for 30 h. To investigate the physiological function of cholinergic axons reaching the SG type I acini, we injected dehydrated ticks with atropine, the mAChR-A antagonist or/and vesamicol, the V AChT inhibitor that reduces ACh uptake into secreting hypostomes. After injection, ticks were maintained under dehydrating conditions of 25% RH for 30 min. They were then placed upside down on double-sided sticky tape and their hypostomes were connected to a glass microcapillary tube (volume 1 μl, length 32 mm, Sigma) filled with water. The biophysiologival function of ChAT and V AChT in tick synganglion.

**Results**

**Organization of the cholinergic gene locus: ChAT and V AChT.** Homology searches were performed using BLAST algorithms from the NCBI (https://www.ncbi.nlm.nih.gov) and Vectorbase (www.vectorbase.org) databases. The search for ChAT revealed uncorrected transcripts corresponding to a putative *Ixodes* ChAT, XM_029980779.1 (*I. scapularis* putative mRNA predicted from genome sequence), ISCW022171-RA (*I. scapularis* transcript, gene set IscaW1.6), ISCI022171-RA (ISE6 cells transcript database, gene set IscaI1.0), and GBBN01014222.1 (assembled transcriptome database of *I. scapularis* female synganglia). All sequences were missing 5’ and 3’ prime ends. Furthermore, multiple discrepancies were found in their protein alignments (see Supplementary Fig. S2). The BLAST search identified a putative *Ixodes* V AChT transcript, ISCW022169-RA. A combination of computational and experimental annotation was used to identify the full-length sequence of the *I. scapularis* cholinergic gene locus (Fig. 1A). BLAST searches of both the putative ChAT and V AChT transcripts against the *I. scapularis* genome sequence confirmed their relationship to the DS910653 scaffold, except for a short ChAT exon 4 which aligned to the DS667170 scaffold (negative reading frame, Fig. 1A). To describe the exon–intron structure of the *I. scapularis* cholinergic gene locus, we employed manual annotation using *I. ricinus* ChAT and V AChT obtained from transcriptomic data (*I. ricinus* transcript project, in-house database). In the *I. scapularis* genome sequence, we identified a total of 16 and 2 exons (including a shared exon) for ChAT and V AChT, respectively (Fig. 1A,B). A shared exon (Fig. 1A) of ChAT and V AChT with a total length of 82 bp was experimentally confirmed by RT-PCR. The second exon of V AChT, encoding the ORF, lies within the first ChAT intron (Fig. 1A). Using RT-PCR, we confirmed that exons 13 and 14 of ChAT are mutually-exclusive spliced exons (one or the other, Fig. 1A, also see Supplementary Fig. S3). The ChAT sequences (Supplementary Fig. S4) were deposited into the GenBank database for *I. scapularis* transcript variant A and B (Accession numbers MT669643 and MT669646, respectively) and for *I. ricinus* transcript variant A and B (Accession numbers MT669641 and MT669642, respectively). V AChT transcripts for both *I. scapularis* and *I. ricinus* were also deposited into GenBank (Accession numbers MT669645 and MT669644, respectively).

Phylogenetic analyses of ChAT and V AChT protein sequences showed a clear evolutionary relationship with other arthropods and/or mammalian orthologues (Fig. 1C,D). The protein sequence of V AChT was predicted to contain 12 putative transmembrane domains typical of the V AChT family (Supplementary Fig. S5). *I. scapularis* and *I. ricinus* protein sequences of ChAT share 97.8% (isof orm A), 97.7% (isof orm B) and V AChT 99.1% identity.
A pair of prominent opistosomal neurons (OsSG)—described as a source of SGs innervation—in the ventral part of the opistosomal ganglion (Fig. 2B). In addition, the same pair of neurons was also recognized by the anti-vacht probe (Fig. 2C). The combination of IHC and ISH on the same synganglion specimen revealed the connections between OsSG neurons and axons entering the OsN (Fig. 2D–F, Videos 1, 2) which subsequently innervate the type I acini (see below Fig. 3). In

Figure 1. (A) Structure of the I. scapularis cholinergic gene locus (scaffold DS910653 is to scale). Horizontal lines represent introns, while vertical lines and black boxes represent exons (numbered). Splicing between exons is indicated with diagonal lines. Exon 1 is shared by both ChAT and VAcT. The second exon of VAcT (that spans its entire ORF) lies within the first intron of ChAT. Exon 4 is located in scaffold DS667170 (for more information see Supplementary Fig. S4). Exons numbered 13 and 14 are the two mutually exclusive exons. Graphical image was generated using the Exon–Intron Graphic Marker version 4 (https://wormweb.org/exonintron). (B) cDNA schematics of ChAT and VAcT indicating the proportional lengths of each exon (numbers). The black color indicates the region encoded by the shared exon. The grey color in ChAT (exons 13 and 14) indicate the two possible ChAT transcript variants (A and B) based on the mutually exclusive exons (one or the other, but not both) shown in (A). ATG and TAG indicate the positions of the initiation and stop codon respectively. (C, D) Phylogenetic relationships of ChAT (C) and VAcT (D) of I. ricinus/I. scapularis. CRAT—carnitine acetyltransferase, VMAT—vesicular monoamine transporter, VGLUT—vesicular glutamate transporter, VGAT—vesicular GABA transporter. GenBank Accession numbers are: Rattus norvegicus ChAT, XM_017600025.1; Mus musculus ChAT, NM_009891.2; Homo sapiens ChAT, AF305907.1; D. melanogaster ChAT, NM_057656.3; I. scapularis ChAT isoform A, MT669643 or I. ricinus ChAT isoform A, MT669641; D. melanogaster CRAT, NM_001300266.1; Caenorhabditis elegans CRAT, NM_064320.4; H. sapiens CRAT, NM_000755.5; R. norvegicus CRAT, NM_001004085.2; M. musculus CRAT, NM_007760.3; Tribolium castaneum VAcT, XM_970406.3; D. melanogaster VAcT, NP_477138.1; Diaphus noxia, XP_015365494.1; I. scapularis VAcT, MT669645 or I. ricinus VAcT, MT669644; Varroa jacobsoni VAcT, XM_022846413.1; Lymnaea stagnalis VMAT, AF484094.1; D. melanogaster VMAT, NM_001273010.1; Ciona intestinalis VGLUT, NM_001128885.1; Aplysia californica VGLUT, NM_001310491.1; C. intestinalis VGAT, NM_001032573.1; D. melanogaster VGAT, NM_137094.3; and C. elegans VGAT, NM_066854.6. The phylogenetic threes in C and D were constructed using neighbor-joining method in MEGA7 software, with 500 bootstrap replications.
addition, the ISH procedure followed by IHC enabled the visualization of the segmental axonal processes exiting each of the pedal lobes I–IV (Fig. 2D–F).

**Cholinergic innervation of salivary glands.** Cholinergic OsSG neurons send their axons via the opistosomal nerves (OsN; Fig. 2A,D–F), and enter the anterior part of *Ixodes* SGs. In each individual SG, the single axon runs along the main salivary duct, and its short branches exclusively reach the type I acini (Figs. 3D, 4B). Cholinergic axon terminals run close together at the basal part of the acinus, but remain further apart in the apical acinar region (Fig. 3D). Performing transmission electron microscopy (TEM) on an entire type I acinus highlighted axons containing several electron-dense neurosecretory vesicles (Fig. 3E–G). Specifically, an axon was found in close association with basolateral infoldings of a central lamellate cell (Fig. 3G). The TEM-immunogold labeling with anti-ChAT antibody confirmed this reaction within the type I acini axoplasm (Fig. 3H).

**Phylogeny and expression pattern of mACHRs.** We experimentally identified the ORFs of both A and B mACHRs. *I. scapularis* and *I. ricinus* protein sequences of both mACHR types share 100% identity. The intronless ORFs of mACHR-A and -B encode 580 and 826 amino acid residues respectively, and contain signatures
for seven transmembrane domains, typical of G protein-coupled receptors (GPCRs) (Fig. 5A,B, see also Supplementary Figs. S6, S7). Phylogenetic analyses comparing vertebrate and arthropod mAChRs suggested clear orthologous clusters of *Ixodes* mAChR-A and mAChR-B in arthropod group of the mAChRs (Fig. 5C). Tissue specific RT-PCR of partially-fed *I. ricinus* females demonstrated *machr-a* expression in the SGs, synganglia, Malpighian tubules, tracheas, ovaries, dorsa, and carcasses, but not in the intestines, whereas *machr-b* was expressed in all examined tissues (Fig. 5D).

**Ligand-receptor interactions.** CHO-K1 cells expressing aequorin and Gα15(16) demonstrated significant mAChR-A activation responses to varying doses of different ligands (Fig. 6A–D). Specifically, among several drugs tested, ACh elicited the highest response, with an EC50 value of 0.236 μM, followed by muscarine with an EC50 value of 0.643 μM. Pilocarpine led to an approximately 25-fold-lower response with an EC50 value of 6.23 μM comparing to ACh (Fig. 6A). When cells were exposed to 1 μM ligand, muscarine generated ~ 85%, pilocarpine ~ 20%, dopamine ~ 8%, octopamine ~ 15%, and epinephrine ~ 10%, of the response of ACh (Fig. 6C,D). The mAChR-A antagonist atropine abolished ACh responses in a dose-dependent manner with an IC50 value of 5.92 μM (Fig. 6E,F). In CHO-K1 cells lacking the Gα15(16) subunit, ACh and muscarine also triggered calcium mobilization upon mAChR-A activation (see Supplementary Fig. S8). CHO-K1 cells transfected with only aequorin and Gα15(16) subunit constructs did not show any responses to ACh, muscarine, or pilocarpine.

mAChR-B expression in CHO-K1 cells either with, or without Gα15(16) subunits, followed by exposure to varying doses of ACh or muscarine, did not elicit any downstream calcium mobilization signal. Similarly, mAChR-B did not inhibit forskolin-mediated cAMP activity in HEK cells as assessed by the GloSensor reporter system (Supplementary Methods).
Role of type I acini in water absorption by dehydrated ticks. To examine the role of cholinergic axon terminals in type I acini, we tested the effect of atropine (an mAChR-A antagonist) and vesamicol (an inhibitor of V AChT) in a forced water absorption assay with dehydrated ticks (Fig. 7A). Specifically, we predicted that reducing synaptic release of ACh by vesamicol, and/or antagonizing the mAChR-A in SGs by atropine, may affect the absorptive activities of type I acini during forced drinking in dehydrated ticks. Indeed, ticks pre-injected with vesamicol ingested significantly less water in the first 30 min compared to the PBS-injected group, while ticks pre-injected with either atropine or an atropine/vesamicol mixture, did not show any differences in ingested volume (Fig. 7B). When left to ingest water for a further 30 min (60 min in total), the same groups of treated ticks did not show any significant differences in water volume absorbed compared to control group (Fig. 7C).

Quantitative RT-PCR of chat, vacht, machr-a, and machr-b in ticks maintained under humid or desiccated conditions. We investigated the variation in transcript levels of chat, vacht, machr-a, and machr-b in synganglia and, machr-a and machr-b in SGs, of unfed Ixodes females when ticks were exposed to severe dehydrating conditions (Fig. 8). The mean transcripts values of chat, vacht, and machr-a in tick synganglia (Fig. 8A), and machr-a in SGs (Fig. 8B), appeared to be elevated in desiccated ticks. However, these differences were not deemed statistically significant, due to high individual variations between biological replicates. No statistically significant differences were observed in machr-b expression levels in either synganglia or SGs when comparing humid and desiccated conditions (Fig. 8A, B).
Discussion

In arthropods, the ACh neurotransmitter is understood to be the primary excitatory compound at the synapse between neurons and their target cells. The cholinergic system plays a vital role in tick physiology, although the specific processes involved in ACh synthesis, packing, and release, along with the effects of cholinomimetic ligands at specific tick body sites, remain largely obscure. Many groups have concentrated on characterizing tick acetylcholinesterases (AChE) as potent targets for organophosphate acaricides. An earlier study confirmed ChAT activity in synganglia extracts from *Rhipicephalus microplus*, however, it is only in this current study that ChAT genomic organization and the full-length ChAT sequence have been characterized.

The *Ixodes* cholinergic gene locus consists of two alternatively-spliced transcripts encoding ChAT and VAChT, in a configuration similar to that identified in other metazoans. Interestingly, among all *chat/vacht* exons, only exon 4 of the *chat* gene appears to be located on an alternative scaffold of the *I. scapularis* genomic sequence. Although we cannot exclude a possible genome assembly error, the presence of transcripts omitting or including exon 4 (two and three...
transcripts, respectively) indicates a possible trans-splicing feature that has already been shown as an important regulatory factor of mRNA processing in insects. The high amino acid identity of ChAT and VAChT protein sequences between *I. ricinus* and *I. scapularis* support their close evolutionary relationship, and suggests a common physiological role for the cholinergic system in these two allopatric hard tick species.

IHC staining of the ChAT protein revealed various types of neurons and their projections within the *Ixodes* synganglion, including prominent pairs of neurons (OsSG) identified as a source of the cholinergic innervation of *Ixodes* SGs (see below). Interestingly, ISH approaches confirmed the exclusive presence of both *chat* and *vacht* mRNA in the somata of OsSG neurons. The discrepancies in the number of neurons visualized by these two techniques can be explained by (i) nonspecific cross-reactivity of *Drosophila* anti-ChAT antibody with unknown protein(s) in some *Ixodes* neuronal cells, or (ii) undetectable *chat* and *vacht* mRNA levels in some cholinergic neurons. In either case, both IHC (for ChAT) and ISH (for *chat* and *vacht*) identified the single pair of opisthosomal neurons as the origin of type I acini innervation in *Ixodes* SGs. In addition, we learnt that performing ISH followed by IHC on synganglia dramatically enhanced immuno-detection in the neurons and their projections within this tissue, possibly as a by-product of enhancing membranes permeabilization during the first procedure. Despite the fact that the ultrastructure of tick SG has been well described, information regarding the axons innervating type I SG acini remains elusive. Based on confocal and TEM approaches, we showed that the highly abundant cholinergic axon terminals in type I acini may target basolateral infoldings of two different cell types: (i) peripheral lamellate cells that are the first cells in contact with these axons entering the acinus and/or (ii) the single central lamellate cell in contact with central and apical parts of axon terminals.

The mAChR has been suggested to play a crucial role in tick SG physiology since the cholinomimetic agent, pilocarpine, was the first pharmacological compound found to stimulate tick SG secretion in vivo. The actions of cholinomimetic drugs have been tested across several species in the ixodid family, and current models suggest that pilocarpine-mediated salivation is linked to putative mAChR activation in tick synganglion, that subsequently stimulates an unidentified secretor-motor nerve directly innervating the SG. In addition, our recent study also showed that pilocarpine induces tick chelicera movement, suggesting that this drug may have a complex effect on *Ixodes* feeding behaviour. The hypothesis that mAChR may be a cholinceptive site for salivation has also been confirmed by effectively blocking pilocarpine-mediated fluid secretion with atropine,

**Figure 6.** Bioluminescent aequorin reporter assays for mAChR-A expressed in CHO-K1 cells alongside the human Gα15(16) subunit. (A) Dose–response curves to various doses of acetylcholine (ACh), muscarine (MUS), and pilocarpine (PIL). (B) Representative 20-s typical luminescent responses to different doses of ligand (muscarine in this case). Inset in (B) shows the integrated relative luminescent values calculated from 40 intervals within the 20 s responses. (C) Luminescent responses to different drugs at 1 μM concentration. Dopamine (DA), octopamine (OCT), and epinephrine (EPI). (D) 20-s luminescent responses to 1 μM drugs. (E) Typical dose–response curve of antagonistic atropine activity. (F) Representative luminescent responses to 10 μM ACh after pre-incubation with different atropine concentrations. The bars in (A,C,E) indicate the standard error for three replicates. In (A,E) some standard error bars are smaller than the symbols used and in these cases, only the symbols are shown.
a typical mAChR-A antagonist33. Here, we identified two types of mAChR (A and B), both expressed in Ixodes synganglion and SG. The Ixodes mAChR-A thought to be linked to the Gq/11 pathway showed high biological affinity to the atropine blocker in our experiments, as has been also reported in Drosophila66. Interestingly, sensitivity to pilocarpine agonist, had approximately 25 × lower activity for this drug compared to ACh, indicating that pilocarpine is a non-potent activator of the receptor. These data also correlate with studies of mammalian mAChR-A orthologues, where pilocarpine also had low biological affinity to the receptor67,68. On the other hand, the potent endogenous mAChR-A agonist ACh, failed to effectively stimulate secretion in ixodid ticks33, likely due to an inability to permeate tick tissue barriers, whereas pilocarpine has a documented high penetration ability64. Although two heterologous systems directly monitoring either calcium or cAMP downstream signals were used, we were unable to detect activation of type B mAChR. In Drosophila, mAChR-B is thought to be linked to the Gαi/o pathway inhibiting cAMP production, and does not appear to be sensitive to atropine blockers66. In contrast, the orthologous mammalian M2 muscarinic receptor is known to be coupled to Gβ/Gγ subunits in heartbeat regulation, thus directly activating the G protein-activated inward rectifier potassium channel GIRK69. Unsuccessful functional expression studies of Ixodes mAChR-B are likely due to downstream receptor incompatibility in our expression system. Thus, taken together, more thorough investigations are required to conclude whether pilocarpine-mediated SG fluid secretion is regulated via mAChR(s) or other system(s).

Despite lacking direct experimental proof, it has been generally believed that type I acini in hard ticks are the source of hygroscopic saliva forming humid-binding crystals onto their hypostome surface70–72. Just recently, two studies proved that hygroscopic saliva is produced by type II/III acini, while ion and water absorptive functions were suggested to be exclusively due to type I acini25,32. In desiccated ticks, ingested water coming from the deliquesced hygroscopic crystals is absorbed via an electrochemical gradient created by Na+/K+-ATPase located on basolateral infoldings of lamellate cells in type I acini25,32. In this article, cholinergic axon terminals reaching the same regions of lamellate cells were described and we predicted that stimulation of putative postsynaptic mAChR, triggers Ca++-mediated activation of protein kinase C, leading to activation of transporters (i.e. Na+/K+-ATPase and possibly V-ATPase) for resorption of Na+ and water in acini type I. Similar mechanism has been described in cockroach SG73. Therefore, we designed an experiment to test whether disrupting synaptic ACh release and/or blocking postsynaptic mAChR in type I acini may affect water ingestion in severely dehydrated ticks. Although we observed substantial variation in the amount of ingested water between individual Ixodes females, significantly less volume was ingested by ticks treated with vesamicol, a drug inhibiting ACh uptake by synaptic vesicles and thus reducing its release into the synapse74. Interestingly, ticks treated with the mAChR-A antagonist, atropine, ingested approximately the same volume of water as control ticks. Similarly, no effect on ingestion volume was observed in ticks treated with the vesamicol/atropine mixture. Here, we question whether the drugs injected into the haemocoele effectively reached their cognate transmembrane proteins in SG, or if the

Figure 7. Effects of vesamicol and atropine on volume of water ingested by desiccated I. ricinus females. (A) Experimental set-up of forced water ingestion by desiccated ticks. Four different groups of Ixodes females (upper panel) were connected to glass microcapillary tubes (arrow, total volume 1 μl) via their hypostomes (lower panel). The black square shows the double-sided adhesive tape to which the ticks were attached upside-down. Inset in lower panel shows detail of the hypostome inserted into the glass microcapillary. (B) Ingested water volume by ticks pre-injected with PBS or different drugs at 30 min. (C) Ingested water volume by the same group of ticks as in (B) at 60 min. Each symbol in (B,C) indicates an individual Ixodes female. The horizontal dotted line represents the mean, and the bars the standard deviation. The experiment represents two biological replicates.
pre-incubation time was sufficient for their maximum efficacy, or if the drugs remained stable during the entire experiment. This assumption is supported by the fact that in our assay, vesamicol effects were evident within the first 30 min, which then started to slow over time. Moreover, the absence of measurable effects from atropine, and possibly from atropine/vesamicol, could be explained by the muscarinic receptor(s) affinity inhibition by Na+/K+-ATPase activity in type I acini, a mechanism that has previously been described in invertebrates. We also cannot exclude the possibility of the mAChR role as an autoreceptor, where the presynaptic mAChR(s) is under the feedback control like the cases shown in insect. Furthermore, systemic effects of injected drugs should be taken into account, since fluid ingestion by ticks involves several primary tick feeding apparatus. In addition we were interested to see if cholinergic synapses within type I acini are active during tick desiccation. Surprisingly, due to large individual variation, we didn’t observe statistically significant differences in cholinergic transcript expressions between desiccated and control ticks in either tick synganglia or SGs. In either case, our data indicate that one of the roles of cholinergic axons in *Ixodes* SG may be to regulate lamellate cell activity in type I acini during the off-host period.

Barker et al. observed that during tick feeding, the mitochondrial dissolution, lipid coalescence and depletion, as well as autophagic structure accumulation in certain type I acini cells, all suggest an important role for type I acini during the on-host period. In addition, a recent study described the important resorptive functions of Na+/K’ pump in forming isosmotic saliva in type I acini during tick feeding. Therefore, we predicted that knocking down elements of the cholinergic synapse in *Ixodes* nymphs may disrupt SGs functionality (and possibly other organs), hindering the feeding and subsequent molting. Neither knock-down of *chat*, *vacht*, *machr-a*, nor *machr-b* genes influenced tick bloodmeal uptake, feeding duration, or molting of nymphs into adults. In addition, only silencing *chat* in synganglia (64.4% knock-down expression compared to control) appeared to be significant, while other tested genes did not show any notable silencing effects after dsRNA injection (Supplementary Figs. S9, S10, Supplementary Methods). Unsuccessful attempts in gene silencing in our experiments are likely due the unknown molecular factors in specific tissue, limiting the silencing efficacy. Thus, more studies are required to understand the obstacles in inefficient or highly variable results of RNA interference in ticks research.

In the current study we investigated the cholinergic pathways in *Ixodes* SG. Our results suggest, that activities of cholinergic synapse in type I acini may play a role in water absorption by desiccated ticks. Although this finding does not replicate previous reports suggesting indirect cholinergic control of secretion activities of tick SGs via the synganglion, it can’t be excluded. Currently, the only candidates that could directly connect

Figure 8. Quantitative RT-PCR showing the transcript levels of *chat*, *vacht*, *machr-a*, and *machr-b* in *I. ricinus* ticks maintained in 98% or 25% relative humidity (RH) for 30 h. (A) Transcript levels of *chat*, *vacht*, *machr-a* and *machr-b* in synganglia. (B) Transcript levels of *machr-a* and *machr-b* in SGs. Error bars indicate the standard error for two biological replicates. Data were normalized using the ribosomal protein S4 transcript, and expression levels of specific transcripts from ticks maintained in 98% RH were assigned a value of 1. Note that comparing the mean to 1 for each transcript using a one-way Student t-test (P ≤ 0.05) did not show any statistically significant differences.
the synganglion with saliva-producing type II and III acini are the neupeptidergic axons, while the origin of InvD1L-expressing axons innervating the same acini types remains to be identified. Whether some of these neurons express cholinergic receptor(s) sensitive to pilocarpine, and thus indirectly activate SG secretion remains poorly understood. Our ongoing research (a current project in Simo’s laboratory) will place additional focus on these aspects. These studies would further establish the localization of the expressed mAChRs proteins in tick synganglion, SG, and possibly other organs, as the mRNA encoding these receptors appears to be present throughout several tick tissues.

The newly revealed innervation of type I acini in our study has filled a missing knowledge gap, and is important in understanding the complex nature of neural mechanisms regulating SGs in Ixodes ticks.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Received: 11 February 2020; Accepted: 10 September 2020
Published online: 29 September 2020

References

1. Hamer, S. A., Tsao, J. I., Walker, E. D. & Hickling, G. J. Invasion of the lyme disease vector Ixodes scapularis: Implications for Borrelia burgdorferi endemcity. EcoHealth 7, 47–63 (2010).
2. Rizzoli, A. et al. Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: New hazards and relevance for public health. Front. Public Health https://doi.org/10.3389/fpubh.2014.00251 (2014).
3. Kocan, K. M., de la Fuente, J. & Coburn, L. A. Insights into the development of Ixodes scapularis: A resource for research on a medically important tick species. Parasit. Vectors https://doi.org/10.1186/s13071-015-1185-7 (2015).
4. Bonnet, S. I. Chapter 4—Arthropod saliva and its role in pathogen transmission: Insect saliva. In Skin and Arthropod Vectors (ed. Boulanger, N.) 83–119 (Academic Press, Cambridge, 2018).
5. Šimo, L., Kazimirova, M., Richardson, J. & Bonnet, S. I. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. Front. Cell. Infect. Microbiol. https://doi.org/10.3389/fcimb.2017.00281 (2017).
6. Sauer, J. R., McSwain, J. L., Bowman, A. S. & Eisenberg, R. C. Tick salivary gland physiology. Annu. Rev. Entomol. 40, 245–267 (1995).
7. Kim, D., Šimo, L., Vancová, M., Urban, J. & Park, Y. Neural and endocrine regulation of osmoregulatory organs in tick: Recent discoveries and implications. Gen. Comp. Endocrinol. 278, 42–49 (2019).
8. Bowman, A. S. & Sauer, J. R. Tick salivary glands: Function, physiology and future. Parasitology 129(Suppl), S67–81 (2004).
9. Binnington, K. C. Sequential changes in salivary gland structure during attachment and feeding of the cattle tick, Boophilus microplus. Int. J. Parasitol. 8, 97–115 (1978).
10. Coons, L. B. & Roshdy, M. A. Fine structure of the salivary glands of unfed male Dermacentor variabilis (Say) (Ixodoidea: Ixodidae). J. Parasitol. 59, 900–912 (1973).
11. Kim, D., Šimo, L. & Park, Y. Orchestration of salivary secretion mediated by two different dopamine receptors in the blacklegged tick Ixodes scapularis. J. Exp. Biol. 217, 3656–3663 (2014).
12. Kaufman, W. The influence of various factors on fluid secretion by in vitro salivary glands of ixodid ticks. J. Exp. Biol. 64, 727–742 (1976).
13. Kaufman, W. R. The influence of adrenergic agonists and their antagonists on isolated salivary glands of ixodid ticks. Eur. J. Pharmacol. 45, 61–68 (1977).
14. Kaufman, W. R. & Harris, R. A. Neural pathways mediating salivary fluid secretion in the ixodid tick Amblyomma hebraeum. Can. J. Zool. 61, 1976–1980 (1983).
15. Lindsay, P. J. & Kaufman, W. R. Potentiation of salivary fluid secretion in ixodid ticks: A new receptor system for gamma-aminobutyric acid. Can. J. Physiol. Pharmacol. 64, 1119–1126 (1986).
16. Koči, J., Šimo, L. & Park, Y. Autocrine/paracrine dopamine in the salivary glands of the black-legged tick Ixodes scapularis. J. Insect Physiol. 62, 39–45 (2014).
17. Šimo, L., Daniel, S. E., Park, Y. & Žitňan, D. The nervous and sensory systems: Structure, function, proteomics and genomics. In Biology of Ticks (eds Sonenshine, D. E. & Roe, R. M.) 309–367 (Oxford University Press, Oxford, 2014).
18. Koči, J., Koči, J., Žitňan, D. & Park, Y. Evidence for D1 dopamine receptor activation by a paracrine signal of dopamine in tick salivary glands. PLoS ONE 6, e16158 (2011).
19. Šimo, L., Žitňan, D. & Park, Y. Two novel neuropeptides in innervation of the salivary glands of the black-legged tick, Ixodes scapularis: Myoinhibitory peptide and SiFamide. J. Comp. Neurol. 517, 551–563 (2009).
20. Šimo, L., Žitňan, D. & Park, Y. Neural control of salivary glands in ixodid ticks. J. Insect Physiol. 58, 459–466 (2012).
21. Kim, D., Šimo, L. & Park, Y. Molecular characterization of neuropeptide eleven and two elevenin receptors, IsElevR1 and IsElevR2, from the blacklegged tick, Ixodes scapularis. Insect Biochem. Mol. Biol. 101, 66–75 (2018).
22. Šimo, L., Koči, J., Kim, D. & Park, Y. Invertebrate specific D1-like dopamine receptor in control of salivary glands in the blacklegged tick Ixodes scapularis. J. Insect Physiol. 522, 2038–2052 (2014).
23. Šimo, L., Slovák, M., Park, Y. & Žitňan, D. Identification of a complex peptidergic neuroendocrine network in the hard tick, Rhipicephalus appendiculatus. Cell Tissue Res. 335, 639–653 (2008).
24. Vancová, M. et al. Ultrastructural mapping of salivary gland innervation in the tick Ixodes ricinus. Sci. Rep. 9, 1–13 (2019).
25. Kim, D., Maldonado-Ruíz, P., Zurek, L. & Park, Y. Water absorption through salivary gland type I acini in the blacklegged tick, Ixodes scapularis. PeerJ 5, e3984 (2017).
26. Needham, G. R., Rosell, R., Greenwald, L. & Coons, L. B. Ultrastructure of type-I salivary-gland acini in four species of ticks and the influence of hydration states on the type-I acini of Amblyomma americanum. Exp. Appl. Acarol. 10, 83–104 (1990).
27. Needham, G. R. & Teel, P. D. Water balance by ticks between bloodmeals. In Morphology, physiology, and behavioral biology of ticks/editors, John R. Sauer and J. Alexander Hair (1986).
28. McMullen, H. L., Sauer, J. R. & Burton, R. L. Possible role in uptake of water vapour by ixodid tick salivary glands. J. Insect Physiol. 22, 1281–1285 (1976).
29. Knulle, W. & Rudolph, D. Humidity Relationships and Water Balance of Ticks (Pergamon Press, Oxford, 1982).
30. Gaede, K. & Knülle, W. On the mechanism of water vapour sorption from unsaturated atmospheres by ticks. J. Exp. Biol. 200, 1491–1498 (1997).
31. Yoder, J. A., Benoit, J. B., Rellinger, E. J. & Tank, J. L. Developmental profiles in tick water balance with a focus on the new Rocky Mountain spotted fever vector, Rhipicephalus sanguineus. Med. Vet. Entomol. 20, 365–372 (2006).
77. Barker, D. M., Ownby, C. L., Krolak, J. M., Claypool, P. L. & Sauer, J. R. The Effects of attachment, feeding, and mating on the morphology of the type I alveolus of salivary glands of the lone star tick, *Amblyomma americanum* (L.). *J. Parasitol.* **70**, 99–113 (1984).

78. Sauer, J. R., Eisenberg, R. C. & Bowman, A. C. Salivary glands in ixodid ticks: Control and mechanism of secretion. *J. Insect Physiol.* **46**, 1069–1078 (2000).

**Acknowledgements**

L. Mateos-Hernández was supported by DIM One Health—Région Île-de-France (Acronym of the project: NeuroPaTick); B. Delafay was supported by the UMR-BIPAR; M. Vancová was supported by MEYS CR (Czech BiolImaging LM2015062); O. Hajdusek and R. Sima were supported by the Centre for Research of Pathogenicity and Virulence of Parasites (no. CZ.02.1.01/0.0/0.0/16_019/0000759), funded by the European Regional Development Fund (ERDF) and the Ministry of Education, Youth, and Sport, Czech Republic (MEYS); H. Attoui was supported by Horizon 2020 PALE-Blu project number 772739-2 and the Institut National de la Recherche pour l'agriculture, l'alimentation et l'environnement (INRAE); L. Šimon was supported by the DIM One Health—Région Île-de-France (Acronym of the project: NeuroPaTick), Projets de Recherche du Conseil Scientifique de l'Ecole Nationale Vétérinaire d'Alfort—Année 2017 and INRAE.

**Author contributions**

L.Š. coordinated the project, prepared the final figures, and wrote the main manuscript. L.Š., L.M.H., B.D., M.V., O.H. and R.S. performed the experiments. Y.P. and H.A. provided the resources. All co-authors reviewed the manuscript and contributed with minor additions and corrections.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-73077-1.

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