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Role of neuronal and non-neuronal acetylcholine signaling in *Drosophila* humoral immunity

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ABSTRACT

Acetylcholine (ACh) is one the major neurotransmitters in insects, whose role in mediating synaptic interactions between neurons in the central nervous system is well characterized. It also plays largely unexplored regulatory functions in non-neuronal tissues. Here we demonstrate that ACh signaling is involved in the modulation of the innate immune response of *Drosophila melanogaster*. Knockdown of ACh synthesis or ACh vesicular transport in neurons reduced the activation of *drosomycin* (*drs*), a gene encoding an antimicrobial peptide, in adult flies infected with a Gram-positive bacterium. *drs* transcription was similarly affected in *Drosophila* α7 nicotinic acetylcholine receptor, nAChRalpha7 (Dα7) mutants, as well as in flies expressing in the nervous system a dominant negative form ($D\alpha7^{DN}$) of this specific receptor subunit. Interestingly, $D\alpha7^{DN}$ elicited a comparable response when it was expressed in non-neuronal tissues and even when it was specifically produced in the hemocytes. Consistently, full activation of the *drs* gene required Dα7 expression in these cells. Moreover, knockdown of ACh synthesis in non-neuronal cells affected *drs* expression. Overall, these findings uncover neural and non-neural cholinergic signals that modulate insect immune defenses and shed light on the role of hemocytes in the regulation of the humoral immune response.

1. Introduction

Acetylcholine (ACh) is a chemical messenger playing important roles in various aspects of animal cell biology and physiological homeostasis. Acting as a neurotransmitter, it mediates communication at neuronal synapses and neuro-muscular junctions, but additional roles in the modulation of multiple physiological activities have been reported, including immunity.

In humans, action potentials traveling in the vagus nerve to the spleen eventually lead to the localized release of acetylcholine, which effectively controls cytokine production by immune cells, thus setting the magnitude of the innate immune response (Pavlov et al., 2018; Tarnawski and Olofsson, 2021). This cholinergic pathway represents the efferent arc of the inflammatory reflex, a neural circuit initiated by sensory signals that are triggered upon pathogen/tissue injury detection and propagated, along the afferent arc, from the periphery to the central nervous system (Pavlov et al., 2018). Thus, the inflammatory reflex relays information on the alterations of the peripheral immune homeostasis to the central nervous system and sends back to relevant tissues/organs appropriate neuro-immunomodulatory signals (Chiu et al., 2012, 2013; Talbot et al., 2016).

The tight interaction between the nervous and immune systems is an ancient regulatory pathway of key-importance for animal survival, as supported by the neural control of immunity observed in one of the simplest organisms with a nervous system, the nematode *Caenorhabditis elegans* (Liu and Sun, 2021).

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In insects, these neuroimmune interactions are very poorly studied, but several lines of evidence suggested the occurrence of a crosstalk between the nervous and immune systems in this arthropod class (Makhijani et al., 2017; Kraus et al., 2021). In particular, the finding that the neonicotinoid clothianidin, which is an agonist of nicotinic acetylcholine receptors (Jeschke et al., 2011; Simon-Delso et al., 2015), has a negative impact on NF-kB signaling in *Drosophila melanogaster* and *Apis mellifera* and impairs the immune response (Di Prisco et al., 2013; Annoscia et al., 2020), suggests that ACh may play an important role in insect immune regulation.

Drosophila has been extensively utilized as a powerful genetic model to unravel the mechanisms underlying the innate immunity in insects (Lemaitre and Hoffmann, 2007). The fruit fly relies on humoral and cell-mediated reactions for its defense against pathogens. In particular, the production of antimicrobial peptides (AMPs) in response to infection, either locally by surface epithelia or systemically by the fat body, plays a very important role (Imler and Bulet, 2005). NF-kB signaling regulates the production of AMPs at transcriptional level, through the Toll pathway, which mainly reacts to Gram-positive bacteria and fungi, and the Immunodeficiency (Imd) pathway, predominantly responding to Gram-negative bacteria (Lemaitre and Hoffmann, 2007). Therefore, AMPs can be used as simple proxy for assessing the impact that any genetic or chemical manipulation can have on the fly immune pathways.

Here, we investigated the occurrence of neuroimmune communication in *Drosophila*, by focusing on ACh signaling. This choice was based both on the central role played by this neurotransmitter in the neural control of the immune system in humans (Andersson and Tracey, 2012; Pavlov et al., 2018) and the immunomodulatory effects reported for neonicotinoids, neurotoxic insecticides targeting the nicotinic acetylcholine receptors (Di Prisco et al., 2013; Annoscia et al., 2020). Moreover, ACh appears to confer protection to lepidopteran larvae against fungal infection (Rajendran et al., 2015). However, the putative role of ACh signaling on insect immunity remains largely unexplored. Here we contribute to fill this research gap.

To ascertain a role for ACh neurotransmission in humoral immunity we analyzed the expression of the AMP Drosomycin (*drs*) upon immune challenge, in genetic conditions of impaired ACh signaling in the nervous system. In these conditions, *drs* expression was reduced and this result was reproduced by altering the nicotinic acetylcholine receptor Dα7 in neuronal cells. Furthermore, we showed that the Dα7 function in the hemocytes and the ACh from non-neuronal cells positively regulate *drs* expression. Taken together, these data uncover a composite scenario where ACh signaling in neuronal and non-neuronal cells is involved in the modulation of *Drosophila* humoral immunity.

2. Material and methods

2.1. Drosophila stocks

Drosophila stocks were maintained on standard cornmeal/yeast medium under 12:12 h light/dark cycle at 25 °C. The following stocks were obtained from Bloomington Drosophila Stock Center: *ChATGAL4* (#6793), *elavGAL4* (#8760), *Dα7*^{PΔEY6} (#24879), *Dα7*^{1} (#24880), *ChAT-RNAi* (#25856), *Dα7-RNAi* (#27251), *VAChT-RNAi* (#27684), *UAS-mCherry* (#35787), *attP2* control line for TRiP RNAi lines (#36303), *w*^{1118} (#5905), *srpHemoGAL4,UAS-GFP* (#78565), *CgGal4* (#7011), *r4GAL4* (#33832). *Dα7*^{Y195T} (*Dα7*^{DN}) and *elavGal80* were kind gifts from Tanja A. Godenschwege and Sergio Casas Tintó, respectively. *Oregon R* and *w*^{1118} flies were used as controls for the *Dα7*^{PΔEY6} and *Dα7*^{1} original strains. The controls for the different transgenes expression were obtained by crossing the specific GAL4 drivers with the strains in which the UAS construct insertions were generated. For *Dα7*^{Y195T} we used the *w*^{1118} strain, while for the different RNAi lines we used the *attP2* strain. Flies of the different genetic contexts analyzed, in which ACh signaling or *Dα7* function were impaired, had a normal developmental time as compared to controls.

2.2. Immune challenge of adult flies

A synchronized cohort of freshly eclosed adult flies was collected in a day, put on new standard cornmeal/yeast medium, kept at 25 °C for 1 more day and transferred at 29 °C for 2 days. The Gram-positive *Micrococcus luteus* bacteria (kind gift from Bruno Lemaitre) was pre-cultured in LB medium. The log phase of bacterial culture was spun down and the resulting pellet resuspended in LB to achieve $OD_{600} = 500$ (BioPhotometer®, Eppendorf AG, Hamburg, Germany). Sharpened needles dipped into this suspension were used to pierce the lateral side of the thorax of a pre-anaesthetized adult fly. Infected adult females were incubated at 29 °C and sampled for analysis at different time points after inoculation.

2.3. Quantitative RT-PCR

Total RNA was isolated from 5 adult females as previously described (Ignesti et al., 2018). RNA was quantified and quality checked with the BioPhotometer® (Eppendorf). HiScript III® RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme Biotech, Nanjing, China) was used according to the manufacturer's protocol to reverse transcribe 350 ng of total RNA in a MJ Research PTC-100 Programmable Thermal Controller (Bio-Rad, Hercules, CA, USA). Gene expression analysis was performed by qRT-PCR using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), according to the manufacturer's protocol in the CFX Connect Real-Time PCR Detection System (Bio-Rad) through the Bio-Rad Manager™ Software Version 3.1. To determine target specificity, dissociation curve analysis was performed. Relative mRNA expression levels were normalized to that of *Rpl32*. In all experiments, the calibrator is given by a biological replicate of the Not Challenged (NC) control. Four biological replicates were used for each experiment.

Primers used for qPCR are listed in Table 1.

2.4. Immunostaining of adult hemocytes

Female flies (*srpHemo* > *GFP*), 3–4 days old, were anaesthetized, and their wings removed. The hemocytes were isolated by fly bleeding (Ghosh et al., 2018). With a needle, a fine incision was made on the lateral side of the thorax, without disturbing the abdomen. The injured region of the thorax was carefully positioned in a 10 μl drop of PBS pH 7.5 (phosphate-buffered saline) on a Polysine™ coated microscope slide (VWR International S.r.l, Radnor, Pennsylvania, USA), in order to collect the hemolymph along with blood cells (12 flies per slide). The hemocytes were allowed to adhere to the glass surface for 20 min inside a moist chamber and then fixed in 3.7% formaldehyde in PBS for 20 min. Incubations and washings were done at room temperature, unless otherwise mentioned. After 3 washes in PBS, hemocytes were incubated with blocking solution (10% Normal Goat Serum (NGS), 0.1% Triton X100 in PBS) for 30 min. Cells were incubated at 4 °C for 24 h with primary antibodies, diluted in 0.1% Triton X100 in PBS supplemented with 10% NGS. After three 5 min washes in 0.1% Triton X100 in PBS, the hemocytes were incubated with fluorescence-tagged secondary

Table 1
Primer sequences used in this study.

Name	Sequence 5'→ 3'
RpL32_FW	GACGCTTCAAGGGACAGTATCTG
RpL32_RV	AAACGCGGTTCTGCATGAG
Drs_FW	CGTGAGAACCTTTTCCAATATGATG
Drs_RV	TCCCAGGACCACCAGCAT
VAChT_FW	CCACGGGCATCCTATTCGC
VAChT_RV	ATCATGGGCAAATCGTAGCCG
ChAT_FW	GCAACATCACACCGAGCGATA
ChAT_RV	CCTTGGGTAGAGTGTCAGGGA
nAChRalpha7_FW	ACGCCCTTCTGGACAACTAC
nAChRalpha7_RV	TGAGTGTTAGTCCGAAGCTCAG

antibodies and 1 μg/ml solution of DAPI (Sigma-Merck, Darmstadt, Germany), diluted in 0.1% Triton X100 in PBS supplemented with 10% NGS, for 30 min at room temperature. After four 5 min washes in 0.1% Triton X100 in PBS, the cells were mounted in Fluoromount G™ (Electron Microscopy Sciences, Hatfield, PA, USA). Hemocytes were analyzed with conventional epifluorescence on a Nikon Eclipse 90i microscope using a 60x immersion-oil objective (Nikon, Japan). Digital images were assembled using Adobe Photoshop (Adobe, San Jose, CA, USA). No biased image manipulations were applied. The following antibodies and dilutions were used: rat anti-Dα7 (1:1000) (kind gift from Hugo J. Bellen); CY3-anti-rat (1:600, Jackson, USA).

For antibody quantification the fluorescence intensities were measured using the Image J software. Cells were outlined to create selections and the Integrated Density (ID) and the area were measured. A region outside of the cell was selected to measure the mean grey value for background normalization. The following equation was used to calculate the Corrected Total Cell Fluorescence (CTCF): CTCF = ID – (Area of selected cell x mean fluorescence of background readings). CTCFs have been estimated for statistical significance using a one-tailed unpaired Student's t-test. The results are reported in the graph as means with standard deviation.

2.5. Statistical analysis

GraphPad Prism version 8 (GraphPad software; San Diego, California, USA) was used for statistical analyses of qRT-PCR results. All results are expressed as mean ± standard deviation (SD). Normality of data was checked by using the Shapiro–Wilk test while homoscedasticity was checked using the F test. Statistical analyses of gene expression results between two genotypes at the same time point were carried out using a two-tailed unpaired *t*-test. Where multiple genotypes were compared, a one-way ANOVA was performed, applying a Tukey post hoc test.

3. Results

3.1. Acetylcholine positively regulates the activation of drosomycin upon septic injury

To investigate whether ACh neurotransmission plays a role in the systemic innate immune response of adult flies, we knocked down by RNAi the *VAChT* gene, which controls loading of ACh into synaptic vesicles (Kitamoto et al., 1998). We expressed the *VAChT-RNAi* in the nervous system using the *elav-GAL4* driver (*elav > VAChT-RNAi*). The efficiency of the *VAChT-RNAi* was checked by RT-qPCR, which showed that *VAChT* transcript level was strongly reduced (Fig. S1A) ($n = 4$; $t = 13.79$; $p < 0.001$). Fly females were challenged with Gram-positive *Micrococcus luteus* bacteria. We performed a time-course analysis, across a 24h time interval, of the *drs* transcription level, as a readout of the immune response against bacterial infection. Knockdown of *VAChT* in the nervous system caused a statistically significant decrease of *drs* transcript level, at 6h and 24h after infection ($n = 4$; $t = 3.610$; $p = 0.015$ and $n = 4$; $t = 4.090$; $p = 0.006$, respectively), as compared to the control (Fig. 1A). This indicates that ACh signaling is required for proper *drs* expression as early as 6h after immune challenge. Next, we analyzed, at 6h post-infection, *drs* expression upon RNAi-mediated silencing of the *choline acetyltransferase* gene (*ChAT*), which is involved in the biosynthesis of ACh (Kitamoto et al., 1998). We expressed a *ChAT-RNAi* transgene in cholinergic neurons using a *ChAT-GAL4* driver (*ChAT > ChAT-RNAi*). The efficiency of the *ChAT-RNAi* was checked by RT-qPCR (Fig. S1B) ($n = 4$; $t = 6.803$; $p = 0.001$). As compared to the control,

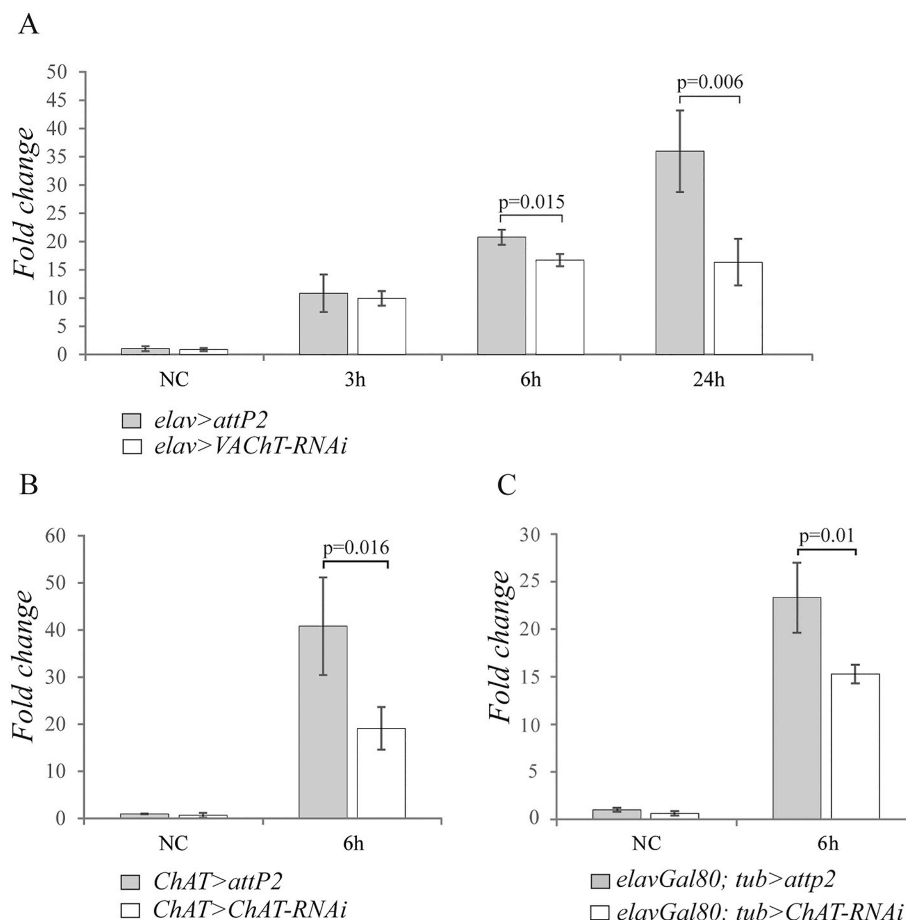

Fig. 1. Effect of neuronal silencing of ACh neurotransmission on *drs* gene expression.
qRT-PCR analysis of *drs* transcript levels at different time points after *Micrococcus luteus* infection of adult flies in which RNAi-mediated knockdown of *VAChT* was induced in the nervous system (*A*) and RNAi-mediated knockdown of *ChAT* was induced in cholinergic neurons (*B*) and in non-neural cells (C) compared to their controls. NC, not challenged. Transcript levels were normalized to *Rpl32*. Data were normalized to the NC control and represent the mean ± SD of 4 biological replicates, each consisting of a pool of 5 flies. Statistical significance for each time point was assessed by two-tailed unpaired Student's t-test and the relative p value reported for significant differences.

ChAT > *ChAT-RNAi* flies showed a significantly reduced level of *drs* expression at 6h post-infection (n = 4; t = 3.327; p = 0.016) (Fig. 1B). This finding further supports a positive effect of ACh on *drs* expression.

Since in mammals ACh is also produced by non-neuronal cells (Zoli et al., 2018b), we hypothesized that this might similarly occur in *Drosophila*. Therefore, we analyzed the potential role of non-neuronal ACh in immunity, by silencing the ChAT gene only in non-neural cells. We induced *ChAT-RNAi* using the ubiquitous *tub-GAL4* driver (*tub* > *ChAT-RNAi*) in flies expressing the GAL4 inhibitor Gal80 in neuronal cells (*elav-Gal80*). In *elavGal80*; *tub* > *ChAT-RNAi* flies, *ChAT* knock down in non-neuronal cells caused a significant reduction in *drs* transcript level (n = 4; t = 3.689; p = 0.0103) (Fig. 1C). Overall, these data indicate that both neural and non-neural cholinergic signals are involved in fly immune regulation.

3.2. The nicotinic acetylcholine receptor α7 is involved in the immune response

Next, we wanted to investigate whether the nicotinic acetylcholine receptors (nAChRs) are involved in the acetylcholine circuit controlling immunity. *D. melanogaster* has ten nAChR subunits (Dα1-Dα7 and Dβ1-Dβ3) and a similar number of subunits has been identified in other insect species (Jones et al., 2007). It has been demonstrated that in humans the α7 nicotinic acetylcholine receptor (nAChRα7) plays a role in maintaining the immunological homeostasis (Andersson and Tracey, 2012). In *D. melanogaster*, the α7 subunit (Dα7) has a high sequence homology with the vertebrate α7 subunit (Grauso et al., 2002; Lansdell and Millar, 2004; Fayyazuddin et al., 2006) and can similarly form homopentameric receptors (Lansdell et al., 2012). We analyzed *drs* expression 3 and 6h after *M. luteus* septic injury in *Drosophila* females homozygous either for a null mutation of the *Dα7* gene (*Dα7*^{PΔEY6}) carrying a deletion of part of the ORF or for a dominant negative allele of the *Dα7* gene (*Dα7*^{1}) (Fayyazuddin et al., 2006), as compared to *Oregon R* and *w*^{1118} females used as controls. A significant effect of the genotype on *drs* expression at 6h after *M. luteus* infection was observed (n = 4; F = 8.776; p = 0.003) (Fig. 2A), and a significant reduction in *drs* expression between the control and mutant flies was observed (Oregon R *vs Dα7*^{PΔEY6} p = 0.030; Oregon R *vs Dα7*^{1} p = 0.010; *w*^{1118} *vs Dα7*^{PΔEY6} p = 0.032; *w*^{1118} *vs Dα7*^{1} p = 0.012). In addition, we generated transheterozygous flies that contained one copy of the *Dα7*^{1} allele and one copy of the wild type *Dα7*$^{+}$ allele derived from the *w*^{1118} control stock. At 6h post-immune challenge, *Dα7*^{1}/*Dα7*$^{+}$ flies had reduced *drs* expression as compared to control flies, thus showing a response similar to homozygous *Dα7*^{1} flies (n = 4; t = 5.696; p = 0.001) (Fig. 2B). This confirms the dominant-negative character of this mutation (Fayyazuddin et al., 2006), and the relevance of the Dα7 nAChR subunit on *drs* activation.

3.3. Dα7 is required in neuronal and non-neuronal cells for proper immune function

In humans the expression of nAChRs has been reported in a variety of non-neuronal cells, including immune cells (Zoli et al., 2018b). To investigate whether neuronal and/or non-neuronal Dα7 function is involved in the modulation of *drs* expression, we took advantage of a transgenic line expressing under *UAS* control a Dα7 protein with the Y195T substitution in the ligand-binding domain (Dα7^{DN}). The expression of this Dα7^{DN} mutant protein causes inhibition of cholinergic responses (Mejia et al., 2013). We expressed this protein in the nervous

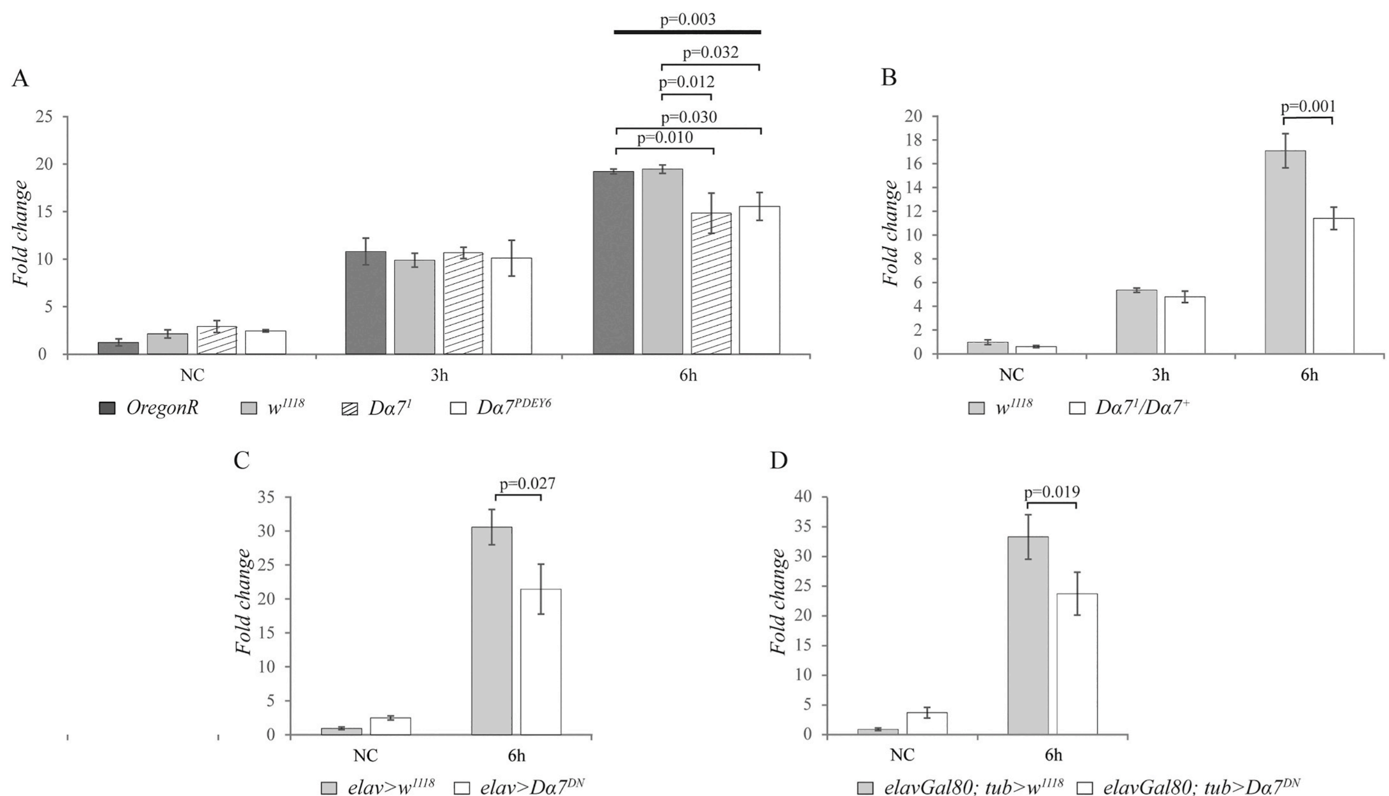

Fig. 2. The nicotinic acetylcholine receptor Dα7 is required for proper *drs* gene expression.
qRT-PCR showing relative expression profiles of *drs* in adult flies (*A*) homozygous for a null mutation of the *Dα7* gene (*Dα7*^{PΔEY6}) or homozygous for a dominant negative allele of the *Dα7* gene (*Dα7*^{1}) and (*B*) heterozygous for the *Dα7*^{1} mutant allele (*Dα7*^{1}/*Dα7*$^{+}$) compared to their controls at 3 and 6h after infection. qRT-PCR analyses in adult flies expressing *Dα7*^{DN} in the nervous system (*C*) and in non-neural domains (*D*). NC, not challenged. Transcript levels were normalized to *Rpl32*. Data were normalized to the NC control and represent the mean ± SD of 4 biological replicates, each consisting of a pool of 5 flies. Statistical significance for each time point was assessed by two-tailed unpaired Student's t-test. Where multiple genotypes were compared (*A*), a one-way ANOVA was performed (bold line), applying a Tukey post-hoc test. The p value is reported for statistically significant differences.

system using the *elav-GAL4* driver (*elav* > *Dα7*^{DN}), and, as shown in Fig. 2C, at 6h after immune challenge *drs* expression level was significantly reduced (n = 4; t = 3.098; p = 0.027). Next, we induced the Dα7^{DN} mutant protein only in non-neuronal cells, by using the system *elavGal80*; *tub-GAL4*. In *elavGal80*; *tub* > *Dα7*^{DN} flies the expression of Dα7^{DN} protein in non-neuronal cells caused a significant reduction in *drs* transcript level (n = 4; t = 3.180; p = 0.019) (Fig. 2D). These results demonstrate a functional role of Dα7 both in neuronal and non-neuronal cells.

3.4. Expression of Dα7^{DN} in the hemocytes affects drosomycin expression

We investigated whether the expression of *Dα7*^{DN} in hemocytes and fat body can affect *drs* expression. We used the *collagen-GAL4* (*Cg*>) driver, which drives expression of GAL4 in hemocytes and fat body (Asha et al., 2003). In *Cg* > *Dα7*^{DN} flies *drs* expression was significantly reduced (n = 4; t = 3.022; p = 0.023) (Fig. 3A). To further support this finding, we analyzed the effect of knocking down the *Dα7* gene in fat body and hemocytes by expressing a *Dα7-RNAi* transgene (*Cg* > *Dα7-RNAi*). We analyzed the RNAi strength of this transgene (Fig. S1C) (n = 4; t = 4.160; p = 0.009) in flies expressing the *Dα7-RNAi* by the enhancer trap driver *Dα7-GAL4* (*Dα7*>) that expresses GAL4 in the pattern of the *Dα7* gene (Fayyazuddin et al., 2006). *Cg* > *Dα7-RNAi* flies showed a similar reduction of *drs* expression (n = 4; t = 3.655; p = 0.015) (Fig. 3B), as detected in *Cg* > *Dα7*^{DN} flies (Fig. 3A), confirming the immune function played by Dα7 in the *Cg-GAL4* territories.

To find whether the fat body and/or the hemocytes were involved in the ACh > Dα7 signaling activating *drs* expression we selectively induced, in these tissues, the expression of *Dα7*^{DN} or *Dα7-RNAi* transgenes. In the fat body, their expression was driven by the *r4-GAL4* driver (*r4*>) (Lee and Park, 2004), while the *srpHemo-GAL4* driver (*srpHemo* > *GFP*) (Brückner et al., 2004) was used to express these transgenes in the hemocytes. As shown in Fig. 4A and B, the expression of either *Dα7*^{DN} (r4> *Dα7*^{DN}) or *Dα7-RNAi* (*r4*> *Dα7-RNAi*) in the fat body did not affect *drs* expression (n = 4; t = 1.262; p = 0.25 and n = 4; t = 1.051; p = 0.33, respectively). Conversely, the expression of these transgenes in the hemocytes (*srpHemo* > *Dα7*^{DN} and *srpHemo Dα7-RNAi*) significantly reduced *drs* expression as compared to control flies (n = 4; t = 4.576; p = 0.010 and n = 4; t = 5.072; p = 0.002, respectively) (Fig. 4C and D). These results indicate that Dα7 is active in the hemocytes and plays in these cells a functional role in the humoral immune response.

To demonstrate the presence of Dα7 in the hemocytes, we performed an immunostaining experiment using the anti-Dα7 antibody on GFP marked hemocytes, obtained from flies expressing the *UAS-GFP* under the control of *srpHemo-GAL4* (*srpHemo* > *GFP*). The hemocytes were easily scored by their green fluorescence, and anti-Dα7 staining was compared in wild type flies and null *Dα7*^{PΔEY6} homozygous mutants (Fig. 5A). In wild type flies the hemocytes anti-Dα7 staining level was significantly higher than the background signal detected in *Dα7*^{PΔEY6} flies (n = 35, 27; p = 0,0003) (Fig. 5B). We also checked the Dα7 expression pattern by crossing the *Dα7*> line (Fayyazuddin et al., 2006) with a *UAS-mCherry* line expressing a brightly fluorescing mCherry protein (Shaner et al., 2004). The *Dα7>mCherry* flies showed a strong red fluorescence in the hemocytes (Fig. 5C and D). These data clearly indicate that Dα7 is expressed in the hemocytes.

4. Discussion

The neural control of immunity has been only limitedly studied in insects. Here we demonstrate a role for ACh in the *Drosophila* humoral immune response. Knockdown of ACh signaling in neuronal cells affects the expression level of *drs* gene triggered by *M. luteus* infection. We found that the Dα7 subtype of nAChRs is involved in the ACh- mediated neuronal-immune system talk. Interestingly, we found that hemocytes are involved in the cholinergic signaling activating the immune response. Indeed, Dα7 is expressed in the hemocytes and its knockdown affects the *drs* expression. Although the involvement of hemocytes in the production of antimicrobial peptides is still not well defined (Kounatidis and Ligoxygakis, 2012; Vlisidou and Wood, 2015), recently it has been shown a role for hemocytes in a local humoral immune response of the fat body and respiratory epithelia that induces the production of the AMP Drosocin (Bosch et al., 2019). Our findings on *drs* expression further support a function of the hemocytes in mediating the AMP production. Using appropriate genetic contexts, we found that silencing the *ChAT* gene in non-neural cells determined a reduction of *drs* expression. The non-neuronal cholinergic system has been poorly studied in insects, while it has been widely described in mammals including humans. The main components of this molecular pathway, ChAT, ACh, and nAChRs have been found in non-neuronal cells. The cholinergic signaling of non-neuronal cells uses the same nAChRs as neuronal cholinergic signaling. Neuronal nAChRs are present in several types of non-neuronal cells including endothelial cells, lung epithelia, immune cells, and cancer cells where they are involved in cell proliferation and differentiation and in the inflammatory responses (Zoli et al., 2018a). In humans, the nACh α7 receptor is needed for ACh-mediated anti-inflammatory effects. Through ACh signaling, a crosstalk between the nervous and the immune system maintains the immunological homeostasis by activating the nACh α7 receptor in spleen macrophages, which in turn reduces cytokines production (Andersson and Tracey, 2012). Critical mediators of this anti-inflammatory reflex are ACh-producing T-cells, which relay functional information transmitted by action potentials originating in the vagus nerve to the spleen (Rosas-Ballina et al., 2011). ACh-producing T cells have been shown to play a role also in the mammalian intestine (Dhawan et al., 2016; Ramirez et al., 2019). In this case, cholinergic signaling has been shown to play a positive effect on the immune response. In fact, genetically modified mice that lacked the potential to secrete ACh in their T cell compartment displayed impaired host defense mechanisms in the gut and increased susceptibility to enteric infections. Our findings, showing that activation of Dα7 receptors positively modulates AMP expression in *Drosophila,* correlate with these latter studies, further substantiating a diverse and expanding role of ACh in animal immune responses. The finding that the block of ACh signaling reduces the level of expression of *drs* gene, further supports that the immune response is regulated at the level of whole

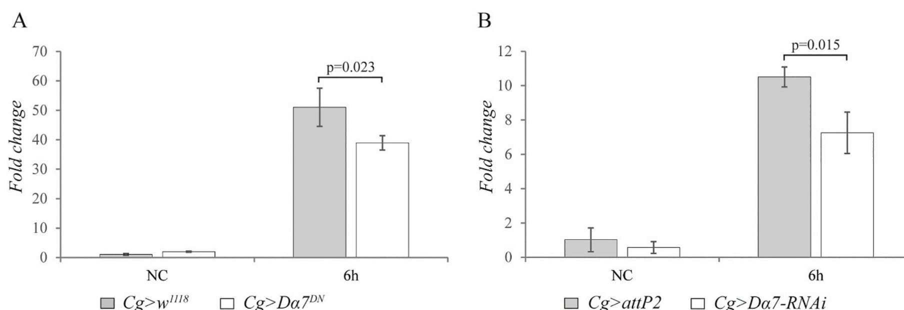

Fig. 3. Expression of *Dα7*^{DN} in the immune cells affects *drs* gene expression.
drs transcript levels as detected by qRT-PCR in adult female flies expressing in hemocytes and fat body the dominant negative *Dα7*^{DN} mutant protein (*A*) and the RNAi-mediated knockdown of *Dα7* (*B*) compared to their controls, at 3 and 6h after immune challenge. NC, not challenged. Transcript levels were normalized to *Rpl32*. Data were normalized to the NC control and represent the mean ± SD of 4 biological replicates, each consisting of a pool of 5 flies. Statistical significance for each time point was assessed by two-tailed unpaired Student's t-test and the relative p value reported for significant differences.

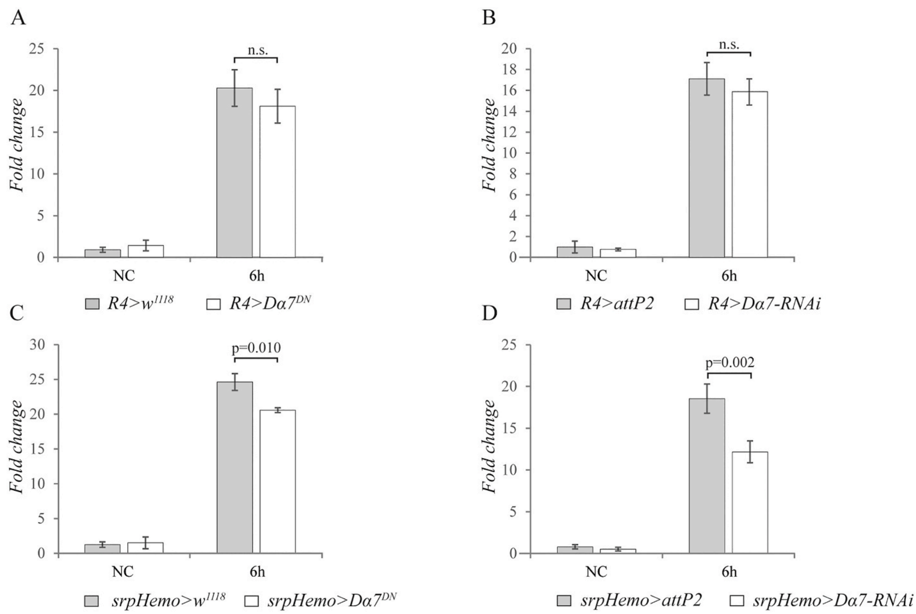

Fig. 4. Expression of the Dα7 receptor in hemocytes is required for proper *drs* expression.
qRT-PCR analysis of *drs* transcript levels after immune challenge in adult flies expressing either the dominant negative $D\alpha7^{DN}$ receptor subunit or *Dα7*-RNAi in fat body or hemocytes compared to their controls. (A and B) Expression of $D\alpha7^{DN}$ and *Dα7*-RNAi in fat body does not affect *drs* gene expression. (C e D) Expression of $D\alpha7^{DN}$ and *Dα7*-RNAi in hemocytes significantly reduces *drs* expression. NC, not challenged. Transcript levels were normalized to *Rpl32*. Data were normalized to the NC control and represent the mean ± SD of 4 biological replicates, each consisting of a pool of 5 flies. Statistical significance was assessed by two-tailed unpaired Student's t-test and the relative p value reported for significant differences. Not significant (n.s.) = p ≥ 0.05.

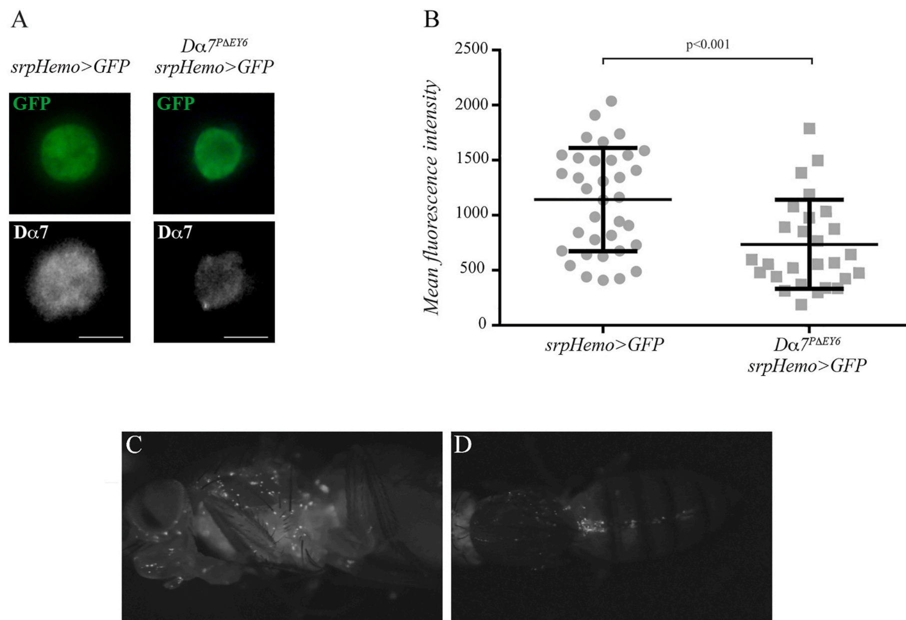

Fig. 5. Dα7 is expressed in the hemocytes.
(A) Fluorescence microscopy images of hemocytes of $D\alpha7^+/D\alpha7^+$; *srpHemo > GFP* and $D\alpha7^{P\Delta EY6}/D\alpha7^{P\Delta EY6}$; *srpHemo > GFP* adult females immunostained with anti-Dα7 antibody. Scale bar: 5 μm. The $D\alpha7^+/D\alpha7^+$ GFP hemocytes analyzed (n = 35) showed a stronger anti-Dα7 staining with respect to $D\alpha7^{P\Delta EY6}/D\alpha7^{P\Delta EY6}$ hemocytes (n = 27). (B) Scattergraph and mean ± SD plotting of quantification of (A). Anti-Dα7 label is significantly higher in hemocytes from $D\alpha7^+$ flies as compared to $D\alpha7^{P\Delta EY6}$ flies. The fluorescence intensities were measured using the Image J software. Statistical significance was assessed by one-tailed unpaired Student's t-test and the relative p value reported for significant differences. Lateral view (C) and dorsal view (D) of *Dα7>mCherry* female flies.

organism (Buchon et al., 2014). The fact that the extent of the reduced level of *drs* expression is similar in the genetic contexts altering the ACh signaling and Dα7 function in both neuronal and non-neuronal cells, as well as in mutants Dα7, suggests that the different players are part of the same molecular mechanism modulating the expression of the *drs* gene expression. This suggests a finely tuned cross-modulation among neural and non-neural cholinergic tissues. In this complex scenario, our data suggest that in the hemocytes the Dα7 receptor could be activated by both neuronal and non-neuronal ACh signaling. However, whether all types of hemocytes or a specific subpopulation of them is involved in *drs* expression, and how the Dα7 signaling targets the activation of *drs* remains to be investigated.

The role of ACh in *Drosophila* immunity, despite the novelty, is not surprising. Indeed, several basic research reports have demonstrated that ACh is virtually ubiquitous in nature, being present in different microorganisms, plants and animals (Sastry and Sadavongvivad, 1978; Wessler et al., 1998; Wessler and Kirkpatrick, 2008). ACh appeared in nature in the very ancestral forms of life and has been maintained throughout evolution, associated with a wealth of different functions only partly understood (Wessler et al., 1999; Horiuchi et al., 2003). The experimental evidence we give in support of an ACh role in insect immunity further confirms the multifunctional role of this signaling molecule in a wealth of very different living organisms. The use of *Drosophila* as a model system to unravel the molecular mechanisms of ACh in the modulation of innate immunity will provide an important additional tool to investigate this interesting area of research. Our results do also provide background information that will allow more focused studies on the risks associated with the use of neurotoxic compounds. The finding of a non-neuronal ACh signaling in *Drosophila* could be relevant for the studies on the effects of neonicotinoids insecticides agonists of nicotinic acetylcholine receptors. A non-neuronal ACh signaling could help in understanding why neonicotinoids have a wide

range of effects on larval and adult development of pollinating bees (Grünewald and Siefert, 2019).

Authors' contributions

Author contributions: G.G. (Giorgia Giordani), G.G. (Giuseppe Gargiulo), V.C., S.G. and F.P. designed research; G.G. (Giorgia Giordani), G. C., A.B., I.DL., V.C., G. DL., S.G. and G.G. (Giuseppe Gargiulo) performed the research; V.C., G.G. (Giuseppe Gargiulo), S.G. and F.P. analyzed the data and wrote the paper.

Declaration of competing interest

The authors declare they have no conflicts of interests.

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Appendix A. Supplementary data

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