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RESEARCH ARTICLE

Neuroligin 1 expression is linked to plasticity of behavioral and neuronal responses to sex pheromone in the male moth *Agrotis ipsilon*

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ABSTRACT

In the moth *Agrotis ipsilon*, the behavioral response of males to the female-emitted sex pheromone increases throughout adult life and following a prior exposure to sex pheromone, whereas it is temporally inhibited after the onset of mating. This behavioral flexibility is paralleled with changes in neuronal sensitivity to pheromone signal within the primary olfactory centers, the antennal lobes. In the present study, we tested the hypothesis that neuroligins, post-synaptic transmembrane proteins known to act as mediators of neuronal remodeling, are involved in the olfactory modulation in *A. ipsilon* males. We cloned a full-length cDNA encoding *neuroligin 1*, which is expressed predominantly in brain and especially in antennal lobes. The level of *neuroligin 1* expression in antennal lobes gradually raised from day-2 until day-4 of adult life, as well as at 24 h, 48 h and 72 h following pre-exposure to sex pheromone, and the temporal dynamic of these changes correlated with increased sex pheromone responsiveness. By contrast, there was no significant variation in antennal lobe *neuroligin 1* expression during the post-mating refractory period. Taken together, these results highlight that age- and odor experience-related increase in sex pheromone responsiveness is linked to the overexpression of *neuroligin 1* in antennal lobes, thus suggesting a potential role played by this post-synaptic cell-adhesion molecule in mediating the plasticity of the central olfactory system in *A. ipsilon*.

KEY WORDS: Antennal lobes, Moth, Mating behavior, *Neuroligin 1*, Olfactory plasticity, Sex pheromone

INTRODUCTION

In most animal species, including insects, the sense of smell plays a crucial role in many aspects of life, such as mate attraction and recognition, navigation, and location of predators and food sources (Brennan, 2010; Galizia and Sachse, 2010; Gadenne et al., 2016). The detection and processing of odors, at the neuronal and behavioral levels, are heavily influenced by extrinsic factors, including photoperiod, temperature or previous chemosensory experiences, but also by intrinsic factors such as the physiological status of the individual (Palmer and Kristan, 2011; Gadenne et al., 2016; Ross and Fletcher, 2019). The neuronal plasticity occurs through the olfactory pathway, from olfactory receptor receptors (ORNs) to the olfactory centers, and relies, in part, on short-term and long-term structural and/or functional alterations in synaptic architecture (Wilson et al., 2004).

Synapses contain cell-adhesion molecules (CAMs) that orchestrate the bidirectional organization of their pre-synaptic and post-synaptic compartments (Dalva et al., 2007). Among CAMs, neuroligins (NLGs) constitute a multigene family encoding post-synaptic transmembrane proteins, which are highly conserved across a wide range of species and even between vertebrates and insects (Knight et al., 2011). The NLG family is composed of four members (*NLGN1*, *NLGN2*, *NLGN3* and *NLGN4*) in mammals, and an additional *NLGN5* gene (also known as *NLGN4Y*) has been characterized on the Y chromosome in humans (Ichtchenko et al., 1995, 1996; Bolliger et al., 2001). Automated annotation of sequenced insect genomes led to the identification of six *NLG* genes (*Nlg1-5*), including two paralogous *Nlg-4* genes in the silkworm *Bombyx mori* (Tsubota and Shiotsuki, 2010), five *NLG* genes (*NLG1-5*) in the honey bee *Apis mellifera* (Biswas et al., 2008) and four *NLG* genes (*dnlg1-4*) in the fruit fly *Drosophila melanogaster* (Banovic et al., 2010; Sun et al., 2011). NLGs are type I transmembrane proteins composed of a large N-terminal extracellular acetylcholinesterase (AChE)-like domain, a single transmembrane domain and a short cytoplasmic tail that contains a post-synaptic density 95/disc-large/zona occludens (PDZ) domain-binding motif (Ichtchenko et al., 1995, 1996) and is exposed to a variety of intracellular regulatory signals (Kim and Sheng, 2004; Meyer et al., 2004). Through their AChE-like domain, NLGs may also interact as ligands with either α-neurexins or β-neurexins, which are CAMs located pre-synaptically, in order to form trans-synaptic bridges that mediate the precise apposition of pre-synaptic and post-synaptic membranes (Varoqueaux et al., 2004; Boucard et al., 2005). A spatiotemporal recognition code between distinct NLGs and neurexins isoforms exists, which is presumed to govern the specification of synaptic connectivity and, thus, to control the balance between excitatory and inhibitory synapses (Prange et al., 2004; Levinson and El-Husseini, 2005; Chih et al., 2006; Siddiqui et al., 2010). The emphasis of NLGs in the development and functioning of neuronal networks in brain has been highlighted in humans, as specific NLG mutations were found to be linked to several neurobiological disorders, such as autism and schizophrenia (Chih et al., 2004; Talebizadeh et al., 2006; Yu et al., 2017). In mammals, the tissular and subcellular expression patterns, as well as the synaptic functions of NLGs, differ according to the protein type

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(Varoqueaux et al., 2004; Chih et al., 2005; Budreck and Scheiffele, 2007). NLGN1 was reported to bind tightly to β-neurexins, but not to α-neurexins (Ichtchenko et al., 1995), and was also shown to be expressed in both the peripheral and central nervous system, with a preferential localization to excitatory glutamatergic synapses (Song et al., 1999; Chih et al., 2005), as well as in a variety of glial cells, including astrocytes and oligodendrocytes (Scheiffele et al., 2000; Sakers and Eroglu, 2019). Accumulating *in vitro* and *in vivo* evidence indicates that NLGN1 plays a role in the formation, maturation and activity of synaptic structures (Hu et al., 2015). *NLGN1* overexpression in primary hippocampal neuronal cultures increased the number of pre-synaptic and post-synaptic contacts, while *NLGN1* silencing by RNA interference led to an inhibition of synaptogenesis (Dean et al., 2003; Chih et al., 2005; Shipman et al., 2011; Burton et al., 2012). In NLGN1 transgenic mice, *NLGN1* overexpression provoked an elevation in synapse density, excitatory/inhibitory ratio and synaptic transmission within hippocampus as well as impairments in memory acquisition (Dahlhaus et al., 2010). Concerning insects, it has been demonstrated in *A. mellifera* that sensory experience- and olfactory learning-induced brain plasticity was accompanied by changes in neuropil volume and synaptic density correlated with increased *NLG1* expression (Biswas et al., 2010; Reinhard and Claudianos, 2012). In addition, in *D. melanogaster*, the loss of *dnlg1* function has been associated with neuromuscular defects characterized by a marked reduction in bouton number, synaptic transmission and post-synaptic apparatus, while increased *dnlg1* expression in mutants promoted ectopic post-synaptic differentiation (Banovic et al., 2010).

Over the past decade, there has been increasing evidence that the noctuid moths are excellent model organisms for studying the plasticity of olfactory system (Anton et al., 2007; Gadenne et al., 2016). In these species, the sexual communication relies on the attraction of males by sex pheromones produced and emitted by conspecific females. The pheromone signal is recognized by ORNs located in the antennae and is integrated first in the primary olfactory centers of brain, the antennal lobes (ALs), which are compartmentalized into spherical neuropil structures, the olfactory glomeruli (Haupt et al., 2010). The ALs consist of two complexes of glomeruli: the macroglomerular complex (MCG), which specializes in the processing of sex pheromone input from ORNs, and the ordinary glomeruli, which are involved in the processing of all the other odors that the male encounters (Haupt et al., 2010). The processed information is then conveyed via projection neurons (PNs) to higher-order brain centers, the mushroom bodies and the lateral horn, ultimately leading to a characteristic orientation behavior of the male towards the pheromone source that is supported by optomotor anemotaxis (Haupt et al., 2010). ALs also contain local interneurons (LNs), which connect many glomeruli and form a network providing transfer of information between glomeruli. LNs have been characterized as GABAergic, cholinergic and glutamatergic, and they help to structure the odor representation in the AL, which ultimately shapes the tuning profiles of PNs (Haupt et al., 2010).

Despite its stereotypy, the orientated flight behavior and the underlying processing steps exhibit high degrees of flexibility. In addition to modulation by circadian rhythm (Groot, 2014), age-, odor experience- and reproductive status-related plasticities in the male response to sex pheromone have been unveiled in noctuid moths, *Spodoptera littoralis* and *Agrotis ipsilon*. Indeed, a brief pre-exposure of *S. littoralis* males to sex pheromone induced a lowering of the threshold for the behavioral response 24 h later. This long-term effect is correlated with a higher sensitivity of ORNs and AL neurons to pheromone signal, accompanied by a volumetric increase in MGC glomeruli and in the calyces of the mushroom bodies (Anderson et al., 2007; Guerrieri et al., 2012; Anton et al., 2016). It is also known that *A. ipsilon* males are apt to elicit the sex pheromone-guided orientation flight only when they reach their full sexual maturity within 3–4 days after emergence (Gadenne et al., 1993; Duportets et al., 1998; Gassias et al., 2018). This increase in behavioral responsiveness is paralleled by a raise in the sensitivity of sex pheromone-responding AL neurons (Gadenne and Anton, 2000). By contrast, newly mated males quickly cease to behaviorally respond to sex pheromone from 15 min after the onset of copulation (Vitecek et al., 2013), and this inhibition is concomitant with a decrease in AL neuron sensitivity (Gadenne et al., 2001). This post-mating refractory period lasts until the end of the scotophase, and the males are able to re-mate only during the following scotophase, when they have fully recovered their sensitivity to sex pheromone and also replenished their sex accessory glands to produce a new spermatophore (Vitecek et al., 2013; Gassias et al., 2018).

In order to decipher the molecular mechanisms underlying the modulation of olfactory system in *A. ipsilon* males, we tested the hypothesis that *NLG1* is involved in mediating the plasticity of behavioral and neuronal responses to sex pheromone. We first cloned a full-length cDNA encoding *A. ipsilon* NLG1 (AiNLG1) and determined its tissue expression profile. We then quantified the level of the expression of *AiNLG1* in ALs as a function of age (immature versus mature), of odor experience (naive versus pre-exposed to sex pheromone) and of reproductive status (virgin versus mated) in relation to the pheromone signal responsiveness. Our results demonstrated that age- and odor experience-related increase in sex pheromone responsiveness is timely correlated with *AiNLG1* overexpression in ALs, thus suggesting a potential role of NLG1 as central organizer of the olfactory plasticity in *A. ipsilon*.

MATERIALS AND METHODS

Chemicals

We prepared a synthetic sex pheromone blend at 1 ng µl^{-1} containing three components identified in natural extracts of the pheromone glands of the female *Agrotis ipsilon* (Hufnagel 1766) (Picimbon et al., 1997; Gemeno and Haynes, 1998): (Z)-7-dodecen-1-yl acetate (Z7-12:OAc), (Z)-9-tetradecen-1-yl acetate (Z9-14:OAc) and (Z)-11-hexadecen-1-yl acetate (Z11-16:OAc), mixed at a ratio of 4:1:4. This blend was proven to be the most attractive to males in field trapping experiments (Causse et al., 1988), and it elicits similar behavior in a wind tunnel as natural extracts of the pheromone gland (Barrozo et al., 2010; Vitecek et al., 2013). The three components were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and diluted in hexane (>98% purity, CAS 110-54-3, Carlo-Erba, Val-de-Reuil, France). A dose of 10 ng of the sex pheromone blend was used in the experiments, and this dose has previously been described as behaviorally and electrophysiologically active (Gadenne et al., 2001; Deisig et al., 2012; Gassias et al., 2018).

Insects and tissue collection

Adult *A. ipsilon* originated from a laboratory colony established in Bordeaux and transferred to Versailles, France. The colony is based on field catches in southern France, and insects were reared on an artificial diet in individual cups until pupation (Poitout and Bues, 1974). Pupae were sexed, and males and females were kept separately on an inversed light:dark cycle (16 h light:8 h dark

photoperiod, with the scotophase starting at 10:00 h) at 22°C. Newly emerged adult males and females were removed from the hatching containers every day and reared in a separate room with access to a 20% sucrose solution *ad libitum*. The day of emergence was considered as day-0. The males were anesthetized with an exposure to carbon dioxide for 10 s, and tissue dissections were performed in the middle of the scotophase (between 13:00 h and 16:00 h), when males respond maximally to the sex pheromone (Barrozo et al., 2010). For the expression profiles of *AiNLG1*, antennae, ALs, brains with excised ALs, thoracic muscles, midguts, fat bodies and testes of males were dissected in Ringer's solution, then immediately flash-frozen in Eppendorf (Montesson, France) vials kept in liquid nitrogen and stored at −80°C until treatment.

RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol reagent (TRI Reagent®, Euromedex, Souffelweyersheim, France), according to the manufacturer's instructions, and quantified by spectrophotometry at 260 nm. DNase treatment was performed with 2 U TURBO™ DNase 1 (Ambion, Villebon-sur-Yvette, France) for 30 min at 37°C, followed by a 10-min inactivation at 75°C. After DNase treatment, single-stranded cDNA was synthesized from total RNAs (1 µg) with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with a deoxynucleotide triphosphate (dNTP) mix, RNase OUT, Oligo(dT) primer and sterile water to a final reaction volume of 20 µl. The mix was heated to 65°C for 5 min before the enzyme was added and then incubated for 1 h at 42°C.

Molecular cloning of *A. ipsilon NLG1*

The amino acid sequence of *Helicoverpa armigera NLG1* was searched against the genome assembly of *A. ipsilon* in the NCBI database (accession number GCA_004193855.1), and a specific primer pair was designed based on the sequence of a putative *AiNLG1* transcript. Polymerase chain reaction (PCR) was carried out on 200 ng brain cDNA with 2.5 U High Expand Fidelity DNA polymerase (Roche, Saint-Quentin Fallavier, France), 0.4 µmol l^{-1} primer pair (NLG1dir, NLG1rev) and 0.25 mmol l^{-1} of each dNTP. Following an initial 5-min denaturation at 94°C, the thermal amplification procedure consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 1 min and then final elongation at 72°C for 10 min. To obtain the full-length cDNA sequence of *AiNLG1*, 5′- and 3′-rapid amplification of cDNA ends (RACE) was conducted using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the manufacturer's instructions. For 5′-RACE, we used the specific reverse primers (NLG15′-RACE1, NLG15′-RACE2) and Universal Primer Mix (UPM; Clontech) as the forward anchor primer; for 3′-RACE, we used UPM as the reverse primer and the specific forward primers (NLG13′-RACE1, NLG13′-RACE2). Touchdown PCR was performed using hot start as follows: 1 min at 94°C, five cycles of 30 s at 94°C, 30 s at 64°C and 1 min at 72°C, then five cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C, then 25 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, then 15 min at 72°C. Specific primers used for reverse transcription (RT)-PCR and RACE reactions are listed in Table S1.

RT-PCR and RACE products were purified by agarose gel electrophoresis (NucleoSpin® Extract II, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and subcloned into PCRII-Topo plasmid (Invitrogen). After colony isolation, DNA minipreps were prepared (NucleoSpin® Plasmid DNA Purification, Macherey-Nagel GmbH & Co. KG) and the DNA clone containing the proper insert was then sequenced (GATC Biotech SARL, Marseille, France).

Quantification of *AiNLG1* expression

Quantitative PCR (qPCR) was performed using a LightCycler 480 Real-Time PCR Detection System (Roche, Saint-Quentin Fallavier, France) according to the manufacturer's instructions. Each 12 µl reaction consisted of 6 µl Absolute Blue SYBR Green Fluor (Thermo Fisher Scientific, Waltham, MA, USA), 4 µl cDNA (25 ng $µl^{-1}$) and 2 µl NLG1dir and qNLG1rev primers at 10 µmol l^{-1} (Table S1). PCR conditions were 35 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 30 s, and the length of the amplified product was 245 bp. Fluorescence measurements over a 55–95°C melting curve confirmed the presence of a single specific peak and the absence of primer-dimer peaks for the used primer pair. Each run included a negative control (water) and a fivefold dilution series of pooled cDNA (from all conditions). The fivefold dilution series (undiluted, 1/5, 1/25, 1/125 and 1/625) was used to construct a relative standard curve to determine the PCR efficiency and for further quantification analysis. In all experiments, the primers gave amplification efficiencies of 90–100%. Each reaction was run in three technical replicates with seven independent biological replicates using the technical platform of The Integrative Biology Institute (Sorbonne University, France). Expression levels were analyzed with LightCycler 480 software (Roche) and the GENORM Visual Basic application for Microsoft Excel (Issy-les-Moulineaux, France) as described by Vandesompele et al. (2002). The cycle threshold (Ct) values were determined for the candidate gene (*AiNLG1*) and the reference gene, the *A. ipsilon Ribosomal protein L8* (*AiRpL8*) gene (accession number JX975720.1), which exhibited the most stable expression levels amongst other tested control genes (*Ribosomal protein L13*, *Glyceraldehyde-3-phosphate dehydrogenase* and *β-actin*). The average Ct value of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of the *AiRpL8* level in the Q-GENE software (Simon, 2003) by using the following formula:

$$\mathrm{NE} = (E_{AiRpL8})^{\mathrm{Ct}_{AiRpL8}} / (E_{AiNLG1})^{\mathrm{Ct}_{AiNLG1}}, \quad (1)$$

where NE is normalized expression, E is PCR efficiency and Ct corresponds to the crossing-point values. Then, mean and s.e.m. were calculated with the NE values for the biological replicates. The sequences of the specific primers RpL8dir and RpL8rev for *AiRpL8* are listed in Table S1.

Behavioral experiments to sex pheromone in a wind tunnel

Behavioral tests were performed using a Plexiglass wind tunnel measuring 190 cm length×75 cm width×75 cm height (VT Plastics, Genevilliers, France). Both ends of the tunnel were enclosed with white synthetic fabric, allowing air to pass through but preventing insect escape. Air movement was provided by an exhaust fan at the downwind end of the tunnel sucking the air at a speed of 0.3 m s^{-1} and evacuating odorized air out of the building. The tunnel was maintained in the dark except for one red visible light source, allowing visual observation. Experiments were performed at 23°C, 40±10% relative humidity, during the middle of the scotophase (between 13:00 h and 16:00 h), when males respond maximally to sex pheromone (Barrozo et al., 2010). A single experimental male was introduced into the wind tunnel and placed on a 36 cm-high platform in the middle of the tunnel width and 160 cm downwind from the pheromone source. After 1 min, during which the male adjusted to the airflow, the pheromone blend was delivered for 3 min. During this time, the following behavioral items were

observed: take-off, partial flight (flight half way between the release site and the pheromone source), complete flight (arrival within 10 cm of the source) and landing on the pheromone source. The partial and complete flights as well as landings were considered as an orientated response towards the sex pheromone. Orientated and random flights were counted together in order to quantify the general flight activity of males, and this activity was ∼95% for all behavioral tests. We also noted the delay in the orientated response of males by measuring the period of time between the onset of pheromone stimulation and the take-off. All experiments were performed double blind to avoid partial observations. On each experimental day, different groups of males were tested, including at least one group of males that were expected to show a high response level to avoid experimental bias.

The pheromone stimulus was delivered with a programmable olfactometer adapted from Party et al. (2009). Air from the building was charcoal filtered and re-humidified, then divided into several equal flows (300±10 ml min^{-1}) using a Y connector (model 1/8″ P514, Upchurch Scientific, Silsden, UK) and a 5-port manifold (model P-115, Upchurch Scientific). Each flow was connected to a miniature electrovalve (model LHDA1233115H, The Lee Company, Westbrook, ME, USA) driven by a Valve-Bank programmer (AutoMate Scientific, Berkeley, CA, USA). Activating the appropriate valve directed the flow to a 4 ml glass vial containing the pheromone source and closed by a septum cork. The inlet and outlet of the source pheromone were made of two hypodermic needles (18-g size) inserted through the septum and connected with polytetrafluorethylene tubing (1.32 mm i.d., 20 cm length). A 10 µl sample of the pheromone blend at 1 ng $µl^{-1}$ was deposited on a filter paper and placed inside the vial. Contaminated air was removed from the set-up by an exhaust fan after each behavioral assay.

Age-related experiments

Males of different ages (1, 2, 3, 4 and 5 days) were collected in the mid-scotophase and transferred to cylindrical plastic containers in a dark box, then transported from the rearing room to the wind tunnel room for quantification of the behavioral response to sex pheromone and response delay. The males were then killed using liquid nitrogen, and the ALs were dissected from frozen heads for analysis of *AiNLG1* expression.

Pre-exposure experiments

Pre-exposure treatments were performed in the wind tunnel at mid-scotophase. Newly emerged males (day-0) were transferred from the rearing room to the wind tunnel room, and placed in cages (maximum of three males per cage) before the end of the photophase. A cage was introduced into the wind tunnel and placed on a 36 cm-high platform in the middle of the tunnel width and 160 cm downwind from the pheromone source. After 1 min, each male, kept within the cage, was subjected to pheromone stimulation during 3 min, after which it was removed from the wind tunnel and transferred to the rearing room. The behavioral response and response delay of the pre-exposed males were analyzed at 2, 24, 48 and 72 h after pre-exposure to sex pheromone. The males were then killed using liquid nitrogen, and ALs were dissected from frozen heads for quantification of *AiNLG1* expression. Control groups were composed of 0-, 1-, 2- and 3-day-old males exposed to hexane for 3 min.

Mating experiments

Mating experiments were performed as previously described (Barrozo et al., 2010). Briefly, virgin 4-day-old sexually mature males and virgin 3-day-old sexually mature females were individually paired in cylindrical plastic containers before the onset of scotophase in a room under an inversed light:dark cycle (16 h light:8 h dark photoperiod) at 22°C. In our photoperiod conditions, the scotophase begins at 10:00 h and ends at 18:00 h and copulation occurs, on average, between 13:30 h and 16:00 h, with a mean duration of 1 h 30 min (Vitecek et al., 2013). In *A. ipsilon* males, the post-mating refractory period, which is characterized by a transient inhibition of behavioral and central nervous responses to sex pheromone, starts at 15 min after the onset of copulation and lasts up to the end of the scotophase (Gadenne et al., 2001; Vitecek et al., 2013; Gassias et al., 2018).

Visual observation of the pairs was done every 5 min during the copulation period in order to detect the onset and the end of the mating. Once copulation had ended, newly mated males were removed from the observation room. The behavioral response to sex pheromone of males and delay response were analyzed immediately (0 h) and at 1 h and 23 h after the end of the copulation. The males were then killed using liquid nitrogen, and ALs were dissected from frozen heads for quantification of *AiNLG1* expression. Control groups consisted of virgin 4- and 5-day-old males, which were paired with virgin 3-day-old females but not allowing them to mate. In order to confirm that mating was successful, all mated females were also dissected to check for the presence of the spermatophore.

Bioinformatics

The protein sequences of insect NLG homologs were aligned using ClustalW in BioEdit 7.0. The signal peptide and the transmembrane domain were predicted with SignalP 4.1 (https://services.healthtech.dtu.dk/service.php?SignalP-4.1) and TMHMM 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). The AChE-like domain was found using the NCBI Conserved Domain Architecture Retrieval Toll (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). PDZ domain-binding motif was predicted with PDZPepInt (Kundu et al., 2014) and EF-hand metal-binding motifs were obtained from Biswas et al. (2008). The phylogenetic tree of insect NLGs was constructed by the neighbor-joining method with Poisson correction of distances using the MEGA 7 program (Kumar et al., 2016) with default settings.

Accession numbers for NLG protein sequences retrieved from GenBank are as follows: the lepidopteran *H. armigera* NLGs (HaNLG1, XP_021194794.1; HaNLG2, XP_021189212.1; HaNLG3, XP_021197425.1; HaNLG4-XL, XP_021196129.1; HaNLG4-YL, XP_021196885.1; HaNLG5, XP_021191347.1); the dipteran *D. melanogaster* NLGs (DmNLG1, NP_731172; DmNLG2, NP_523496; DmNLG3, NP_001036685; DmNLG4, NP_001163661); the coleopteran *Tribolium castaneum* NLGs (TcNLG1, XP_008192968.1; TcNLG2, XP_015835928.1; TcNLG3, XP_001810887.1; TcNLG4, EFA03840; TcNLG5, EFA03175); and the hymenopteran *A. mellifera* NLGs (AmNLG1, ACM48186; AmNLG2, XP_006571554.2; AmNLG3, NP_001139208; AmNLG4, NP_001139209; AmNLG5, NP_001139211).

Statistical analysis

The means of *AiNLG1* expression level and of delay response were compared using one-way ANOVA followed by a Tukey's multiple comparison *post hoc* test. $P<0.05$ was accepted as statistically significant. For behavioral experiments, statistical differences between groups of males were evaluated using an R×C test of independence by means of a G-test and also by applying the Williams correction (Sokal and Rohlf, 1995).

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Animal ethics

Adequate measures were taken to minimize pain or discomfort of animals. Experiments were conducted according to international standards on animal welfare and were compliant with local and national regulations. All applicable international guidelines for the care and use of animals were followed.

RESULTS

Cloning and sequence analysis of *A. ipsilon* NLG1

By searching the nucleotidic sequence of *H. armigera* NLG1 against the genome database of *A. ipsilon*, we designed a pair of specific primers based on the sequence of a putative *NLG1* transcript and succeeded in amplifying a partial fragment of 919 bp via RT-PCR on brain total RNA extracted from 5-day-old males. The remaining 5′ and 3′ ends of this fragment were then obtained by RACE-PCR using gene-specific primers. The nucleic acid sequences for the 5′-RACE and 3′-RACE reaction products were assembled with the original fragment to generate a full-length cDNA named *AiNLG1*, which was deposited in the GenBank database under the accession number MZ423824. The *AiNLG1* cDNA extends over 5047 bp and contains a 5′-untranslated region (5′-UTR) of 180 bp, an open reading frame (ORF) of 4401 bp and a 3′-UTR of 466 bp, with a polyadenylation signal upstream of the poly(A) tail (Fig. S1). The *AiNLG1* ORF was translated into a predicted protein sequence of 1467 amino acids (Fig. S1), which is composed of the typical functional regions of NLG protein family: an N-terminus cleaved signal sequence, a large extracellular AChE-like domain comprising two EF-hand metal-binding motifs, followed by a single transmembrane domain and a C-terminus PDZ domain-binding motif (Fig. 1). Comparison of the predicted protein of AiNLG1 with NLG1 characterized in other insect species showed a high percentage of amino acid identity with the lepidopteran *H. armigera* (HaNLG1; 89.5%), followed by the coleopteran *T. castaneum* (TcNLG1; 28.1%), the dipteran *D. melanogaster* (DmNLG1; 23.1%) and the hymenopteran *A. mellifera* (AmNLG1; 20.8%) (Fig. 1). A neighbor-joining phylogenetic tree of insect NLGs was also created, and, as expected, its analysis revealed that AiNLG1 belongs to the cluster of lepidopteran NLG1 (Fig. 2).

Tissue pattern expression of *AiNLG1*

In order to gain some preliminary information about the functions of *AiNLG1*, a tissue distribution screen was performed by evaluating

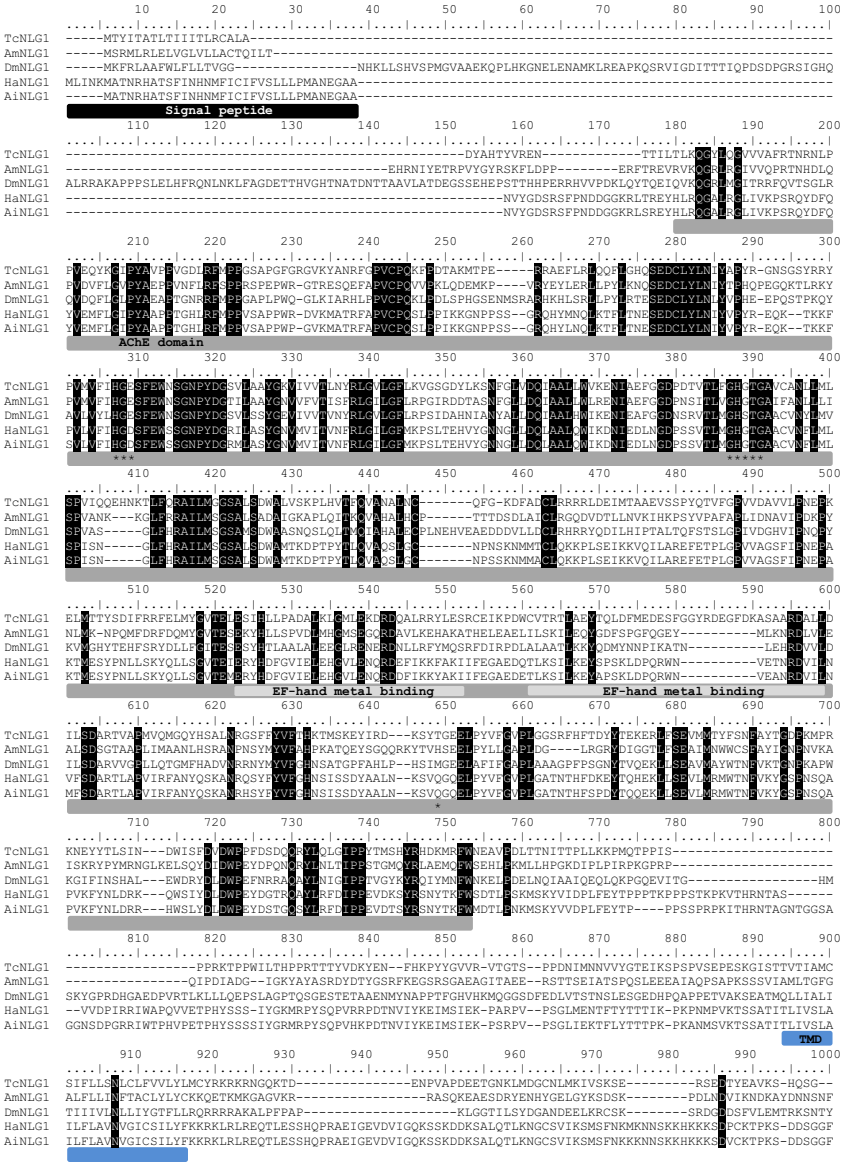

Fig. 1. Analysis and comparison of the protein sequence of *Agrotis ipsilon* neuroligin 1 (AiNLG1) with those of other insect NLG1s. The deduced amino acid sequence of AiNLG1 was aligned with sequences of NGL1 from *Tribolium castaneum* (TcNLG1), *Apis mellifera* (AmNLG1), *Drosophila melanogaster* (DmNLG1) and *Helicoverpa armigera* (HaNLG1). GenBank accession numbers of the proteins are listed in the Materials and Methods. The amino acid position is indicated above the sequences for each decimal, and dashes represent gaps introduced to maximize alignment scores. Amino acids conserved are printed in white letters on a black background. The signal peptide, the acetylcholinesterase (AChE) domain, the EF-hand metal-binding motifs, the transmembrane domain (TMD) and the post-synaptic density 95/disc-large/zona occludens (PDZ) domain-binding motif are also indicated. The number at the end of each sequence represents the percentage identity (ID) with AiNLG1.

Journal of Experimental Biology (2021) 224, jeb243184. doi:10.1242/jeb.243184

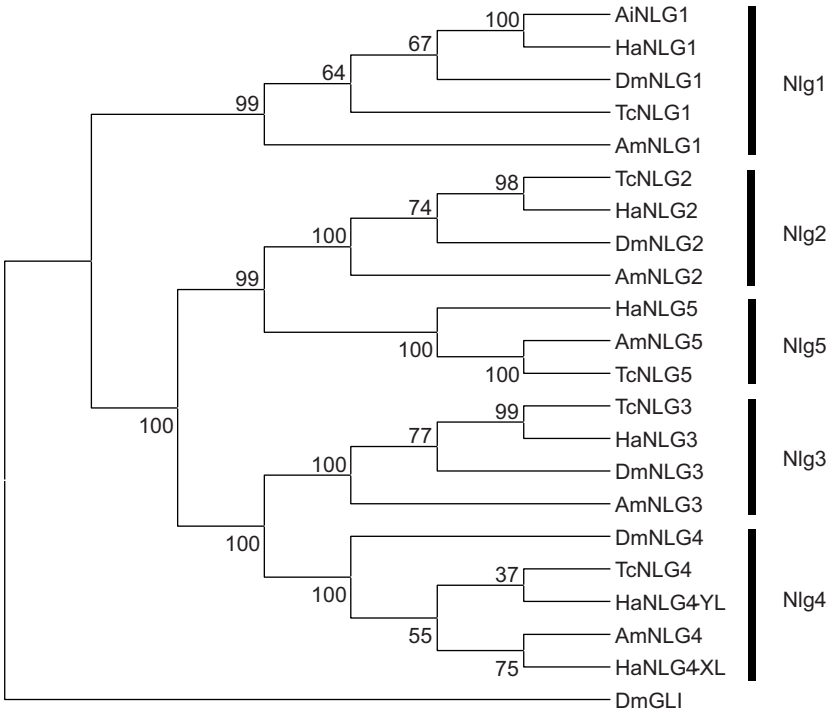

Fig. 2. Neighbor-joining phylogenetic tree of NLG homologs from *A. ipsilon* and various insect orders. The number at each branch point represents the bootstrap probability. *Drosophila melanogaster* Gliotactin (DmGLI; GenBank accession number NP_476602) was used as the outgroup to root the tree. The abbreviations and accession numbers of NLG sequences are listed in the Materials and Methods.

the expression level of *AiNLG1* transcript by qPCR in the antenna, ALs, brain with excised ALs, thoracic muscle, midgut, fat body and testis in 5-day-old males. As shown in Fig. 3, *AiNLG1* is expressed at higher levels in both ALs and the rest of the brain than in peripheral tissues including the thoracic muscle and midgut. No amplification of the *AiNLG1* transcript was detected in the antenna, fat body and testis (Fig. 3).

Age-related changes in the behavioral response to sex pheromone and *AiNLG1* expression in ALs

In order to explore the possible role of *AiNLG1* in age-dependent olfactory plasticity, we evaluated the proportion of sex pheromone-guided orientated flight and response delay as well as the level of *AiNLG1* mRNA in ALs in 1- to 5-day-old males. The percentage of orientated behavioral response was only ∼14% and 21% in 1- and 2-day-old sexually immature males and significantly raised at the age of 3 days to reach ∼78% and 75% in 4- and 5-day-old sexually mature males, respectively (Fig. 4A). The response delay of 1- and 2-day-old males (97±11 s and 91±9 s, respectively) was higher than that of 4- and 5-day-old males (45±10 s and 39±12 s, respectively) (Fig. 4B). The expression of *AiNLG1* mRNA in ALs was detected at day-1, and gradually increased from day-2 until day-4 and then decreased slightly at day-5 (Fig. 4C). The level of *AiNLG1* mRNA in ALs of 5-day-old sexually mature males was approximatively threefold higher than that in ALs of 1-day-old sexually immature males (Fig. 4C).

Pre-exposure-related changes in the behavioral response to sex pheromone and *AiNLG1* expression in ALs

In order to ascertain the possible involvement of *AiNLG1* in odor experience-dependent olfactory plasticity, newly emerged males (day-0) were pre-exposed to sex pheromone blend for 3 min, and then the proportion of sex pheromone-guided orientated flight and response delay as well as the level of *AiNLG1* mRNA in ALs were determined at 2, 24, 48 and 72 h after sex pheromone pre-exposure. The percentage of orientated behavioral response of males at 24 h

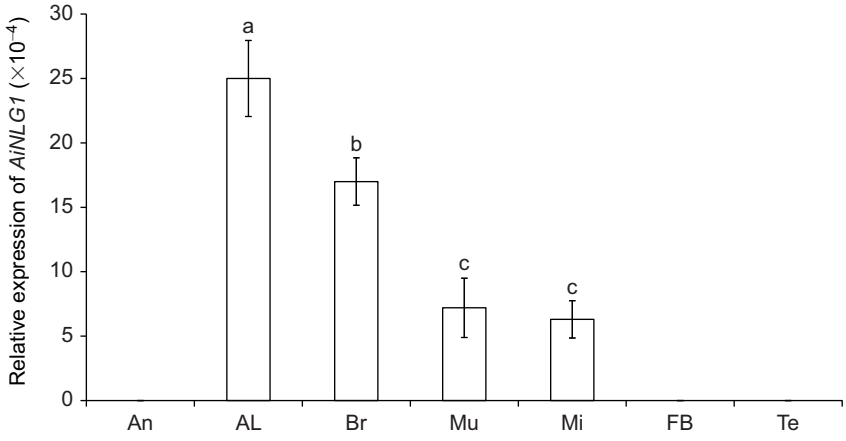

Fig. 3. Tissue expression pattern of *AiNLG1* in 5-day-old *A. ipsilon* males. Total RNA from antennae (An), antennal lobes (AL), brains without antennal lobes (Br), thoracic muscles (Mu), midguts (Mi), fat bodies (FB) and testes (Te) were reverse transcribed to cDNAs for *AiNLG1* expression analysis by qPCR. Each qPCR was run in three technical replicates with seven independent biological replicates. The bars represent means±s.d.; different letters indicate a statistically significant difference (one-way ANOVA; Tukey's test; $P<0.05$).

Journal of Experimental Biology (2021) 224, jeb243184. doi:10.1242/jeb.243184

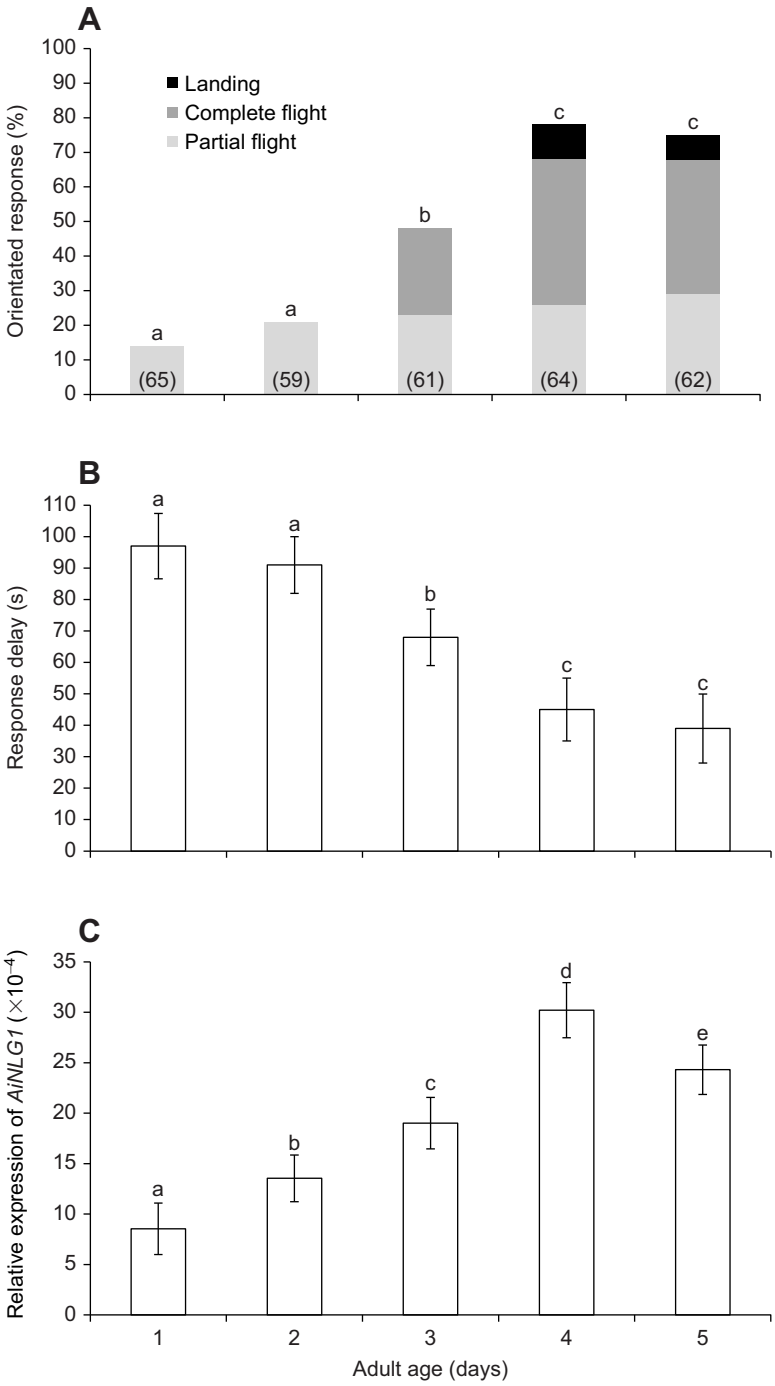

Fig. 4. Effect of age on the sex pheromone response and level of AL *AiNLG1* expression in *A. ipsilon* males. (A–C) The percentage of sex pheromone-guided orientated flight (A), the response delay (B) and the amount of *AiNLG1* mRNA in ALs (C) were evaluated in 1- to 5-day-old males. Each qPCR was run in three technical replicates and seven independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a statistically significant difference (G-test; $P<0.05$). For the response delays and the AL *AiNLG1* mRNA levels, the bars represent means±s.d.; different letters indicate a statistically significant difference (one-way ANOVA; Tukey's test; $P<0.05$).

(PE-24h), 48 h (PE-48h) and 72 h (PE-72h) following pre-exposure was significantly higher than that of day-1, day-2 and day-3 control males (C1, C2 and C3), respectively (Fig. 5A). This increase in the behavioral responsiveness to sex pheromone was accompanied by a decrease in response delay and a rise in the amount of AL *AiNLG1* mRNA in PE-24h, PE-48h and PE-72h males compared with C1, C2 and C3 males, respectively (Fig. 5B,C). By contrast, there was no significant difference in the behavioral response, response delay and AL *AiNLG1* mRNA level between PE-2h and CO males (Fig. 5A–C). The quantity of *AiNLG1* mRNA in ALs of PE-72h males was ∼1.5-fold higher than that in ALs of C3 males (Fig. 5C).

Mating-related changes in the behavioral response to sex pheromone and *AiNLG1* expression in ALs

In order to assess the possible function of *AiNLG1* in mating-dependent olfactory plasticity, virgin 4-day-old sexually mature males were individually paired with virgin 3-day-old sexually mature females, and then we determined the proportion of sex pheromone-guided orientated flight and response delay as well as *AiNLG1* mRNA expression level in ALs of mated males at the end of the copulation, and at 1 h and 23 h later. The percentage of orientated behavioral response of males at 0 h (PM-0h) and 1 h (PM-1h) post-mating was low in comparison with that of virgin 4-day-old sexually mature males (C4) and then increased sharply at

Journal of Experimental Biology (2021) 224, jeb243184. doi:10.1242/jeb.243184

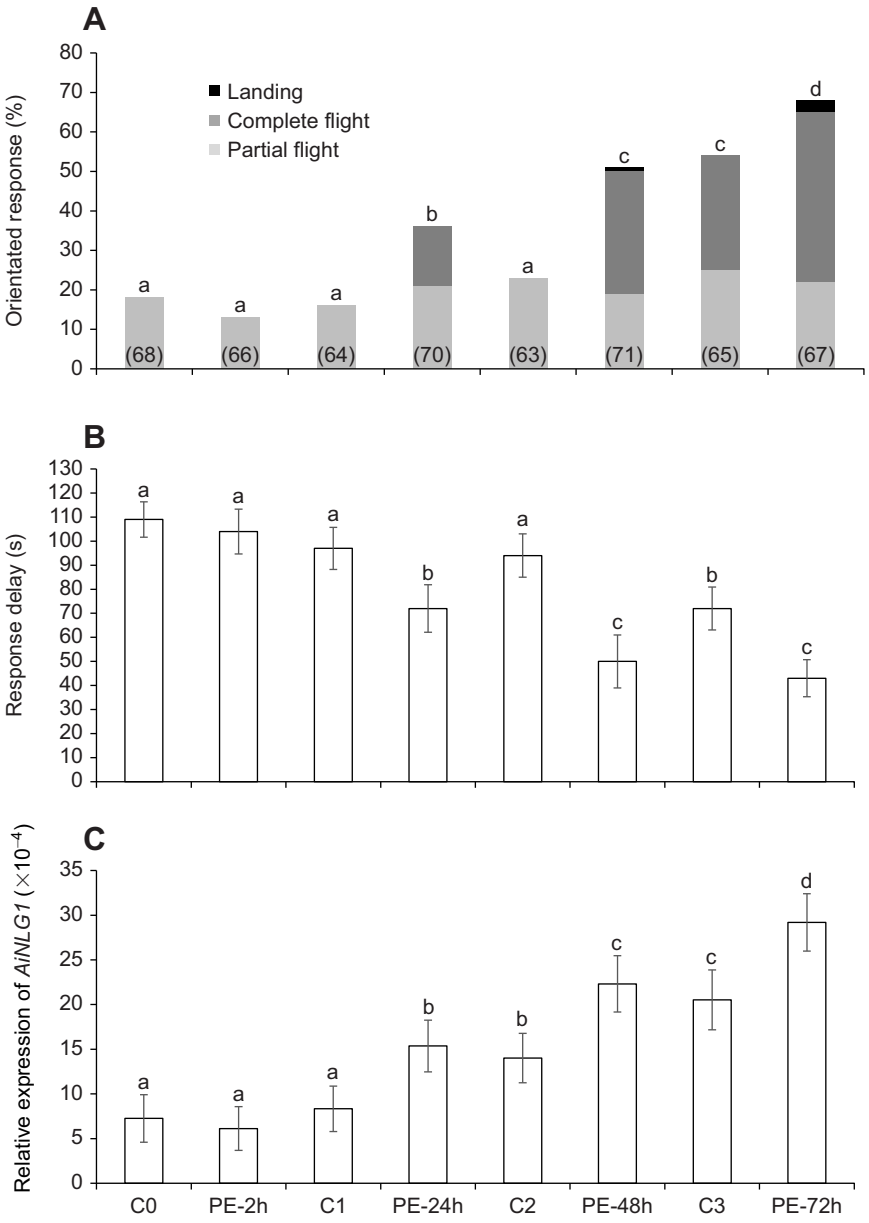

Fig. 5. Effect of pre-exposure on the sex pheromone response and level of AL *AiNLG1* expression in *A. ipsilon* males. Newly emerged males (day-0) were pre-exposed to the sex pheromone blend for 3 min. (A–C) The proportion of sex pheromone-guided orientated flight (A), the response delay (B) and the amount of *AiNLG1* mRNA in ALs (C) were determined at 2, 24, 48 and 72 h after sex pheromone pre-exposure (PE). Unexperienced 0-, 1-, 2- and 3-day-old males (C0, C1, C2 and C3, respectively) were used as controls. Each qPCR was run in three technical replicates and seven independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a statistically significant difference (G-test; $P<0.05$). For the response delays and the AL *AiNLG1* mRNA levels, the bars represent means±s.d.; different letters indicate a statistically significant difference (one-way ANOVA; Tukey's test; $P<0.05$). The statistical analyses were carried out through a global comparison followed by individual comparisons between unexperienced and pre-exposed males at each post-exposure time.

23 h (PM-23h) to reach that of virgin 5-day-old sexually mature males (C5) (Fig. 6A). PM-0h and PM-1h males responded behaviorally more slowly to sex pheromone than C4 males, whereas PM-23h males responded as quickly as C5 males (Fig. 6B). The AL *AiNLG1* mRNA level was not significantly different in PM-0h and PM-1h males compared with C4 males, and in PM-23h compared with C5 males (Fig. 6C).

DISCUSSION

In this study, we examined whether NLG1 might be a mediator of the plasticity of the olfactory system in insects. In order to test this hypothesis, we turned to the long-living noctuid moth *A. ipsilon*, a model organism for which the behavioral and neuronal response to the female emitted-pheromone signal are known to be dependent on age, odor experience and reproductive status of the male.

AiNLG1 belongs to the NLG1 subfamily

We first succeeded in isolating a single 5047 bp cDNA from the nervous tissue of the adult male *A. ipsilon*, and this clone was found to encode a predicted AiNLG1 protein that exhibits all the signature motifs of the NLG family. The large extracellular AChE-like domain of AiNLG1 has sequence homology to the typical α/β-hydrolase fold structure of the carboxyl-cholinesterase superfamily, but it is devoid of the active site residues for enzymatic activity (Varoqueaux et al., 2004; Boucard et al., 2005) and contains two EF-hand metal-binding motifs, which are Ca^{2+}-binding sites that are presumed to be essential for the interaction of NLG with its cognate receptor neurexin. The C-terminus PDZ domain-binding motif of AiNLG1 is known to be critical for synaptic protein recruitment through the binding of NLG to PDZ domain-containing scaffolding proteins, which, in turn, cluster together post-synaptic transmitter receptors, ion channels and signaling proteins (Irie et al., 1997; Kim and Sheng, 2004; Meyer et al., 2004). Finally, the overall protein sequence of AiNLG1 was found to exhibit a degree of similarity with NLG1 proteins previously identified in the lepidoptera *H. armigera*, with more than 89% identity, and also those identified in other insect orders including Diptera, Hymenoptera and Coleoptera. These homology sequence data were further supported by our

Journal of Experimental Biology (2021) 224, jeb243184. doi:10.1242/jeb.243184

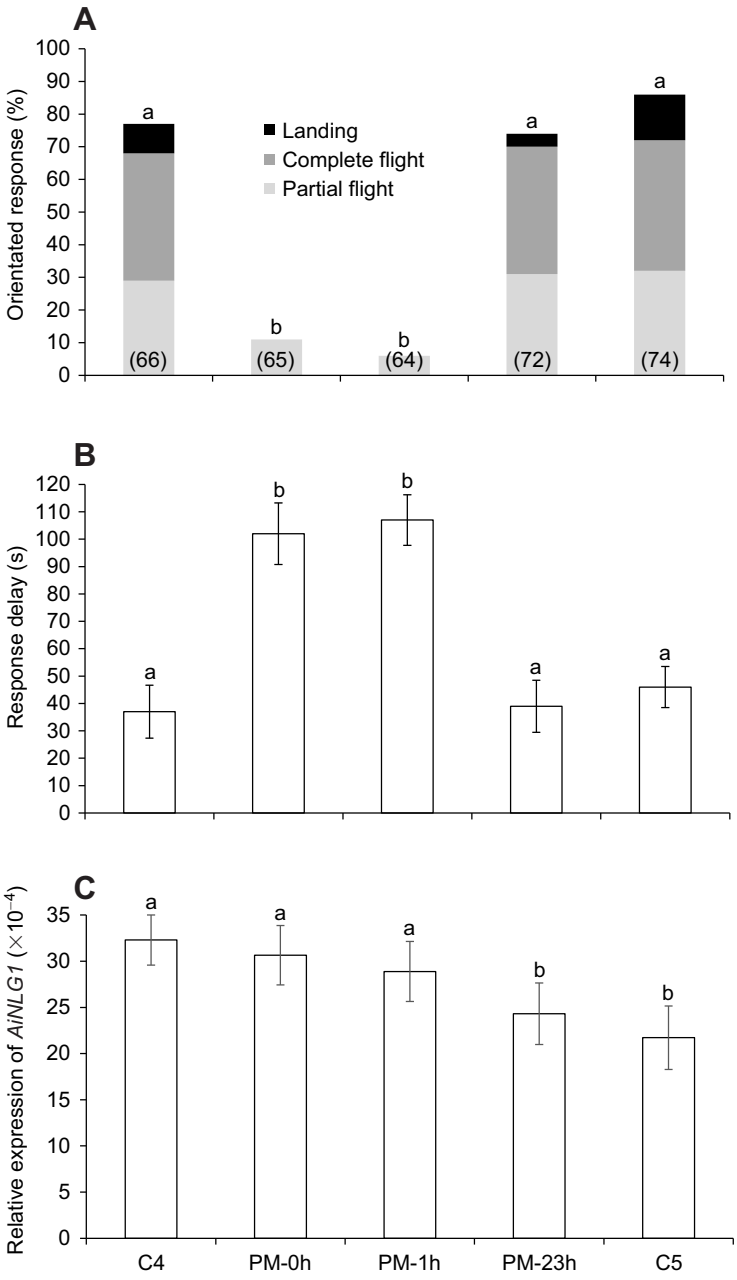

Fig. 6. Effect of mating on the sex pheromone response and level of AL *AiNLG1* expression in *A. ipsilon* males. Virgin 4-day-old sexually mature males were individually paired with virgin 3-day-old sexually mature females. Once copulation had ended, mated males were isolated from females. (A–C) The proportion of sex pheromone-guided orientated flight (A), the response delay (B) and the amount of *AiNLG1* mRNA in ALs (C) were determined immediately (0 h) and at 1 h and 23 h post-mating (PM). Control groups consisted of virgin 4- and 5-day-old sexually mature males (C4 and C5, respectively). Each qPCR was run in three technical replicates and seven independent biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a statistically significant difference (G-test; $P<0.05$). For the response delays and the AL *AiNLG1* mRNA levels, the bars represent means±s.d.; different letters indicate a statistically significant difference (one-way ANOVA; Tukey's test; $P<0.05$).

phylogenetic analysis demonstrating that AiNLG1 belongs to the cluster of the lepidopteran NLG1s. In addition, our phylogenetic tree revealed that NLG sequences diversified independently during evolution in insects from a common ancestral NLG sequence as observed for vertebrate NGLs (Knight et al., 2011). It is also interesting to note that two *Nlg4* forms have been characterized in *H. armigera* (Pearce et al., 2017) as well as in *B. mori* (Tsubota and Shiotsuki, 2010), *Plutella xyllostella* (You et al., 2013) and *Danaus plexippus* (Zhan and Reppert, 2013), indicating the existence of a duplication event of the *Nlg4* gene that would have recently occurred in the Lepidoptera lineage.

AiNLG1 is expressed within the peripheral and central nervous system in adult *A. ipsilon*

As for all the members of the NGL1 subfamily, the transcriptional activity of *AiNLG1* is tissue dependent (Chih et al., 2005; Biswas et al., 2008; Durand et al., 2016), measured here in the brain and peripheral organs at adult stage. Elevated levels of the *AiNLG1* transcript were detected more specifically in ALs, the analogs of the vertebrate olfactory bulb, thus suggesting a possible involvement of the AiNGL1 protein in the proper assemblage and activity of neuronal networks within the primary olfactory neuropiles in *A. ipsilon*. This is consistent with previous reports of *NLGN1* mRNA abundance in the mitral cells of the rat olfactory bulb, which are functional equivalents of insect PNs, during early post-natal development (Clarris et al., 2002). We cannot rule out the possibility that AiNLG1 also acts in other brain areas, as evidenced by the observation that its mRNA was detected in brain after excision of the ALs. This speculation is supported by *in situ* hybridization experiments performed in *A. mellifera* demonstrating that the expression of *NLG*s, and especially *AmNLG3*, is not restricted to ALs, but is also present at high levels within higher-order brain regions, and more precisely in the Kenyon cells of mushroom bodies (Biswas et al., 2008), which are known to be

essential for multisensory integration as well as learning and memory. We plan in future studies to perform *in situ* mapping of *AiNLG1* expression in the entire brain of adult *A. ipsilon*.

It is interesting to note that, in addition to its expression in the brain, *AiNLG1* mRNA is present in the midgut. It is well established that the general organization and the roles of the enteric nervous system (ENS) of insects are clearly analogous to those of the vertebrate ENS. Thus, the insect ENS is formed by interconnected networks of ganglia and diffuse nerve plexuses that extend superficially on the layers of longitudinal and circular striated muscles surrounding the gut epithelium, and many of the vital functions of this organ, including rhythmic propulsive movements, ingestion, digestion, absorption and defecation, are controlled by the activity of the intrinsic neurons of the ENS (Copenhaver, 2007). In the human ENS, NLGN1 is enriched within the post-synaptic boutons of excitatory glutamatergic synapses that are inserted between the longitudinal and circular muscle layers of the intestine. Reduced abundance of NLGNI was associated with altered synaptic development, leading to intestinal contractile dysfunction characterized by obstructions and the toxic megacolon syndrome in patients with Hirschsprung's disease (Wang et al., 2015). Similar to the action of its human NLGN1 homolog, AiNLG1 might be an actor of the excitatory synaptic transmission within the ENS in the control of gut motility in *A. ipsilon* males.

We also observed minor expression of *AiNLG1* in skeletal muscles of *A. ipsilon*. Unlike the cholinergic excitatory innervation of the vertebrate skeletal muscle, the insect skeletal muscle is innervated by glutaminergic excitatory motor neurons and may receive an additional nerve supply by GABAergic inhibitory neurons and axonal terminals of neuromodulatory neurons including octopaminergic neurons (Pflüger and Duch, 2011; Wolf, 2014). In *D. melanogaster*, it has been observed that the neuromuscular junctions establish on all muscles of *dnlg1* mutants in a usual timing, but exhibit marked deficits in synapse density, synaptic transmisssion and post-synaptic apparatus, including scaffolding proteins and glutamate receptors, and that restoration of *dlng1* expression in mutants induced an ectopic post-synaptic differentiation (Banovic et al., 2010). It seems reasonable to hypothesize that, like *Drosophila dlgn1*, *AiNLG1* is probably not required for initiating the formation of the excitatory glutaminergic neuromuscular synapses but is rather indispensable for their functional maturation by promoting the post-synaptic specializations.

Finally, this tissue-specific gene expression analysis highlights that AiNLG1 may potentially serve as an organizer for central and peripheral excitatory trans-synaptic signaling involved in the functioning of the olfactory system, as well as intestinal and neuromuscular motor control, in *A. ipsilon* males.

AiNLG1 overexpression in ALs is concomitant with increase in sex pheromone responsiveness throughout adulthood

We noticed that *AiNLG1* mRNA in ALs is at higher amounts in old sexually mature males than in young sexually immatures males. This result points to the existence of an age-dependent upregulation of *AiNLG1* expression within ALs, which is concomitant with the age-dependent increase in neuronal and behavioral responses to the pheromone signal. Such a correlation led us to assume that increased *AiNLG1* expression reflects an anatomical and functional shaping of synaptic connections, notably through the maturation of excitatory glutaminergic synapses that takes place within AL glomeruli, and thus facilitates the processing of pheromone information and the emergence of orientated flight behavior. In concordance with this assumption, it has been demonstrated in male rats that the expression of the NLGN1 protein in neocortex, and especially within the primary olfactory cortex, is timely upregulated with the synaptogenesis accompanying the pubertal maturation of reproductive behavior (Song et al., 1999). In order to prove a functional involvement of *AiNLG1* in age-related plasticity of sex pheromone responsiveness, we will evaluate the effects of its knockdown using RNA interference technology, which has been shown to be highly efficient for gene silencing in *A. ipsilon* brain, on both the activity of PNs in the MCG by intracellular recordings and the behavioral response to the pheromone signal. In line with this experimental perspective, *nlg-1* mutants of *Caenorhabditis elegans* were found to have deficits in the processing of chemosensory cues (Hunter et al., 2010).

In mammals, it is known that steroid hormones play a major role in fetal and neonatal brain development by controlling many neurodevelopmental processes, including apoptosis, growth, neuronal differentiation and synaptogenesis, via the activation of GABAergic and glutaminergic systems (González et al., 2007; McCarthy, 2008; Konkle and McCarthy, 2011). A series of recent reports has shown that *NLG*s are steroid-regulated genes (Kang et al., 2004; Bethea and Reddy, 2012) and that altered prenatal steroidogenic activity is associated with a decline in the brain *NLG* expression levels, thus contributing to the appearance of autistic disturbances in childhood (Sellers et al., 2015; Baron-Cohen et al., 2020). In *A. ipsilon*, growing evidence has accumulated these past years about the involvement of a steroidal system, and in particular 20-hydroxyecdysone (20E), the main insect steroid hormone, in male sexual development. Indeed, it has been uncovered that 20E is necessary to enhance high sensitivity in sex pheromone responsiveness, and that this stimulatory action is at least part transduced by the binding of 20E to the Ecdysone Receptor (EcR)/Ultraspiracle (USP) complex in ALs and, ultimately, engenders the upregulation of synaptic plasticity-related proteins such as synaptotagmins (Duportets et al., 2013; Abrieux et al., 2014; Bozzolan et al., 2015). Other interesting data are that age-dependent increases in the circulating level of 20E match fluctuations in *AiNLG1* expression amount in ALs (Vitecek et al., 2013). By taking into account the above-mentioned data, it seems reasonable to suggest the existence of a possible functional interplay between the 20E- and NLG1-initiated signaling pathways that operate in ALs. Thus, we propose that the activation of 20E–EcR–USP signaling in response to the elevation of circulating 20E level is responsible for the increased *AiNGL1* expression, which is probably linked to the synaptic plasticity occurring in ALs during male sexual maturation in *A. ipsilon*. To verify whether *AiNLG1* is a potential molecular target of the 20E genomic signaling pathway, we will examine the impact of 20E on the transcriptional activity of *AiNGL1* in ALs by injecting 20E into young sexually immature males, in which the circulating hormone level is low, or by removing the testes, which are recognized as the primary organs for steroidogenesis in male moths (Loeb et al., 1988; Yamamoto et al., 2017), in old sexually mature males.

AiNLG1 overexpression in ALs is paralleled with increase in sex pheromone responsiveness after odor pre-exposure

We observed that young sexually immature males displayed an increase in their behavioral response to sex pheromone at 24, 48 and 72 h after a brief exposure to this odor, and this long-term effect was associated with a pronounced expression of the *AiNLG1* mRNA in ALs compared with that observed in naive males. Histological and neurophysiological experiments conducted on *S. littoralis* have unveiled that the increased behavioral response of males to sex

pheromone after odor pre-exposure coincided with an increase in both the sensitivity of AL neurons and the size of MCG glomeruli (Anton et al., 2016). It has been uncovered that the expansion of AL glomeruli in young *Drosophila* females through olfactory pre-exposure is in part due to an increase in the number of neuronal branching and synaptic contacts (Devaud et al., 2001, 2003). Taken together, all the above data lead us to assume that the short pre-exposure of young *Agrotis* males to the female sex pheromone induces early *AiNLG1* overexpression within AL glomeruli, thus triggering NLG1-mediated signaling that participates in the shaping of synaptic architecture and consequently promoting pheromone information processing and the expression of orientated flight behavior. This assumption is strengthened by earlier data in *A. mellifera* demonstrating that sensory experience- and odor associate learning-driven brain plasticity is accompanied by upregulation of *AmNLG1* expression (Biswas et al., 2010; Reinhard and Claudianos, 2012).

A recent study reported that, in young worker bees exposed to queen mandibular pheromone (QMP), QMP-evoked physiological and behavioral effects are in part initiated by augmentation in the circulating level of ecdysteroids, including 20E, via regulation of biosynthesis rate (Trawinski and Fahrbach, 2018). An interesting observation in *A. ipsilon* is that young males pre-exposed to sex pheromone exhibit higher amounts of 20E in both hemolymph and brain than do naive males (E. Gassias, unpublished data). This raises the possibility that sex pheromone exposure evokes an elevation of circulating 20E that might result in the activation of AL NLG1-mediated signaling via 20E-induced *NLG1* overexpression, thus contributing to the emergence of sex pheromone-guided flight behavior in young *A. ipsilon* males. In order to ascertain the existence of such an interaction between 20E and NLG1 signals through early olfactory experience, in future investigations we will examine whether 20E deprivation, through chirurgical castration, prevents the sex pheromone-induced effects on the level of *AiNLG1* expression in ALs as well as on the sex pheromone behavioral response in pre-exposed males.

AiNLG1 expression in ALs is not linked to the transient post-mating inhibition of sex pheromone responsiveness

As in many other species, *A. ipsilon* males have a post-ejaculatory refractory period, preventing them from re-mating until the next scotophase, by which time they replenish their sex accessory glands for a potential new ejaculate (Gadenne et al., 2001; Vitecek et al., 2013). This transient inhibition of male sexual behavior results from a decline in the sensitivity of sex pheromone-responding AL neurons: most PNs have much higher pheromone response thresholds after mating (Barrozo et al., 2010). Our results showed that the transcriptional activity of *AiNLG1* in ALs of mated males is unchanged at 0, 1 and 23 h after copulation compared with that of virgin males. Thus, there is a high probability that mating-triggered switch off of the central processing of pheromone information during the refractory period, as well as the restoration of sex pheromone responsiveness on the following night, are not driven by alterations in the AL NLG1-initiated trans-synaptic signaling through modulation of *AiNLG1* expression. In concordance with this, we observed no significant variation in the amounts of transcripts encoding other synaptic plasticity-related proteins, including NLG3, which is known to be present at both glutamatergic and GABAergic synapses, and synaptotagmin I, in ALs of *A. ipsilon* mated males (E. Gassias, unpublished data). All these data are also supported by the fact that this mating-related functional plasticity of the central olfactory system occurs rapidly, within 15 min after copulation (Vitecek et al., 2013), and probably without requiring any long-lasting structural modifications (i.e. glomerular size, synapse number) compared with the other forms of olfactory plasticity in relation to sexual development and odor experience in *A. ipsilon* adult males.

Finally, the present study reveals that, in long-living male moths, the increased behavioral and neuronal sensitivity to sex pheromone through sexual maturation and odor experience is linked to the upregulation of the expression of *NGL1* within the primary olfactory neuropiles and thus highlights a role played by this post-synaptic CAM in mediating the plasticity of the central olfactory system, for which the underlying molecular mechanisms appear to be highly conserved between insects and vertebrates.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.D., P.A., E.D., T.B., F.B., S.D.; Methodology: P.A., E.D., T.B., F.B.; Validation: N.D., P.A., S.D.; Formal analysis: N.D., P.A., T.B., F.B.; Investigation: F.B., S.D.; Writing - original draft: N.D., S.D.; Visualization: F.B.; Supervision: S.D.

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Data availability

Agrotis ipsilon neuroligin 1 has been deposited in GenBank, accession no. MZ423824.1.

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TGGTCGTACCTGCATCGTCGTCACACATCGCTCATTCTACCTTGCGACACATCTCTTGGCTCGTCACACTCTCATATCGCTCGGGTACTCGACTTTCGTCGACACAA
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 181 - ATGGCCACTAACCGGCACGCGACTTCGTTTATAAATCACAACATGTTTATTTGTATTTTCGTCTCGCTCTTGTTGCCGATGGCGAACGAAGGTGCCGCG
   1 -  M  A  T  N  R  H  A  T  S  F  I  N  H  N  M  F  I  C  I  F  V  S  L  L  L  P  M  A  N  E  G  A  A

 280 - AATGTGTACGGTGACAGCCGAAGTTTTCCGAACGATGACGGAGGGAAAAGATTGTCGAGGGAATACCACCTGAGACAGGGTGCGCTTAGAGGATTGATA
  34 -  N  V  Y  G  D  S  R  S  F  P  N  D  D  G  G  K  R  L  S  R  E  Y  H  L  R  Q  G  A  L  R  G  L  I

 379 - GTGAAACCAAGTCGGCAGTATGATTTTCAGTATGTGGAAATGTTTCTTGGCATACCGTATGCTGCTCCGCCGACGGGGCACCTAAGGTTTATGCCACCA
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 577 - TCAGGTCGGCAGCACTACTTGAACCAGCTGAAGACATTCCTCACTAATGAATCTGAAGACTGCCTGTACTTGAATATTTATGTTCCTTATAGAGAACAA
 133 -  S  G  R  Q  H  Y  L  N  Q  L  K  T  F  L  T  N  E  S  E  D  C  L  Y  L  N  I  Y  V  P  Y  R  E  Q

 676 - AAAACAAAGAAGTTTTCCGTCCTGGTGTTCATACACGGTGACTCCTTCGAGTGGAGTTCTGGCAACCCTTATGATGGACGGATGCTGGCCTCGTACGGG
 166 -  K  T  K  K  F  S  V  L  V  F  I  H  G  D  S  F  E  W  S  S  G  N  P  Y  D  G  R  M  L  A  S  Y  G

 775 - AATGTCATGGTCATCACTGTCAACTTCAGACTGGGAATTTTGGGTTTCATGAAGCCAAGCCTAACAGAACACGTGTATGGTAACAACGGGTTGCTAGAT
 199 -  N  V  M  V  I  T  V  N  F  R  L  G  I  L  G  F  M  K  P  S  L  T  E  H  V  Y  G  N  N  G  L  L  D

 874 - CAGTTGGCAGCACTCCAGTGGATCAAGGATAATATTGAGGATCTAAACGGAGATCCATCATCAGTTACCTTGATGGGACATGGCACTGGAGCAGCCTGT
 232 -  Q  L  A  A  L  Q  W  I  K  D  N  I  E  D  L  N  G  D  P  S  S  V  T  L  M  G  H  G  T  G  A  A  C

 973 - GTTAATTTCTTGATGCTTTCTCCTATATCAAATGGGTTATTCCATCGAGCAATACTGATGTCGGGTTCTGCGCTATCAGACTGGGCAATGACAAAGGAC
 265 -  V  N  F  L  M  L  S  P  I  S  N  G  L  F  H  R  A  I  L  M  S  G  S  A  L  S  D  W  A  M  T  K  D

1072 - CCAACGCCGTATACACTACAAGTGGCCCAGAGTTTGGGCTGCAATCCTAGCTCTAAGAATATGATGGCTTGCTTGCAGAAGAAACCATTATCAGAAATC
 298 -  P  T  P  Y  T  L  Q  V  A  Q  S  L  G  C  N  P  S  S  K  N  M  M  A  C  L  Q  K  K  P  L  S  E  I

1171 - AAAAAAGTCCAGATCTTAGCCCGAGAGTTTGAGACACCACTGGGTCCTGTTGTAGCTGGCTCGTTCATACCGAATGAGCCAGCCAAGACTATGGAGTCT
 331 -  K  K  V  Q  I  L  A  R  E  F  E  T  P  L  G  P  V  V  A  G  S  F  I  P  N  E  P  A  K  T  M  E  S

1270 - TATCCGAATCTGTTGAGCAAATATCAACTTCTAAGCGGTGTGACAGAAATGGAAAGGTATCACGACTTCGGCGTAATTGAGTTAGAGCATGGAGTGCTA
 364 -  Y  P  N  L  L  S  K  Y  Q  L  L  S  G  V  T  E  M  E  R  Y  H  D  F  G  V  I  E  L  E  H  G  V  L

1369 - GAGAACCAAAGAGATGACTTTATCAAGAAGTATGCTAAGATCATCTTTGAAGGTGCAGAGGATGAAACGCTGAAGTCTATTTTGAAAGAATATGCACCA
 397 -  E  N  Q  R  D  D  F  I  K  K  Y  A  K  I  I  F  E  G  A  E  D  E  T  L  K  S  I  L  K  E  Y  A  P

1468 - TCGAAGTTAGACCCCCAGCGCTGGAACGTGGAGGCCAACCGAGACGTTATTCTGAACATGTTCAGTGACGCTCGCACTTTGGCCCCCGTCATCCGCTTC
 430 -  S  K  L  D  P  Q  R  W  N  N  V  E  A  N  R  D  V  I  L  N  M  F  S  D  A  R  T  L  A  P  V  I  R  F

1567 - GCCAACTATCAGTCGAAGGCCAACCGTCATTCCTACTTCTATGTCTTTGGACATAATTCTATCAGCTCGGATTACGCTGCGCTCAACAAAAGCGTACAA
 463 -  A  N  Y  Q  S  K  A  N  R  H  S  Y  F  Y  V  F  G  H  N  S  I  S  S  D  Y  A  A  L  N  K  S  V  Q

1666 - GGTCAGGAATTGCCCTATGTATTCGGTGTTCCACTTGGAGCTACGAACACGCACTTCAGTCCTGACTACACCCAGCAGGAGAAGTTACTCAGTGAAGTC
 496 -  G  Q  E  L  P  Y  V  F  G  V  P  L  G  A  T  N  T  H  F  S  P  D  Y  T  Q  Q  E  K  L  L  S  E  V

1765 - CTCATGAGGATGTGGACCAACTTCGTCAAATATGGGTCTCCCAACTCACAGGCTCCAGTCAAGTTCTACAATTTGGATAGAAGACATTGGAGCCTCTAT
 529 -  L  M  R  M  W  T  N  F  V  K  Y  G  S  P  N  S  Q  A  P  V  K  F  Y  N  L  D  R  R  H  W  S  L  Y

1864 - GATCTTGACTGGCCAGAATACGACAGTACTGGACAGTCATACTTAAGGTTCGACATTCCACCTGAAGTAGATACGTCATACCGGTCAAACTATACCAAG
 562 -  D  L  D  W  P  E  Y  D  S  T  G  Q  S  Y  L  R  F  D  I  P  P  E  V  D  T  S  Y  R  S  N  Y  T  K

1963 - TTTTGGATGGACACATTACCAAACAAGATGAGCAAATACGTAGTGGATCCTCTATTTGAGTACACACCACCTCCTTCCTCGCCGAGGCCCAAAATCACT
 595 -  F  W  M  D  T  L  P  N  K  M  S  K  Y  V  V  D  P  L  F  E  Y  T  P  P  P  S  S  P  R  P  K  I  T

2062 - CACAGAAATACTGCAGGAAATACGGGTGGGAGTGCAGGAGGAAATTCAGACCCAGGCCGTAGAATTTGGACACCGCATGTGCCAGAAACGCCACACTAT
 628 -  H  R  N  T  A  G  N  T  G  G  S  A  G  G  N  S  D  P  G  R  R  I  W  T  P  H  V  P  E  T  P  H  Y

2161 - TCCTCATCATCAATATATGGGAGAATGCGTCCGTATTCGCAGCCAGTACATAAGCCAGACACCAATGTTATATACAAGGAGATTATGTCTATCGAGAAG
 661 -  S  S  S  S  I  Y  G  R  M  R  P  Y  S  Q  P  V  H  K  P  D  T  N  V  I  Y  K  E  I  M  S  I  E  K

2260 - CCTTCGCGACCTGTTCCATCAGGATTAATTGAAAAAACCTTCCTATATACTACTACACCCAAACCTAAAGCCAACATGTCCGTAAAAACGTCCAGCGCG
 694 -  P  S  R  P  V  P  S  G  L  I  E  K  T  F  L  Y  T  T  T  P  K  P  K  A  N  M  S  V  K  T  S  S  A

2359 - ACCATTACATTGATAGTCTCGTTAGCTATTCTCTTTCTCGCCGTCAACGTTGGAATTTGCTCTATATTGTATTTCAAAAAACGGAAGCTCAGACTACGG
 727 -  T  I  T  L  I  V  S  L  A  I  L  F  L  A  V  N  V  G  I  C  S  I  L  Y  F  K  K  R  K  L  R  L  R

2458 - GAACAAACACTCGAGAGTTCTCATCAGCCGCGCGCTGAAATAGGCGAAGTGGATGTAATCGGTCAAAAGAGCTCGAAGGACGACAAAAGCGCTTTGCAA
 760 -  E  Q  T  L  E  S  S  H  Q  P  R  A  E  I  G  E  V  D  V  I  G  Q  K  S  S  K  D  D  K  S  A  L  Q

2557 - ACTCTAAAAAACGGCTGCAGCGTCATCAAGTCGATGAGTTTTAATAAAAAGAAAAATAATTCTAAGAAACATAAAAAGAAATCGGATGTCTGTAAAACG
 793 -  T  L  K  N  G  C  S  V  I  K  S  M  S  F  N  K  K  K  N  N  S  K  K  H  K  K  K  S  D  V  C  K  T

2656 - CCGAAGTCTGATGACTCAGGTGGATTCAGAGAGAGATTTAAGCTAAGACGGCACTTATCAACGAGTACTCTGGACGCTCATACTAAAGTTAGGGACTGG
 826 -  P  K  S  D  D  S  G  G  F  R  E  R  F  K  L  R  R  H  L  S  T  S  T  L  D  A  H  T  K  V  R  D  W

2755 - ATTGCCAATGAAATGATGCATCGATGTTCCCCAGGTATACTAAGAAAATCAAACTCTGATTTAAACGAAAAGCATTCGACTGTGACAAAGCCTTTCACT
 859 -  I  A  N  E  M  M  H  R  C  S  P  G  I  L  R  K  S  N  S  D  L  N  E  K  H  S  T  V  T  K  P  F  T

2854 - AGATCGGAAGAACTACTTTCAGACACAAAGAAAAACAAAAAAGATACCATACTGATGAATGACAGCGTAAATTCGAAGAGTTTTGCGTCAAAAACATAC
 892 -  R  S  E  E  L  L  S  D  T  K  K  N  K  K  D  T  I  L  M  N  D  S  V  N  S  K  S  F  A  S  K  T  Y
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2953 - AGTAAAACTAGAACTTCTGAGAAAACTACATCCACGTCGGCATTAGGCTCGCATTCATCCATAGAAAGTCATAAGCAATCACTCAACAGTATTAAACAA
 925 -  S  K  T  R  T  S  E  K  T  T  S  T  S  A  L  G  S  H  S  S  I  E  S  H  K  Q  S  L  N  S  I  K  Q

3052 - ACAAGTAAATCAAATGAATCCATAAAAAGTAAAGCTACTGAAAGTATTAAGTCTAAAAAAGTGTCAGTAGCTATCGATGCTACACCTGCAGCTCGAACT
 958 -  T  S  K  S  N  E  S  I  K  S  K  A  T  E  S  I  K  S  K  K  V  S  V  A  I  D  A  T  P  A  A  R  T

3151 - CACTCCATATTAAATCAAGAACCAATCGAAATATCGAAATCATTCGACGCTGGTGACCAAATACATTCCAATAAAGTCGAAAAATCAACGGAAGTTGAA
 991 -  H  S  I  L  N  Q  E  P  I  E  I  S  K  S  F  D  A  G  D  Q  I  H  S  N  K  V  E  K  S  T  E  V  E

3250 - TTTAAAGCTCCAAGTACTATCGATCCAATGAAGAATGAACAAACGTTCACAAATGTTGTAACTCTATCAGTTCAAGATAAACCTTTGGTAATTACTCAC
1024 -  F  K  A  P  S  T  I  D  P  M  K  N  E  Q  T  F  T  N  V  V  T  L  S  V  Q  D  K  P  L  V  I  T  H

3349 - AAGCATTCCTCATCTGATCCAGTTACTGATGTGAATTATGATAAATTACTAGAAAAGATGGAGTCTGCTAATCTTGCAAACATCTTACCACCTGTCACT
1057 -  K  H  S  S  S  D  P  V  T  D  V  N  Y  D  K  L  L  E  K  M  E  S  A  N  L  A  N  I  L  P  P  V  T

3448 - TTCAGAAATGATATAAATGTGACTTCTCGTGAAGAAAGTTCACAAGTTAATCCAATGACAGCTGAAGAAGCCTTATTGACTATCAAAAAGAGGAATTTT
1090 -  F  R  N  D  I  N  V  T  S  R  E  E  S  S  Q  V  N  P  M  T  A  E  E  A  L  L  T  I  K  K  R  N  F

3547 - CCTAAAGTATTACCTGATTTGCCAAAGGCGCAAAAGCGTTTATCACTTCAACCGGCGTCGTTGCAAACTTTTCGTGGATATTCGTCCATGGATGATCGT
1123 -  P  K  V  L  P  D  L  P  K  A  Q  K  R  L  S  L  Q  P  A  S  L  Q  T  F  R  G  Y  S  S  M  D  D  R

3646 - CCTAAGGTCCCACCGCAGCCCCCGCCGAGAACTACGACTTTAGAGAGACGTCTTGCATACAAAAACACGAAACCATTATCTTCGTTTGATGCTTCCGGT
1156 -  P  K  V  P  P  Q  P  P  P  R  T  T  T  L  E  R  R  L  A  Y  K  N  T  K  P  L  S  S  F  D  A  S  G

3745 - GTTAAAAGAATAAATGAAGACACTGTTAAAAATTATGAAAACATCGATAGTTTGTCACCTTACACGTATTCTGCAGCGACATCGCGTAATGCTTCCTTC
1189 -  V  K  R  I  N  E  D  T  V  K  N  Y  E  N  I  D  S  L  S  P  Y  T  Y  S  A  A  T  S  R  N  A  S  F

3844 - GACAGTAGTAAAGCGGAAACGTCTATTATATTGACAAAAGACAGAGAGATTCCTCGAGTCATAATAGCATCTAGTGACTTGCCTTCCGCGCAAGAACCC
1222 -  D  S  S  K  A  E  T  S  I  I  L  T  K  D  R  E  I  P  R  V  I  I  A  S  S  D  L  P  S  A  Q  E  P

3943 - AGAATAGTAATCACACCATCGCCCAGCCAGGTCGAGATACCTACAAATGCGCCCAGGGTTCGTCTACCTGCTGATTTCCACGTCCAAGCTGGATCACTC
1255 -  R  I  V  I  T  P  S  P  S  Q  V  E  I  P  T  N  A  P  R  V  R  L  P  A  D  F  H  V  Q  A  G  S  L

4042 - ACGTCTTTCTCATCGTTTTGCTCTGATGAAGATGACGATGACGACATTGATATTGATGAAGAATTTGAAGATAAACTCATACAAGAATTGGTTGGTGGC
1288 -  T  S  F  S  S  F  C  S  D  E  D  D  D  D  D  I  D  I  D  E  E  F  E  D  K  L  I  Q  E  L  V  G  G

4141 - GTTGATTCACCAACAAACATGGATGACGTATTAGATTCTAAGGGCCCGGACTCAATTTTTGAGAAGAAATTGGAAATTGTACCTGTAAAAATTAATCCC
1321 -  V  D  S  P  T  N  M  D  D  V  L  D  S  K  G  P  D  S  I  F  E  K  K  L  E  I  V  P  V  K  I  N  P

4240 - GGTATAGTAGTTCCTAAAAAGAAAGAAGATTACGTATGTGTTAGTGATTTATTTTTACCAGACGCTGGTGATTTAGAAAAAACTGCCCAAATAAAACCA
1354 -  G  I  V  V  P  K  K  K  E  D  Y  V  C  V  S  D  L  F  L  P  D  A  G  D  L  E  K  T  A  Q  I  K  P

4339 - AATTTTGGTTTAAACAAACTGCAGCATAGGACAGATAGCATAAATAGGCCACCAGAAAAAGCAGTTAAAATGAAACAAAGGAAGAGCATAAGAACGCCT
1387 -  N  F  G  L  N  K  L  Q  H  R  T  D  S  I  N  R  P  P  E  K  A  V  K  M  K  Q  R  K  S  I  R  T  P

4438 - TCTATTTTGAACCGTAGTGCTAAAGGTAGAGGTAGTGATAAAAGTAGAGATGATTCAGTGAAACTGGAACATTCTAATTCAGGTTCTTCCAATGATACT
1420 -  S  I  L  N  R  S  A  K  G  R  G  S  D  K  S  R  D  D  S  V  K  L  E  H  S  N  S  G  S  S  N  D  T

4537 - GAAACAAGCACAGGGACTGTGAAAAAGGTAGAATTCAAGAAAGGT
1453 -  E  T  S  T  G  T  V  K  K  V  E  F  K  K  G

TAAATCTCGTCGACACATGCCGTCTGAACAATGCTCGTGCAACATGCGTCAACGTCTGCTCTCGTCGAACATGCTCGACAATGCTCATCGTCGAACTTGCTGCCTGA
ACACACTCGTCGTCAACTACACTGCTCATGCTACTCGTCAACCGTCTCGGCGAGACATCGTCGTCGAACACTCTCGAGACAACTGCTCAACACACTCTCGTCACATC
TCTCAACTCTGCGAGAGACATACAACTCGAACTGCACACTGCTCAGTCGTCAACACAGAGAGTCGTCGATAGCACTGC AATAA GCTCGTCTTACACTCGAGTCGACA
ATGCTCGATCGCTCACACTGCTCGAGAGTCTGCGTACGTCGCACATGGTCACATCTCATCTAACAATTGCACATTCGAGCATCGTCTGCCACATGTCGTCAAGTCGA
CATGCACACTGCTGCTCACTCGAAAAAAAAAAAAAAAA
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Fig. S1. Nucleotide and deduced amino acid sequences of *A. ipsilon* NLG1. Nucleotide (upper line) and amino acid (lower line) numbers are given on the left. The *AiNLG1* cDNA of 5047 bp contains a 180-bp 5'-untranslated region, an open reading frame of 4401 bp and a 466-bp 3'-untranslated region with a polyadenylation signal upstream of the poly(A) tail. The *AiNLG1* ORF was translated into a 1467 amino acid sequence.

Table S1. List of the primers used in the study.

Primer name	Sequence
NLG1dir	5’-TACGGGTGGGAGTGCAGGAGG-3’
NLG1rev	5’-GCCTAATGCCGACGTGGATGT-3’
NLG15’-RACE1	5’-GCGAATACGGGACGCATTCTCC-3’
NLG15’-RACE2	5’-CCAGTCTGATAGCGCAGAACCC-3’
NLG13’-RACE1	5’-CCATACTGATGAATGACAGCG-3’
NLG13’-RACE2	5’-GCGCCCAGGGTTCGTCTACCTG-3’
qNLG1dir	5’-CCTCCTTCCTCGCCGAGGCCC-3’
qNLG1rev	5’-GGAACAGGTCGCGAAGGCTTC-3’
RpL8dir	5’-CCAGTTTGTCTACTGCGGCAA-3’
RpL8rev	5’-GCTTAACCCTAGTACGCTTGGCA-3’