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RESEARCH ARTICLE

A critical role for Dop1-mediated dopaminergic signaling in the plasticity of behavioral and neuronal responses to sex pheromone in a moth

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ABSTRACT

Most animal species, including insects, are able to modulate their responses to sexual chemosignals and this flexibility originates from the remodeling of olfactory areas under the influence of the dopaminergic system. In the moth *Agrotis ipsilon*, the behavioral response of males to the female-emitted sex pheromone increases throughout adult life and after a prior exposure to pheromone signal, and this change is accompanied by an increase in neuronal sensitivity within the primary olfactory centers, the antennal lobes (ALs). To identify the underlying neuromodulatory mechanisms, we examined whether this age- and experience-dependent olfactory plasticity is mediated by dopamine (DA) through the Dop1 receptor, an ortholog of the vertebrate D1-type dopamine receptors, which is positively coupled to adenylyl cyclase. We cloned *A. ipsilon* Dop1 (AiDop1), which is expressed predominantly in brain and especially in ALs; its knockdown induced a decrease in AL cAMP and altered sex pheromone-orientated flight. The levels of DA, *AiDop1* expression and cAMP in ALs increased from the third day of adult life and at 24 and 48 h following pre-exposure to sex pheromone, and the dynamic of these changes correlated with the increased responsiveness to sex pheromone. These results demonstrate that Dop1 is required for the display of male sexual behavior and that age- and experience-related neuronal and behavioral changes are sustained by DA–Dop1 signaling that operates within ALs, probably through cAMP-dependent mechanisms in *A. ipsilon*. Thus, this study expands our understanding of the neuromodulatory mechanisms underlying olfactory plasticity, mechanisms that appear to be highly conserved between insects and mammals.

KEY WORDS: Sexual behavior, Dopamine, Dop1 receptor, Olfactory plasticity, Neuromodulation, Insects

INTRODUCTION

Many animal species, including insects, have developed a sophisticated olfactory system allowing them to display a wide range of vital behaviors such as locating food sources and mating partners (Brennan, 2010; Galizia and Sachse, 2010; Gadenne et al., 2016). Given that the performance of these odor-guided behaviors requires a considerable amount of time and energy, the responsiveness to biologically active chemical cues, at the behavioral and neuronal level, must occur only under certain situations in which it is beneficial. As a direct consequence, the coding and processing of odor-related information is highly malleable and appears to be dependent on external factors, previous experience and coordination with physiological state (Palmer and Kristan, 2011; Gadenne et al., 2016; Ross and Fletcher, 2019). For instance, responses to sexual chemosignals are restricted to periods of fertility in order to optimize the production of offspring. This olfactory plasticity is known to be achieved through anatomical and functional modification of the neuronal network under the influence of hormonal factors and biogenic amines (Dacks et al., 2012; Duportets et al., 2013; Schellino et al., 2016; Harvey and Heinbockel, 2018; Verlinden, 2018), and the interactions between these neuromodulators as well as their action mechanisms are still not fully explored.

In general, the organization of the monoaminergic system of insects has a high similarity with that of vertebrates (Sotnikova and Gainetdinov, 2009). The most notable difference is that in insects, dopamine (DA) is the only physiologically relevant catecholamine present at relatively large amounts throughout the peripheral tissues, sense organs and central nervous system (Blenau and Baumann, 2001; Donly and Caveney, 2005; Mustard et al., 2005). Thus, DA can act as neurohormone, neurotransmitter and neuromodulator to regulate multiple physiological functions as well as innate and cognitive behaviors. DA was found to have potential effects on the maturation of reproductive organs in eusocial hymenopterans (Sasaki et al., 2009; Sasaki and Harano, 2010) and to promote male courtship behavior in the fruit fly *Drosophila melanogaster* (Wicker-Thomas and Hamann, 2008) and behavioral phase changes from solitariness to gregariousness in the migratory locust, *Locusta migratoria* (Ma et al., 2011). Moreover, dopaminergic signaling is involved in regulating olfactory learning and memory within a wide range of insect species (Unoki et al., 2005; Mizunami et al., 2009; Agarwal et al., 2011; Dacks et al., 2012; Keleman et al., 2012; Klappenbach et al., 2013; Awata et al., 2016; Lenschow et al., 2018; Lim et al., 2018; Vinauger et al., 2018).

The neuromodulatory actions of DA are attributed to the multiplicity of DA receptors linked to distinct intracellular signaling pathways, a complex innervation of all major areas of the brain by clusters of DA neurons and dynamic changes in the brain levels of DA (Blenau and Baumann, 2001; Yamamoto and Vernier, 2011; Verlinden, 2018). Dopaminergic signaling is mediated through the activation of G protein-coupled receptors (GPCRs) belonging to the rhodopsin-like subfamily. In vertebrates,

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List of abbreviations

20E	20-hydroxyecdysone
AiDop1	*Agrotis ipsilon* Dop1
AL	antennal lobe
BCA	bicinchoninic acid
cAMP	cyclic adenosine monophosphate
DA	dopamine
DRD1	dopamine D1 receptor
dsRNA	double-stranded RNA
EXL	extracellular loop
GPCR	G protein-coupled receptor
I_{KCa}	Ca^{2+}-dependent K^+ current
INL	intracellular loop
JH	juvenile hormone
MCG	macroglomerular complex
ORN	olfactory receptor neuron
PCA	perchloric acid
PKA	protein kinase A
PKC	protein kinase C
QMB	queen mandibular pheromone
qPCR	real-time PCR
RNAi	RNA interference
SK	Ca^{2+}-dependent K^+ channel

five DA receptor subtypes have been characterized and further classified into two major groups based on their structural and pharmacological properties: D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors (Callier et al., 2003; Beaulieu and Gainetdinov, 2011). In invertebrates, specifically in insects, three distinct groups of DA receptors were identified and referred to as Dop1, Dop2 or invertebrate-type DA receptor, and Dop3 according to the vertebrate DA receptor classification system (Mustard et al., 2005). Members of Dop1 and Dop2 groups are most closely related to the vertebrate D1-like DA receptors and stimulate the activity of adenylyl cyclase, resulting in increased intracellular cyclic adenosine monophosphate (cAMP) levels (Hill et al., 2016; Himmelreich et al., 2017; Xu et al., 2017), whereas Dop3 receptors are functionally D2 like and repress adenylyl cyclase (Beggs et al., 2011; Verlinden et al., 2018; Xu et al., 2017). In addition, a fourth group of DA receptors, DopEcR, was initially discovered in *D. melanogaster* and this receptor has a binding affinity for both DA and 20-hydroxyecdysone (20E) (Srivastava et al., 2005; Evans et al., 2014).

These DA receptor subtypes exhibit significant divergence in their sequence, structure and function (Xu et al., 2017; Verlinden et al., 2018). Recent studies on the functional characterization of the Dop1 receptor have been performed in a few insect species. In *D. melanogaster*, Dop1R1 has been shown to be involved in the dopaminergic pathways controlling various aspects of sexual behavior such as persistence copulation, male–male courtship, and courtship learning and memory (Keleman et al., 2012; Lim et al., 2018). In the mosquito, *Aedes aegypti*, it has been demonstrated that the female has the ability to learn odors associatively with aversive stimuli, thus contributing to shifts in host preferences, and that this plasticity is modulated by DA and requires Dop1 (Vinauger et al., 2018). More recently, *L. migratoria* Dop1 was shown to facilitate the olfactory preference for gregarious volatiles and that this effect was mediated through the inhibition of microRNA-9a maturation leading to the upregulation of adenylyl cyclase expression (Guo et al., 2018).

Over the last decade, evidence has accumulated that the noctuid moths are excellent model organisms for studying the plasticity of the olfactory system (Anton et al., 2007; Gadenne et al., 2016). In

these species, sexual communication relies on the attraction of males by sex pheromones produced and emitted by conspecific females. The pheromone signal is recognized by olfactory receptor neurons (ORNs) located in the antennae and is integrated first in the primary olfactory centers of the brain, the antennal lobes (ALs), which are compartmentalized into spherical neuropil structures, the olfactory glomeruli. The ALs consist of two complexes of glomeruli, the macroglomerular complex (MCG) that is devoted to processing of sex pheromone input from ORNs and the ordinary glomeruli that is involved in processing of all the other odors that the male encounters (Haupt et al., 2010). The processed information is then conveyed by different pathways 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, which is supported by optomotor anemotaxis (Haupt et al., 2010). Despite its stereotypy, this behavior and the underlying processing steps exhibit high degrees of flexibility.

In addition to modulation by circadian rhythm (Groot, 2014) and mating status (Gadenne et al., 2001; Kromann et al., 2015), age- and experience-related plasticity in the male response to sex pheromone has 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 was correlated with a higher sensitivity of ORNs and AL neurons to pheromone signal accompanied by a volumetric increase of MGC glomeruli and of the calyces of the mushroom bodies (Anderson et al., 2007; Anton et al., 2016). It is also known that *A. ipsilon* males are apt to elicit the sex pheromone-mediated orientation flight only when they reach their full sexual maturity within 3–4 days after emergence (Gadenne et al., 1993; Duportets et al., 1998). This increase in behavioral responsiveness is paralleled by a rise in the sensitivity of sex pheromone-responding AL neurons (Gadenne and Anton, 2000). This modulation of sex pheromone perception was found to be under the control of juvenile hormone (JH) (Anton and Gadenne, 1999; Gadenne and Anton, 2000) and 20E (Duportets et al., 2013; Bozzolan et al., 2015), which act in concert with octopamine (Jarriault et al., 2009) and DA, respectively (Abrieux et al., 2014). Moreover, *A. ipsilon* DopEcR was recently identified as a mediator of the combined actions of both 20E and DA in the central nervous system (Abrieux et al., 2013).

To further unveil the molecular mechanisms underlying olfactory modulation by the dopaminergic system in *A. ipsilon* males, we examined whether the age- and experience-related plasticity of behavioral and neuronal responses to sex pheromone is mediated by DA through the Dop1 receptor, which is positively coupled to adenylyl cyclase. We first cloned *A. ipsilon* Dop1 (AiDop1) and determined its tissue expression profile. We then quantified the levels of DA, *AiDop1* expression and intracellular cAMP in the ALs as a function of age (immature versus mature) and experience (naive versus pre-exposed to sex pheromone) in relation to pheromone signal responsiveness. Finally, we examined the effects of the deficiency of Dop1 signaling caused by RNAi-knockdown on AL cAMP levels and the behavioral response to sex pheromone. Our results highlight a major requirement of DA–Dop1 signaling in the flexibility of male sexual behavior, probably by mediating the neuronal plasticity within ALs through cAMP-dependent mechanisms in the moth *A. ipsilon*.

MATERIALS AND METHODS
Insects and tissue collection
Adult *Agrotis ipsilon* (Hufnagel 1766) originated from a laboratory colony established in Bordeaux and transferred to Versailles,

France. The colony is based on field catches in southern France and wild insects are introduced each spring. 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 inverse light:dark cycle (16 h light:8 h dark photoperiod, with scotophase starting at 10:00 h) at 22°C. Newly emerged adults were removed from the hatching containers every day and were given access to a 20% sucrose solution *ad libitum*. The day of emergence was considered as day 0. All 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 profile of *AiDop1*, antennae, ALs, brains with excised ALs, sex accessory glands, thoracic muscles, testes, fat bodies, malpighian tubules and midguts of males were dissected under Ringer's solution, then immediately flash-frozen in Eppendorf (Montesson, France) vials kept in liquid nitrogen and stored at −80°C until treatment. For the collection of ALs, brains were first dissected and ALs were then cut from the protocerebrum with a pair of fine scissors.

cDNA synthesis and cloning of *AiDop1*

Total RNA was extracted with 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 of 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 RNA (1 µg) with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with 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.

Degenerate DNA primers Dop1Gdir1 and Dop1Grev1 were designed against the highly conserved amino acid sequences LIFLSVA and IIYSIFN located in the TM1 and TM7 transmembrane domains of *Bombyx mori* and *Chilo suppressalis* (Table S1). PCR was carried out on 200 ng brain cDNA with 2.5 U of High Expand Fidelity DNA polymerase (Roche, Saint-Quentin Fallavier, France), 0.4 µmol l^{-1} Dop1Gdir1 and Dop1Grev1 primer and 0.25 mmol l^{-1} 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 62°C for 30 s, elongation at 72°C for 1 min 30 s and then final elongation at 72°C for 10 min. The 5′ and 3′ regions of the corresponding cDNA were obtained by 5′- and 3′-RACE (SMART RACE cDNA Amplification Kit, Clontech, Mountain View, CA, USA) following the manufacturer's instructions. For 5′-RACE, we used the specific reverse primer Dop15′-RACE and Universal Primer Mix (UPM, Clontech) as the forward anchor primer (Table S1); for 3′-RACE, we used UPM as the reverse primer and the specific forward primer Dop13′-RACE (Table S1). 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 67°C and 1 min at 72°C, then five cycles of 30 s at 94°C, 30 s at 64°C and 1 min at 72°C, then 25 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C, then 15 min at 72°C.

PCR 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). By merging the overlapping sequences obtained from 5′- and 3′-RACE, a putative full-length cDNA of 2113 bp was generated and named *AiDop1*.

Quantification of *AiDop1* expression

Real-time PCR (qPCR) was performed on cDNA preparations using the ICycler IQTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Each 12 µl reaction consisted of 6 µl Absolute Blue SYBR Green Fluor (Thermo Scientific, Waltham, MA, USA), 4 µl cDNA (25 ng µl^{-1}) and 2 µl of 10 µmol l^{-1} qDop1dir and qDop1rev primers (Table S1). PCR conditions were 35 cycles of 95°C for 30 s, 67°C for 30 s and 72°C for 30 s, and the length of the amplified product was 216 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 pairs. Each run included a negative control (water) and a 5-fold dilution series of pooled cDNA (from all conditions). The 5-fold dilution series were used to construct a relative standard curve to determine 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 at least five independent biological replicates using the technical platform of The Integrative Biology Institute (Sorbonne University, France). Expression levels were analyzed with ICYCLER IQ software (Bio-Rad, Marnes-la-Coquette, France) and the GENORM Visual Basic application for Microsoft EXCEL (Issy-les-Moulineaux, France) as described by Vandesompele et al. (2002). Cycle threshold (Ct) values were determined for the candidate gene 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). The sequences of the specific primers RpL8dir and RpL8rev for *AiRpL8* are given in Table S1.

Measurement of DA levels in ALs

In the middle of scotophase, the brains of males were removed in ice-cold saline solution and then the ball-shaped ALs were cut from the protocerebrum under a dissecting microscope. The ALs were immediately transferred into Eppendorf vials kept on ice and were sonicated in 100 µl of 0.1 mol l^{-1} perchloric acid (PCA) with antioxidants (0.2 g l^{-1} sodium metabisulfite, 0.05 g l^{-1} EDTA). PCA extraction has an excellent recovery (over 90%) and therefore no internal standard was used in this procedure. Eppendorf vials contained 10 ALs (from 5 males) and 10 vials were prepared for each experimental group. After centrifugation at 20,000 *g* for 30 min at 4°C, the supernatant was collected and stored at −20°C until analysis and the protein content of the pellets was measured using Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Samples (15 µl) of supernatant were analyzed with high-performance liquid chromatography (HPLC) using a BAS 460 MICROBORE-HPLC system with electrochemical detection (Bio-analytical Systems Inc., West Lafayette, IN, USA), with a Uniget C-18 reverse phase microbore column as the stationary phase and connected to a computer equipped with chromatography data station software (model D-7000 HPLC system software manger version 2.0, Merck-Hitachi). The mobile phase was pumped with a flow rate

of 0.8 ml min^{-1} and consisted of 0.16 mol l^{-1} monochloroacetic acid, 0.5 mmol l^{-1} EDTA (adjusted to pH 3.4 with glacial acetic acid), 1.4 mmol l^{-1} sodium octylsulfonate, 10 nmol l^{-1} sodium acetate and 8% acetonitrile. The electrochemical detector with a glassy carbon electrode was set to an oxidation potential of +0.85 V relative to an Ag/AgCl reference electrode. Under these conditions, the detection limit for DA was determined by running the known concentrations of DA in the HPLC system and was about 20 fmol. The DA peak was identified by the retention time and quantified by the area peak as related to the external standard (20 pmol). The DA levels in the AL samples were expressed as nmoles per milligram of protein.

Measurement of cAMP levels in ALs
cAMP concentration was assayed using the cAMP Direct Immunoassay Kit (ab65355; Abcam, Cambridge, UK) according to the manufacturer's instructions. Once dissected, ALs were transferred into Eppendorf vials and rapidly homogenized on ice in 100 μl of 0.1 mol l^{-1} hydrochloric acid. The homogenate was then centrifuged at 10,000 g for 30 min at 4°C and the resulting supernatant was directly used in the assay. Using 2-fold serial dilutions, a set of standard solutions of cAMP was prepared for the calibration curve. A standard reaction solution was added to 100 μl of the diluted cAMP standard solution or 100 μl of the supernatant and cAMP levels were then determined by ELISA with absorbance read at 260 nm. cAMP levels, expressed as nanograms per milligram of protein, were obtained from the luminescence measurements by reference to the cAMP standard curve. The protein content of the centrifugation pellets was measured using Pierce™ BCA Protein Assay Kit. cAMP and total proteins were quantified in a sample prepared from 10 ALs for each group with 10 biological replicates.

Age-related experiments
Males of different ages (0, 1, 2, 3, 4 and 5 days) were collected in 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. The males were then killed using liquid nitrogen, and the ALs were dissected from frozen heads for quantification of DA and cAMP and analysis of *AiDop1* expression (see above).

Pre-exposure experiments
Pre-exposure treatments were performed in the wind tunnel at mid-scotophase under the same light and temperature conditions as for behavioral assays. Newly emerged males (day 0) were transferred from the rearing room to the wind tunnel room, and placed in plastic cages (maximum of 3 males per cage) before the end of photophase. A cage was introduced into the wind tunnel. After 30 s, during which the males adjusted to the airflow, a filter paper containing the stimulus was placed 160 cm upwind from the cage. Pheromone stimulation was performed with an artificial pheromone blend containing (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) (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio of 4:1:4. For pre-exposure, we used a dose of 10 ng pheromone blend, which was previously found to elicit the optimum behavioral response (Barrozo et al., 2010). Each male, within the cage inside the wind tunnel, was subjected to pheromone stimulation for 3 min, after which it was removed from the wind tunnel and transferred to the rearing room. The behavioral response to sex pheromone of pre-exposed males was analyzed at 4, 24 and

48 h after the pre-exposure treatment. The males were then killed using liquid nitrogen, and the ALs were dissected from frozen heads for the quantification of DA, cAMP and *AiDop1* expression (see above). Control groups were composed of 0, 1 and 2 day old sexually immature males exposed to hexane for 3 min.

RNA interference (RNAi) experiments
Double-stranded RNA (dsRNA) production and injection conditions were established following protocols used in *A. ipsilon* for the silencing of AiDopEcR (Abrieux et al., 2013). The dsRNAs targeting *AiDop1* (Dop1-1-dsRNA, 375 bp; and Dop1-2-dsRNA, 411 bp) and bacterial *LacZ* (372 bp; control) were produced with MEGAscript® T7 High Yield Transcription Kit (Ambion) according to the manufacturer's instructions. PCR was performed on 1 μl plasmid (50 ng μl^{-1}) with specific primers for each target gene (Dop1-1 T7 dir/Dop1-1 T7 rev, Dop1-2 T7 dir/Dop1-2 T7 rev and LacZ T7 dir/LacZ T7 rev) (Table S1) under the following program: 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min. PCR products were purified with a Nucleospin® extract II kit (Macherey-Nagel GmbH & Co. KG) and quantified by spectrometry. The transcription reaction was then performed overnight at 37°C on 1 μg of PCR product in a reaction mix containing 2 μl of each nucleotide, 2 μl of T7 RNA polymerase enzyme and 2 μl T7 10× buffer in a final volume of 20 μl. After spectrometry quantification and gel dock analysis, the reaction mix was incubated for 15 min at 37°C with 2 U of Turbo DNase (Ambion). dsRNA precipitation was performed by addition of 30 μl diethylpyrocarbonate (DEPC)-treated water and 20 μl LiCl, storage of the mixture at −20°C for 2 h, and then centrifugation for 30 min at 16,000 g. The pellet was washed with 1 ml 75% ethanol, centrifuged and dried before elution in 11 μl DEPC water. Samples were denatured at 95°C for 5 min followed by a rehybridization step of 1 h 30 min at room temperature. dsRNA integrity was checked by loading on an agarose gel. Before injection, dsRNA was diluted to 0.5 μg μl^{-1} in saline solution (7.1 mmol l^{-1} CaCl$_2$, 22 mmol l^{-1} Na$_2$ β-glycerophosphate, 13.5 mmol l^{-1} MgSO$_4$, 26.9 mmol l^{-1} MgCl$_2$, 29.5 mmol l^{-1} KCl, 23.9 mmol l^{-1} glucose, pH 6.8).

Day 2 males were anesthetized with carbon dioxide and injected with 1 μg dsRNA in the lateral region of the intersegment membrane between the second and third abdominal segments. The injections were performed with a Hamilton syringe with a 32-gauge needle. On day 5, the behavioral response of males to sex pheromone was tested in a wind tunnel. The ALs were then dissected for *AiDop1* expression analysis and cAMP quantification (see above). Control groups were composed of non-injected, bacterial LacZ dsRNA-injected and saline-injected males.

Behavioral response to sex pheromone in a wind tunnel
For the wind tunnel experiments, we prepared a pheromone blend diluted in hexane at 1 ng μl^{-1} containing (Z)-7-dodecenyl acetate (Z7-12:Ac), (Z)-9-tetradecenyl acetate (Z9-14:Ac) and (Z)-11-hexadecenyl acetate (Z11-16:Ac) at a ratio of 4:1:4, which has been used successfully in field trapping experiments (Picimbon et al., 1997; Gemeno and Haynes, 1998). Behavioral tests were performed using a 190 cm-long flight tunnel under red light illumination as described above. Experiments were performed during the middle of scotophase when males respond maximally to the sex pheromone (Barrozo et al., 2010). Environmental conditions during the bioassay were held constant at 23°C, 50±10% relative humidity and a wind speed of 0.3 m s^{-1}. A single experimental male was introduced into the wind tunnel and placed in a cage. After 30 s, during which the male adjusted to the airflow, the pheromone blend,

positioned 150 cm upwind from the cage, was delivered for 3 min. During this period, the behavior of males (AiDop1 dsRNA-injected males and control males) was observed, and partial flight (half of the distance between the source and the cage), complete flight (within 5 cm of the source) and landing on the pheromone source were considered as an orientated response towards the sex pheromone. Orientated as well as random flights were all counted together in order to quantify the general flight activity of insects. 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-gauge size) inserted through the septum and connected with polytetrafluorethylene tubing (1.32 mm i.d., 20 cm length). A 10 µl sample of the blend pheromone at 1 ng µl^{-1} was deposited on filter paper and placed inside the vial. This dose (10 ng) was shown to give the best behavioral results with sexually mature virgin males (Barrozo et al., 2010). Contaminated air was removed from the set-up by an exhaust fan after each behavioral assay.

Bioinformatics

The molecular mass of AiDop1 protein was determined with the Compute pI/MW application (http://www.expasy.org/). The amino acid sequences of DA receptors were aligned with MAFFT program (https://mafft.cbrc.jp/alignment/software/). The transmembrane domains were predicted by TMHMM (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). The potential N-linked glycosylation sites (N-x-[S/T]) and potential phosphorylation sites by protein kinase C (PKC: [S/T]-x-[R/K]) were determined using NetNglyc (http://www.cbs.dtu.dk/services/NetNGlyc) and NetPhos (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1), respectively.

Accession numbers (IDs) for protein sequences of dopamine receptors retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) are as follows: the lepidopterans B. mori Dop1 (BmDop1) ID: NP_001108459.1 and C. suppressalis Dop1 (CsDop1) ID: KP784317; the dipteran D. melanogaster Dop1 (DmDop1) ID: CAA54451.1; the coleopteran Tribolium castaneum Dop1 (TcDop1) ID: NP_001280543.1; the hymenopteran Apis mellifera Dop1 (AmDop1) ID: NP_001011595.1; and the orthopteran L. migratoria Dop1 (LmDop1) ID: AKD41292.1.

Statistical analysis

Mean AiDop1 expression and DA and cAMP levels in ALs were compared using one-way analysis of variance 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).

RESULTS

Cloning and sequence analysis of AiDop1

On the basis of the amino acid sequences of Dop1 receptors cloned in the lepidopterans B. mori and C. suppressalis, we designed a pair of degenerate oligonucleotide primers from highly conserved regions in the TM1 and TM7 transmembrane domains and succeeded in amplifying a partial fragment of 864 bp via RT-PCR on brain total RNA extracted from 5 day old adult males. The missing 5′ and 3′ ends of this fragment were then obtained by RACE using gene-specific primers. The 5′-RACE and 3′-RACE reaction products were assembled with the original fragment to generate a full-length cDNA named AiDop1 and deposited in the GenBank database under accession number MN164482. The cDNA extends over 2113 bp and contains a 5′-UTR of 169 bp, an ORF of 1227 bp and a 3′-UTR of 717 bp, with a polyadenylation signal upstream of the poly(A) tail (Fig. S1). The AiDop1 ORF was translated into a predicted protein sequence of 409 amino acids with a calculated molecular mass of 46.5 kDa.

Hydrophobicity analysis of the AiDop1 protein revealed the expected conformation of GPCRs, with seven transmembrane domains (TM1–7) connected with three intracellular loops (INL1–3) and three extracellular loops (EXL1–3), an extracellular N-terminal region and a cytoplasmic C-terminal region (Fig. 1). A series of functionally critical amino acid residues and sequence motifs for the aminergic GPCRs were also identified (Fig. 1). The AiDop1 receptor possesses two aspartate residues (D_{82} in TM2 and D_{116} in TM3) that are shared between all catecholamine receptors and two conserved cysteine residues (C_{109} and C_{195}) that are involved in the formation of a disulfide bond between EXL1 and EXL2. As in all dopaminergic receptors, three serine residues (S_{207}, S_{208} and S_{211} in TM5) with a conserved spacing pattern in the motif S_{207}-S-x-x-S are present in AiDop1. Additionally, AiDop1 harbors the consensus sequence D_{133}RY located at the N-terminus of INL2. In TM6, the motif F_{285}-x-x-CW-x-PFF is highly conserved and the pair of phenylamine residues (F_{291}, F_{292}) represents a unique feature of aminergic receptors. Finally, the characteristic motif N_{325}P-x-x-Y located at the end of TM7 is commonly found in all members of the biogenic amine receptor family. It is known that aminergic GPCRs are subject to regulatory events involving post-translational modifications, such as phosphorylation, palmitoylation and glycosylation. AiDop1 contains two potential sites for N-linked glycosylation, N_{16}ET in the extracellular N-terminal region and N_{249}RT in INL3, and nine potential sites for phosphorylation by PKC such as T_{63}DR in INL1, T_{149}RK in INL2, T_{186}QK in EXL2, S_{236}IR and S_{253}VR in INL3, and T_{345}SR, S_{356}VR, T_{393}PR and S_{400}VR in the intracellular C-terminal region (Fig. 1).

Comparison of the full protein sequence of AiDop1 with insect Dop1 orthologs revealed a high percentage of amino acid identity, especially with the lepidopterans B. mori Dop1 (BmDop1; 96%) and C. suppressalis Dop1 (CsDop1; 95%) followed by the coleopteran Tribolium castaneum Dop1 (TcDop1; 67%), the hymenopteran A. mellifera Dop1 (AmDop1; 60%), the orthopteran L. migratoria Dop1 (LmDop1; 59%) and the dipteran D. melanogaster Dop1 (DmDop1; 53%) (Fig. 1).

Tissue expression pattern of AiDop1

In order to gain some preliminary information about the functions of AiDop1, an initial tissue distribution screen of AiDop1 mRNA in 5 day old males was performed by qPCR. AiDop1 is expressed at higher levels in both the ALs and the rest of the brain than in peripheral tissues including the sex accessory gland, testis and midgut (Fig. 2). No amplification was detected in other investigated tissues (antenna, thoracic muscle, fat body and malpighian tubule) (Fig. 2).
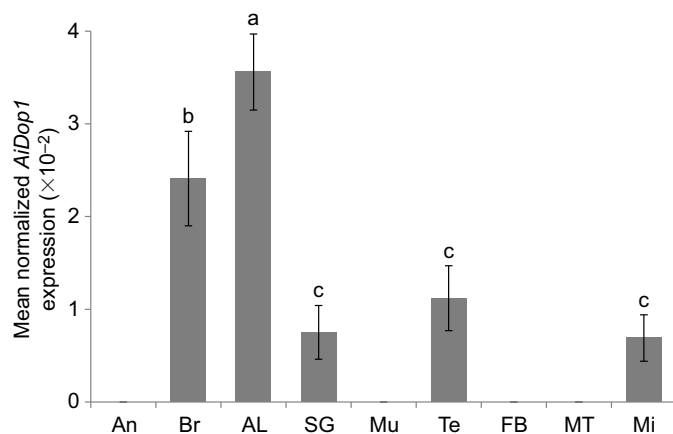
Fig. 1. Analysis and comparison of the protein sequence of *Agrotis ipsilon* Dop1 with other insect D1-like dopamine receptors. The deduced amino acid sequence of AiDop1 was aligned with sequences of orthologous receptors from *Bombyx mori* (BmDop1), *Chilo suppressalis* (CsDop1), *Drosophila melanogaster* (DmDop1), *Tribolium castaneum* (TcDop1), *Apis mellifera* (AmDop1) and *Locusta migratoria* (LmDop1). GenBank accession numbers of the proteins are given in Materials and Methods. The amino acid position is indicated on the right and dashes represent gaps introduced to maximize alignment scores. Amino acid residues conserved between all sequences are printed in white letters on a black background. The transmembrane domains (TM1–7) are delineated by bold black lines and the intracellular (INL) and extracellular loops (EXL) are indicated. Amino acid residues belonging to the binding domain of dopamine (DA) are indicated by squares. Potential N-linked glycosylation sites and potential phosphorylation sites by protein kinase C (PKC) are indicated by stars and circles, respectively. The number at the end of each sequence represents the percent identity (ID) with AiDop1.

Effect of age on the sex pheromone response and DA, *AiDop1* mRNA and cAMP levels in ALs

To ascertain the possible involvement of Dop1-mediated dopaminergic signaling in age-related olfactory plasticity, we evaluated the percentage of sex pheromone-guided orientated flight as well as the levels of DA, *AiDop1* mRNA and cAMP in the ALs of 0–5 day old males. The proportion of the orientated behavioral response was low (below 20%) in 0–2 day old males and significantly increased at the age of 3 days to attain about 75% in 4–5 day old males (Fig. 3A). DA, *AiDop1* mRNA and cAMP were present in ALs in the first days of adult life, and increased

significantly from day 3, remaining high at day 5 (Fig. 3B–D). The level of DA, *AiDop1* mRNA and cAMP in ALs of 5 day old sexually mature males was, respectively, approximately 4.6-, 3.1- and 3-fold higher than in 1 day old sexually immature males (Fig. 3B–D).

Effect of pre-exposure on the sex pheromone response and DA, *AiDop1* mRNA and cAMP levels in ALs

To explore the possible role of Dop1-mediated dopaminergic signaling in experience-induced olfactory plasticity, newly emerged males (day 0) were pre-exposed to sex pheromone for 3 min and we then determined the percentage of sex pheromone-guided orientated

Fig. 2. Tissue expression pattern of *AiDop1* in 5 day old *A. ipsilon* males. Total RNA from antennae (An), brains without antennal lobes (Br), antennal lobes (AL), sex accessory glands (SG), thoracic muscles (Mu), testes (Te), fat bodies (FB), malpighian tubules (MT) and midguts (Mi) were reverse transcribed to cDNA for *AiDop1* expression analysis by real-time PCR (qPCR). Each qPCR was run in three technical replicates with six independent biological replicates. Error bars represent standard errors; different letters indicate a significant difference (analysis of variance; Tukey's test; $P<0.05$).

flight as well as the levels of DA, *AiDop1* mRNA and cAMP in ALs at 4, 24 and 48 h after the pre-exposure. The percentage of males performing an orientated response at 24 h (PE-24 h) and 48 h (PE-48 h) following pre-exposure was significantly higher than that of day 1 and day 2 control males (C1 and C2), respectively (Fig. 4A). AL DA, *AiDop1* mRNA and cAMP in PE-24 h and PE-48 h males were at higher levels than in C1 and C2 males, respectively (Fig. 4B–D). By contrast, there was no difference in the behavioral response and the levels of AL DA, *AiDop1* mRNA and cAMP between PE-4 h and C0 males (Fig. 4). The quantity of DA, *AiDop1* mRNA and cAMP in ALs of PE-48 h males was approximately 3-, 1.9- and 2.4-fold higher than in C2 males, respectively (Fig. 4B–D).

Effect of *AiDop1* silencing on AL cAMP level and the behavioral response to sex pheromone

Using RNAi technology, we tested the impact of *AiDop1* knockdown on both the cAMP level in ALs and the behavioral response of males to sex pheromone. Injection of Dop1-1-dsRNA into 2 day old males resulted in a decrease in *AiDop1* mRNA of approximatively 67% in ALs 3 days later as compared with the three control groups (saline-injected, LacZ dsRNA-injected and non-injected males) (Fig. 5A). The Dop1-1-dsRNA-injected males exhibited a sharp decline in AL cAMP levels and the orientated behavioral response to sex pheromone as compared with control males (Fig. 5B,C). The general flight activity of Dop1-1-dsRNA-injected males (around 85%) was not statistically different from that of control males (around 92%), showing that *AiDop1* silencing did not alter the flight ability of moths (Fig. 5D). In order to consolidate our RNAi results, we also tested the effect of a second AiDop1 dsRNA (Dop1-2-dsRNA). Injection of Dop1-2-dsRNA caused a decrease in AL *AiDop1* mRNA expression and cAMP levels (Fig. S2A,B) and the resulting behavioral phenotype was similar to that induced in the Dop1-1-dsRNA-treated males (Fig. S2C).

DISCUSSION
AiDop1 belongs to the Dop1 group of insect DA receptors
We cloned a full-length cDNA fragment encoding a predicted AiDop1 protein that exhibits the structural features of GPCRs,

including seven hydrophobic transmembrane domains, an extracellular N-terminus and a cytoplasmic C-terminal region. AiDop1 also contains several highly conserved amino acid residues and sequence motifs among the catecholamine receptors. The two aspartate residues (D_{82}, D_{116}) are thought to act as counter-ions in binding the protonated amine group of DA (Fraser et al., 1988; Strader et al., 1988) and the three serine residues (S_{207}, S_{208}, S_{211}) within the signature motif S-x-x-SS are predicted to form hydrogen bonds with the DA ring hydroxyl groups (Strader et al., 1989, 1995). The formation of a disulfide bond between EXL1 and EXL2 by the two cysteine residues (C_{109}, C_{195}) is believed to maintain the receptor under its active conformation (Karnik and Khorana, 1990). The characteristic $D_{133}RY$ and F_{285}-x-x-CW-x-PFF sequences, which are presumed to play a key role in both receptor activation and interaction with the aromatic ring of DA (Kobilka, 2007), are also present in AiDop1. The consensus motif $N_{325}P$-x-x-Y was additionally found in TM7 of AiDop1 and this sequence is known to be critical for ligand binding, G protein coupling and sequestration of the inactive state of the receptor (Fritze et al., 2003; Kalatskaya et al., 2004; Borroto-Escuela et al., 2011). As for the aminergic GPCRs, AiDop1 has a number of consensus motifs for N-glycosylation sites and PKC phosphorylation sites. Thus, potential glycosylation and phosphorylation of AiDop1 might be involved in cell surface expression of the receptor as well as in its desensitization mediated by an uncoupling of the associated G protein (Defea, 2008). Finally, the overall protein sequence of AiDop1 was found to share a high degree of similarity with Dop1 receptors previously identified in the lepidopterans *B. mori* and *C. suppressalis*, with more than 94% identity, but also in other insect orders including Coleoptera, Orthoptera, Diptera and Hymenoptera. Based on all these bioinformatic data, AiDop1 can clearly be assigned to the Dop1 group of insect DA receptors, which is closely related to vertebrate D1-like receptors.

AiDop1 is expressed within the central nervous, digestive and reproductive systems
A single form of Dop1 seems to be present in *A. ipsilon* as reported in the moths *B. mori* (Mitsumasu et al., 2008), *C. suppressalis* (Xu et al., 2017) and *M. sexta* (Dacks et al., 2012), the honey bee *A. mellifera* (Blenau et al., 1998) and the cricket *G. bimaculatus* (Watanabe et al., 2013), whereas two splice variants encoding Dop1R1 and Dop1R2 isoforms have been characterized in the fruit fly *D. melanogaster* (Sugamori et al., 1995; Han et al., 1996). As for all Dop1-type receptors, *AiDop1* exhibits a wide tissue distribution with major expression in the brain (Watanabe et al., 2013; Xu et al., 2017). This elevated transcriptional activity of *AiDop1* was measured more specifically in the ALs, indicating that this receptor is a potential target for any modulatory actions of DA within the central olfactory system. This is consistent with previous studies performed in the moth *M. sexta* and the mosquito *A. aegypti*, demonstrating that *Dop1* is highly expressed in the entire glomerular volume of ALs (Dacks et al., 2012; Vinauger et al., 2018).

It is interesting to note that *AiDop1* was also detected in the organs of the male reproductive tract, and especially in the testes and the sex accessory glands, which produce proteinaceous material that is associated with the production and transport of sperm within a spermatophore, transferred into the female during copulation. In *A. mellifera*, *Dop1* is expressed in different parts of the male reproductive apparatus such as the testes, mucus glands and seminal vesicles in which the high expression of *Dop1* is coupled to increased cAMP levels in response to DA (Matsushima et al., 2019). Thus, it was proposed that DA might promote, via Dop1, the
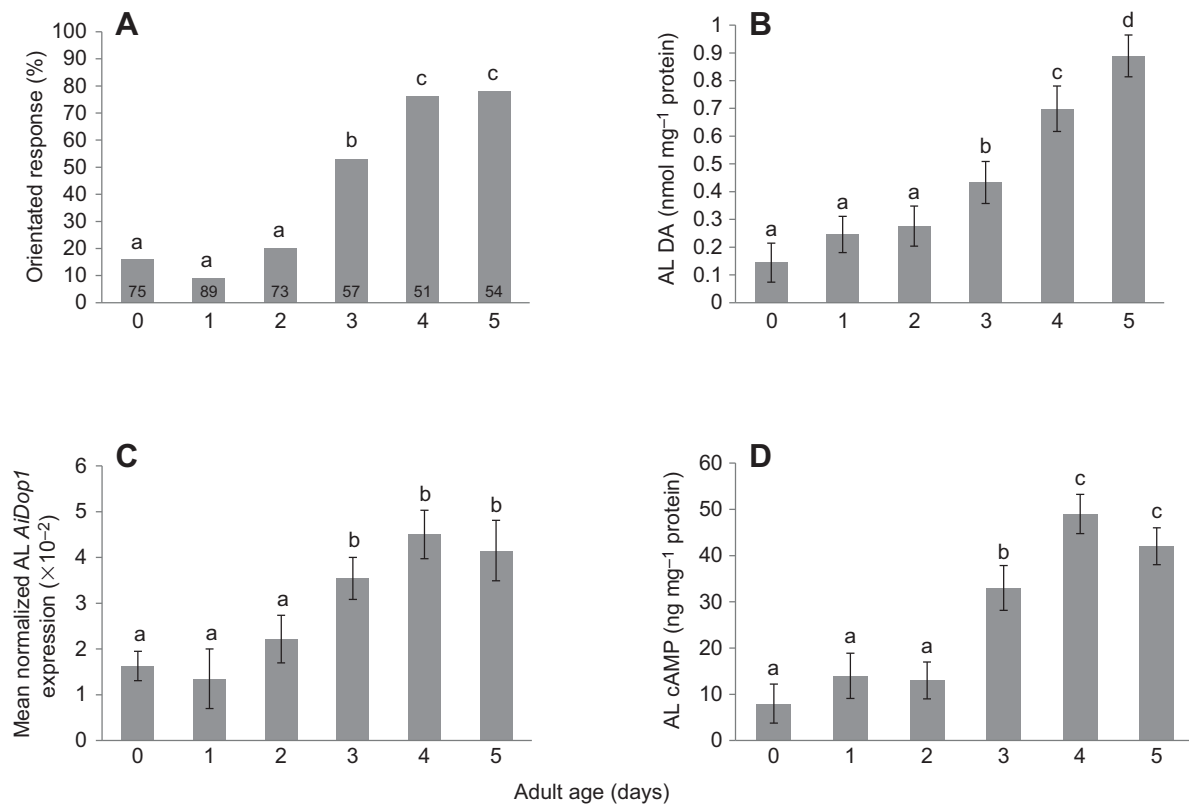
Fig. 3. Age-related plasticity in the sex pheromone response and levels of AL DA, *AiDop1* mRNA and cAMP in *A. ipsilon* males. The behavioral response to sex pheromone and the levels of DA, *AiDop1* mRNA and cAMP in ALs were evaluated for 0–5 day old males. (A) Percentage of orientated responses of males. (B) AL DA level. (C) Relative expression of *AiDop1*. (D) cAMP level. Each qPCR was run in three technical replicates with six independent biological replicates. DA and cAMP levels were quantified from 10 ALs for each experimental group with 10 biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a significant difference (*G*-test; $P<0.05$). For the DA, *AiDop1* mRNA and cAMP levels, the bars represent means±s.d.; different letters indicate a significant difference (analysis of variance; Tukey's test; $P<0.05$).

activities of seminal vesicles including sperm transfer and storage. In the mammalian testis, DA originates from nerve innervation, the bloodstream and local synthesis by Leydig cells (Romeo et al., 2004; Davidoff et al., 2005) and the dopamine D1 receptor (also known as DRD1) has been localized in germ cells and spermatogonia nearest the basal laminal of seminiferous tubules, thus suggesting paracrine actions of DA in the spermatogenic process through the activation of DRD1 (González et al., 2015).

As described in *G. bimaculatus* and *C. suppressalis* (Watanabe et al., 2013; Xu et al., 2017), *A. ipsilon Dop1* is expressed in the midgut. In insects, and especially in moths, it is known that the midgut receives catecholamine-containing nerve endings from the ventral nerve cord along its longitudinal axis (Klemm, 1979). In *L. migratoria*, DA was found to repress the spontaneous and phasic contractions of the foregut (Lange and Chan, 2008). Similarly, it has been revealed in the mouse, *Mus musculus*, that the inhibitory action of DA on the contractility of the small intestine is achieved through a DRD1/adenylyl cyclase-dependent signaling, resulting in altered calcium mobilization and myosin ATPase activity (Zizzo et al., 2010).

Finally, this tissue-specific expression analysis of *AiDop1* highlights that the dopaminergic system is widely distributed in *A. ipsilon* and that Dop1 is probably a molecular mediator of central and peripheral dopaminergic signaling pathways modulating olfactory functions, midgut mobility and several aspects of male reproductive physiology, and notably spermatogenesis and seminal fluid production.

AiDop1 is required for the display of sex pheromone-orientated flight

In this study, we observed that injection of dsRNAs targeting various regions of *AiDop1* induced an approximately 65–75% reduction in mRNA expression levels in ALs, and led to similar effects on both AL cAMP levels and the sex pheromone behavioral response in dsRNA-treated males. These results consolidated our RNAi experiments even though we could not verify the decline of the corresponding protein in the ALs of *Dop1*-deficient males because the antibody against this receptor is not available.

AiDop1 silencing resulted in decreased AL cAMP levels and altered sex pheromone-orientated flight, with a lower percentage of responding males. Given that *AiDop1* is not exclusively expressed in ALs but is also present in other brain regions as well as in the reproductive organs such as the testes and sex accessory glands, it is likely that *AiDop1* knockdown in these tissues also contributes to the disruption of the sex pheromone behavioral response in dsRNA-treated males. Our RNAi data provide evidence that *AiDop1* potentiates the orientated response of males to sex pheromone in *A. ipsilon*, probably through the activation of the cAMP signaling pathway in ALs. In accordance with this finding, *Dop1R1* has been shown to be involved in many aspects of *D. melanogaster* sexual activity including male–male courtship (Chen et al., 2012) and copulation duration (Crickmore and Vosshall, 2013). Moreover, it has been demonstrated in rats that the copulatory behavior of males and their preference for female pheromones depend on the activation of DRD1 signaling in the mesolimbic dopaminergic system (Beny-Shefer et al., 2017).
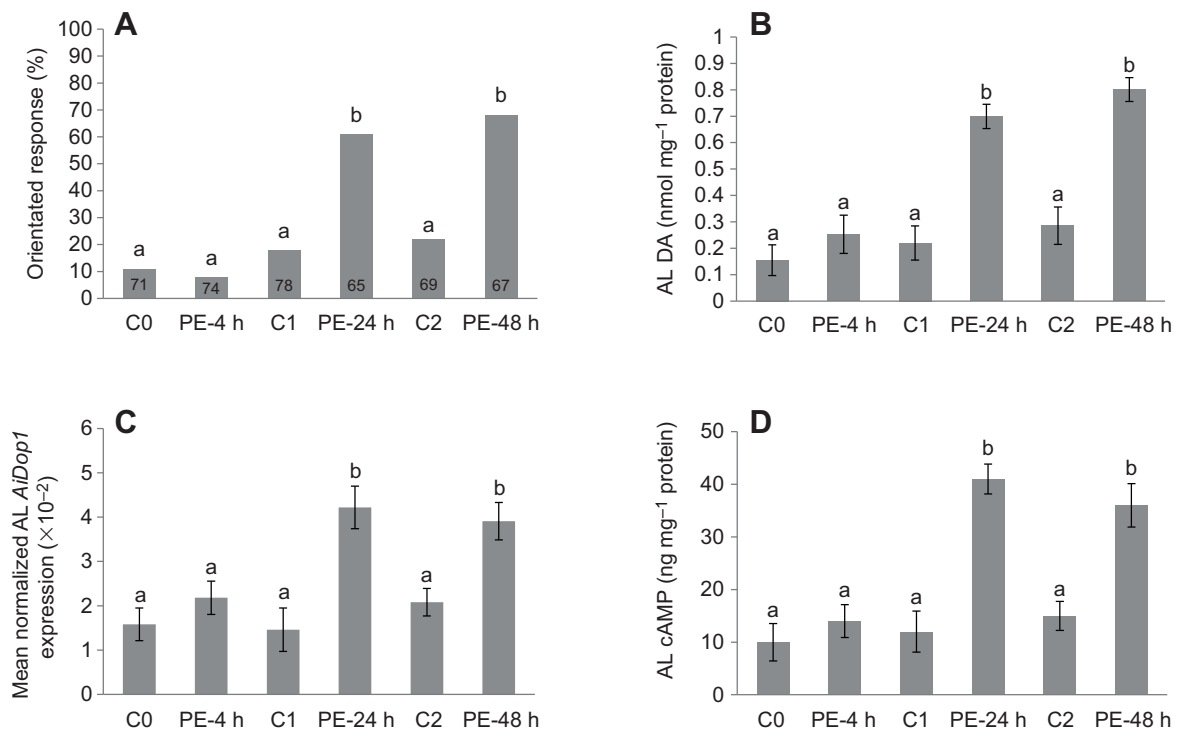
Fig. 4. Pre-exposure-related plasticity in the sex pheromone response and levels of AL DA, *AiDop1* mRNA and cAMP in *A. ipsilon* males. Newly emerged males (day 0) were pre-exposed for 3 min to the blend pheromone. The behavioral response to sex pheromone and the levels of DA, *AiDop1* mRNA and cAMP in ALs were determined 4, 24 and 48 h later after pre-exposure (PE). (A) Percentage of orientated responses of males. (B) AL DA level. (C) Relative expression of *AiDop1*. (D) cAMP level. Unexperienced 0, 1 and 2 day old males (D0, D1 and D2) were used as controls. Each qPCR was run in three technical replicates with seven independent biological replicates. DA and cAMP levels were quantified from 10 ALs for each experimental group with 10 biological replicates. For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a significant difference (*G*-test; $P<0.05$). For the DA, *AiDop1* mRNA and cAMP levels, the bars represent means±s.d.; different letters indicate a significant difference (analysis of variance; Tukey's test; $P<0.05$).

In moths, it is well described that the dopaminergic innervation is extended across the brain with a high density in all the ordinary glomeruli and the proximal region of the MCG within ALs as well as in the lateral protocerebrum, including the mushroom bodies (Dacks et al., 2012). Recent studies have revealed that DA facilitates male sexual behavior in *A. ipsilon* (Abrieux et al., 2014) and affects both the response (i.e. amplitude and temporal dynamic) and the sensibility of AL neurons to odors, thereby inducing changes in the proportion of neurons participating in the encoding of olfactory stimuli, in *M. sexta* (Dacks et al., 2012). Considering all the above data, it is likely that *AiDop1* mediates some modulatory effects of DA on the sex pheromone-driven responses of AL neurons, thus contributing to the elicitation of sexual attraction behavior in *A. ipsilon* males. In order to decipher these central actions of DA and AiDop1, we plan in future studies to evaluate the effects of DA treatment and *AiDop1* silencing on the neuronal response to pheromone signal, and in particular in the *Agrotis* MCG, using intracellular recordings.

We cannot rule out the possibility that DA receptors other than Dop1 might be involved in the dopaminergic modulation of the sex pheromone responsiveness of the AL. Indeed, DopEcR has been shown to act as an up-regulator of sex pheromone perception by transmitting DA signals within the *A. ipsilon* AL (Abrieux et al., 2013, 2014) and, in addition to *Dop1*, expression of *Dop2* and *Dop3* has been detected in the *M. sexta* AL (Dacks et al., 2012). It seems reasonable to hypothesize that all these receptors may co-exist in the *A. ipsilon* AL with a distinct and/or complementary distribution pattern. Given that their activation is either negatively or positively

coupled to intracellular cAMP levels, these receptors might be potential mediators of enhancing and suppressive effects of DA on the AL neuronal responses, thus modulating the central processing of pheromone information in moths. This speculation is strengthened by recent data demonstrating in rats that dopaminergic modulation plays a role in odor detection and discrimination through opposing effects of D1- and D2-type receptors within the olfactory bulb (Yue et al., 2004; Escanilla et al., 2009). There is also a strong possibility that *AiDop1* could act in brain areas other than the primary olfactory neuropiles. This is supported by the observation that *AiDop1* was detected in the brain after excision of ALs. In addition, several studies have shown that Dop1 is present in second-order olfactory centers, and more precisely in the Kenyon cells of mushroom bodies (Mustard et al., 2005; Hamada et al., 2009; Dacks et al., 2012) and that its silencing impaired the ability to discriminate, learn and memorize odors (Lim et al., 2018; Vinauger et al., 2018).

DA–Dop1 signaling in AL is potentially related to the increase in sex pheromone responsiveness throughout adulthood

Our results indicate that DA and *AiDop1* mRNA as well as intracellular cAMP in ALs are at higher levels in old sexually mature males than in young sexually immature males. This revealed an age-dependent upregulation of DA levels and *AiDop1* expression coupled to cAMP within ALs, and which coincided with the age-dependent increase in neuronal and behavioral responses to the pheromone signal. Such a correlation leads us to suggest that DA–Dop1 signaling is positively related to the neuronal plasticity
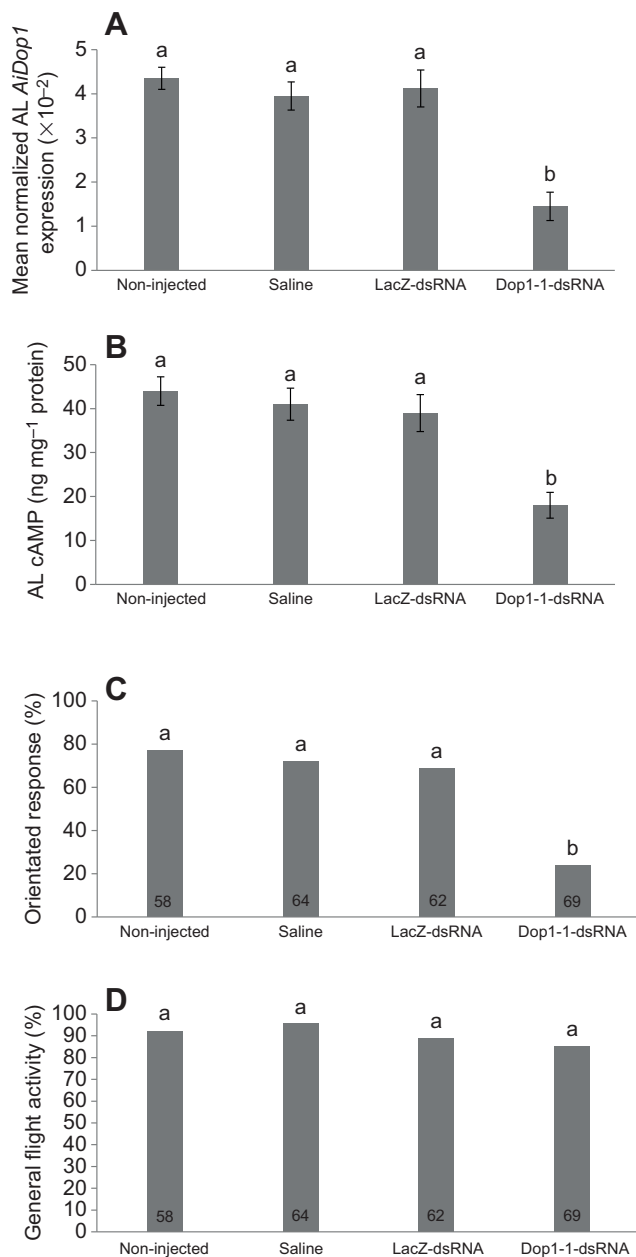
Fig. 5. Efficiency of double-stranded RNA (dsRNA)-mediated *AiDop1* silencing and effect on both AL cAMP levels and the behavioral response to sex pheromone in *A. ipsilon* males. Males (2 days old) received an injection of saline solution, LacZ-dsRNA or Dop1-1-dsRNA, or no injection (non-injected). For each treatment and 3 days after injection, the relative expression of *AiDop1* mRNA (A) and cAMP level (B) were quantified in ALs, and the percentage of orientated responses (C) and of general flight activity (D) were determined in wind tunnel experiments. Each qPCR was run in three technical replicates with five independent biological replicates. cAMP levels were quantified from 10 ALs for each experimental group with 10 biological replicates. For the *AiDop1* mRNA and cAMP levels, the bars represent means±s.d.; different letters indicate a significant difference (analysis of variance; Tukey's test; $P<0.05$). For the behavioral tests, numbers within bars indicate the number of tested males; different letters indicate a significant difference (*G*-test; $P<0.05$).

that takes place within ALs and contributes to the emergence of sex pheromone-orientated flight behavior in sexually mature males. Similarly, a coordinated increase in both DA levels and *Dop1* expression in the adult brain was correlated with the activating

effects of DA on the sexual maturation and mating flight of male honeybees (Sasaki et al., 2012). In addition, it has been described in male hamsters that the DA and DRD1 levels increase gradually in various brain areas throughout the pubertal transition and this molecular plasticity of the dopaminergic system is associated with the neuronal remodeling that accompanies the maturation of reproductive behavior (Romeo et al., 2002).

There is a growing body of evidence that biogenic amines can themselves be modulated through functional interactions with endocrine factors such as sexual hormones, and this second-order modulation, termed metamodulation, has been particularly described in the context of male reproduction (Mesce, 2002). In mammals, testosterone is known to be essential for the display of male sexual behavior by enhancing the processing of relevant stimuli and this action has been shown to be driven through alterations in the synthesis, release and receptor expression of neurotransmitters such as DA and serotonin (Hull et al., 1999, 2004). In insects, JH was found to be a critical signal in the emergence of DA-mediated mating flight of *A. mellifera* drones by simultaneously increasing DA and *Dop1* expression levels in the brain (Akasaka et al., 2010; Sasaki et al., 2012). In the *A. ipsilon* male, it was observed that the levels of JH in the hemolymph of old sexually mature males are higher than those of young sexually immature males and that this difference is in part due to the elevation of JH biosynthetic activity by the corpora allata, the hormone-producing gland, with aging (Duportets et al., 1998). By manipulating JH hemolymph titer, we have previously shown that the presence of this hormone is needed for the maturation of the sex pheromone responses of males. Indeed, the injection of JH into immature males resulted in an increased sensitivity of the sex pheromone-responding AL neurons, which was sufficient to elicit the anemotactic zigzagging flight. In contrast, the neuronal and behavioral responsiveness to sex pheromone was suppressed in mature males if they were deprived of JH by surgical removal of the corpora allata (Anton and Gadenne, 1999; Gadenne and Anton, 2000; Jarriault et al., 2009). It is interesting that the behavioral changes resulting from RNAi-mediated *Dop1* silencing are similar to those caused by JH deprivation in mature males. It is also noteworthy that the age-related dynamics of DA and *AiDop1* expression levels in ALs match fluctuations of JH hemolymph titer. Taken together, these observations point to a possible functional interplay between JH signaling and the dopaminergic system operating in ALs. Thus, DA might be a mediator of the enhancing effects of JH on sex pheromone responses through the activation of AL DA–Dop1 signaling via JH-evoked concomitant increases of DA and Dop1 protein in response to the elevation of circulating hormone levels throughout the adulthood of *A. ipsilon* male. To explore the existence of such a regulation of the AL dopaminergic system by JH, experiments are in progress to examine the impact of JH administration in young males and of JH deprivation in old males on the levels of DA and *AiDop1* expression in ALs.

DA–Dop1 signaling in ALs is potentially related to the increase in sex pheromone responsiveness after odor pre-exposure

We noticed that young sexually immature males exhibited an increase in their behavioral response to sex pheromone 24 and 48 h after a brief pre-exposure to this odor and this long-term effect was accompanied by elevated amounts of DA, *AiDop1* mRNA and intracellular cAMP in ALs as compared with naive males. In accordance with these results, it has been reported in *A. mellifera* that exposing young worker bees to queen mandibular pheromone (QMB) induced fluctuations in

the levels of DA, dopaminergic receptors and cAMP in the brain, and these central effects of QMB on the DA pathways strengthened their attraction towards this pheromone (Beggs et al., 2011). Histological and neurophysiological investigations performed in the moth *S. littoralis* indicated that the behavioral increase in the response of males to sex pheromone after odor pre-exposure was paralleled by a rise in both the sensitivity of AL neurons and the volume of glomeruli belonging to the MCG (Anton et al., 2016). The enlargement of AL glomeruli observed in young *Drosophila* females through olfactory pre-exposure has been found to originate from an increase in the number of neuron branching and synaptic connections, and these structural modifications of the glomerular network appear to be dependent on the cAMP cascade (Devaud et al., 2001, 2003). By taking into account the above-mentioned data, it seems reasonable to assume that the brief pre-exposure of *A. ipsilon* males to the female sex pheromone, early in their adult life, causes concomitant increases in DA and *AiDop1* expression levels within ALs, thus triggering DA–Dop1 signaling, which in turn governs the anatomical and functional reshaping of neuronal circuits within glomeruli processing pheromone information and consequently facilitates the expression of orientated flight behavior. This assumption is backed up by recent data in rats that the binding of DA to the DRD1 receptor contributes to the enhancement of copulatory behavior in males pre-exposed to an estrous female, probably through the involvement of adenylyl cyclase–cAMP–protein kinase A (PKA) signaling in the medial preoptic area (McHenry et al., 2012).

A series of reports demonstrated that in young worker bees exposed to QMP, the pheromone-evoked behavioral effects were mediated by changes in the circulating JH levels via a modulation of JH biosynthesis rate, and these were correlated with changes induced in the brain dopaminergic system (Kaatz et al., 1992; Pankiw et al., 1998; McQuillan et al., 2014). An interesting observation in *A. ipsilon* is that young males pre-exposed to sex pheromone have higher levels of circulating JH than do naive males (E.G., unpublished data). This raises the possibility that sex pheromone pre-exposure provokes an elevation of circulating JH, which might affect the functioning of the AL dopaminergic system through DA–Dop1 signaling activation, and thus promoting the emergence of sex pheromone-guided flight behavior in young *A. ipsilon* males. In order to ascertain the existence of such an interaction of JH with DA signals through early olfactory experience, in future studies we will examine whether JH deprivation prevents the sex pheromone-induced effects on the levels of DA and *AiDop1* expression in ALs as well as on the sex pheromone behavioral response in pre-exposed males.

Hypothetical model for Dop1-mediated DA actions in *A. ipsilon* AL

It is well established that D1-like receptors have the ability to activate numerous signaling cascades, via a physical and functional coupling to heterotrimeric G proteins, including the canonical adenylyl cyclase–cAMP–PKA pathway, which has been found in mammals to regulate the excitability of neurons as well as their synaptic communication by modulating the trafficking and activity of ion channels (Neve et al., 2004; Undieh, 2010; Yang et al., 2013). *In vitro* studies performed on honeybee AL neurons provided convincing evidence that DA modifies the temporal dynamic of their odor-driven responses by attenuating the amplitude of Ca^{2+}-dependent K^+ currents (I_{KCa}), thus reducing the duration of the post-excitatory inhibition and consequently increasing the cell excitability (Ellen and Mercer, 2012). Given that there are numerous reports showing that the inhibition of Ca^{2+}-dependent K^+ channels (SK) also facilitates the growth and differentiation of

neurons and synaptic plasticity (Faber and Sah, 2007; Ohtsuki et al., 2012), the downregulation of SK conductance by DA would be expected to play a role in developmental events occurring within ALs. Taken together, these data lead us to propose a hypothetical model of Dop-1-mediated DA modulation in *A. ipsilon* ALs. After binding to DA, Dop1 activates the catalytic activity of adenylyl cyclase by the G subunit and subsequently increases cAMP to initiate a putative cAMP–PKA signaling pathway which might repress SK channel activity, and the resulting reduction of I_{KCa} would contribute to structural and functional development of MCG glomeruli, thus promoting the neuronal and behavioral responses to pheromone signal. In *A. ipsilon* males, the response of MCG neurons to sex pheromone has recently been characterized by extracellular recordings and it exhibited the post-excitatory inhibition phase (called I) which was presumed to be generated by an intrinsic SK conductance (Martinez et al., 2013). In order to test the hypothesis that SK channels are targets of DA–Dop1 signaling, as proposed above, it would be interesting to examine whether the magnitude of the I phase in the sex pheromone response of *A. ipsilon* MCG neurons is affected in Dop1-deficient males. A recent study highlighted a new molecular mechanism of action of Dop1 in the migratory locust adult brain. Dop1 activation was found to inhibit the maturation of microRNA-9a, leading to an increase in the expression of adenylyl cyclase coupled with the enhancement of gregarious behavior (Guo et al., 2018). It is conceivable that Dop1 mediates its modulatory actions in *A. ipsilon* AL by targeting adenylyl cyclase through regulation of the expression of its mRNA via microRNA-9a inhibition and of its activity via G protein activation. This double regulation of adenylyl cyclase in DA–Dop1 signaling might strengthen the activation of the downstream cascade, thus inducing long-lasting changes in the MCG network.

In summary, we show here that in moths, Dop1 signaling in ALs mediates the behavioral response of males to the female sex pheromone, probably by promoting the central processing of the pheromone signal. Moreover, our results reveal an age- and experience-related upregulation of DA and *AiDop1* expression levels coupled to cAMP in ALs, which is temporally correlated with the upregulation of neuronal and behavioral sensitivity to sex pheromone. This relationship provides evidence that age- and experience-dependent changes in sex pheromone responsiveness are sustained by DA–Dop1 signaling, which operates in the primary olfactory centers, presumably through cAMP-dependent mechanisms. Finally, the present study expands our understanding of the mechanisms underlying olfactory plasticity, which appear to be highly conserved between insects and mammals.

Data availability
The full-length cDNA for *Agrotis ipsilon* Dop1 (*AiDop1*) has been deposited in GenBank under accession number MN164482.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.211979.supplemental

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