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Synaptotagmin I, a molecular target for steroid hormone signaling controlling the maturation of sexual behavior in an insect

Françoise Bozzolan¹, Line Duportets^{1,2}, Denis Limousin³, Marie-Anne Wycke³, Elodie Demondion³, Adrien François^{4,5}, Antoine Abrieux⁶ and Stéphane Debernard¹

1 Département d'Ecologie Sensorielle, UMR 1392, Institut d'Ecologie et des Sciences de l'Environnement de Paris, Université Paris VI, France

2 Service d'Enseignement de Biologie Animale, Université Paris-Sud, Orsay, France

3 Département d'Ecologie Sensorielle, UMR 1392, Institut d'Ecologie et des Sciences de l'Environnement de Paris, INRA, Versailles, France

4 INRA, UR1197, Jouy-en-Josas, France

5 Université de Versailles, France

6 Laboratoire Récepteurs et Canaux Ioniques Membranaires (RCIM), Université d'Angers, UPRES-EA 2647 USC INRA 1330, France

Keywords

insect; olfactory plasticity; sexual behavior; steroid signaling pathway; synaptotagmin I

Correspondence

S. Debernard, UMR 1392, Institut d'Ecologie et des Sciences de l'Environnement de Paris, Université Paris VI, Bâtiment A, 7 quai Saint Bernard, 75005 Paris, France
Fax: +33 1 4427 6509
Tel: +33 1 4427 3839
E-mail: stephane.debernard@snv.jussieu.fr

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As in vertebrates, the insect steroid hormones, especially 20-hydroxyecdysone (20E), initiate and regulate sexual behavior by acting on the central nervous system. This 20E action is, in part, triggered by transcriptional events mediated through the binding of 20E to a heterodimer comprising the ecdysone receptor (EcR) and ultraspiracle (USP). However, to date, our knowledge about this genomic steroid pathway remains incomplete. In moths, males detect female sex pheromones, eliciting stereotyped sexual behavior. In *Agrotis ipsilon* males, the behavioral response and the neuronal sensitivity to sex pheromone in the olfactory center, the antennal lobe (AL), increase with age. We recently showed that 20E controlled this age-dependent olfactory plasticity via the activation of an EcR/USP-dependent pathway in the AL. Here, we cloned the gene encoding *A. ipsilon* synaptotagmin I (*AisytI*), a presynaptic vesicle protein known to act as a calcium sensor in neurotransmitter release. *AisytI* was expressed in the AL, where its amount increased with age, whereas its knockdown inhibited the sex pheromone-oriented flight of males. 20E administration to males induced AL *AisytI* expression in a dose-dependent and time-dependent manner. Moreover, *A. ipsilon* EcR silencing caused decreases in AL *AisytI* expression and the behavioral response to sex pheromone. Our results show that the synaptotagmin I gene is a target gene for the genomic steroid signaling that controls the expression of insect sexual behavior by acting on central sex pheromone processing. This study thus represents a significant advance in our understanding of the steroid actions that influence neural functions, and thereby behavioral plasticity, in various organisms.

Database

The nucleotide sequence of *Agrotis ipsilon* synaptotagmin I is available in the DDBJ/EMBL/GenBank databases under the accession number [KJ863735](#).

Abbreviations

20E, 20-hydroxyecdysone; AiEcR, *Agrotis ipsilon* EcR; AisytI, *Agrotis ipsilon* synaptotagmin I; AL, antennal lobe; d.f., degrees of freedom; DIG, digoxigenin; EcR, ecdysone receptor; IN, intravesicular N-terminal domain; qPCR, quantitative PCR; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; sytl, synaptotagmin I; TR, transmembrane domain; USP, ultraspiracle.

Introduction

During insect metamorphic molting, the steroid hormones, through their main active form 20-hydroxyecdysone (20E), are known to influence the fine-tuning of neuronal circuits by controlling cell proliferation, differentiation, and apoptosis, and neurite remodeling [1,2]. In addition to its canonical role in development, 20E has been reported to regulate some physiological and cognitive functions throughout adult life [3,4], as well as the expression of behavioral patterns essential for sexual interactions. For instance, in the fruit fly *Drosophila melanogaster*, a reduction in the circulating levels of 20E caused defects in the display of some components of male courtship behavior, especially regarding gender orientation [5].

Many of the biological effects of 20E are elicited through its binding to a nuclear heterodimeric complex composed of the ecdysone receptor (EcR) and its partner ultraspiracle (USP), the insect homolog of the vertebrate retinoid X receptor [6]. The 20E–EcR–USP complex then directly triggers the sequential expression of genes encoding transcription factors that ultimately regulate the activity of target genes [7]. In *D. melanogaster*, EcR in fruitless-expressing neurons was found to be critical for the performance of male courtship behavior by mediating the establishment of male-specific neuronal circuits within the primary olfactory center, the antennal lobe (AL) [8]. There is now increasing evidence for the involvement of membrane-initiated steroid signaling in neuronal and behavioral plasticity, via a G-protein-coupled receptor, DopEcR [9], which shows affinity for both 20E and dopamine *in vitro* [10]. In the noctuid moth *Agrotis ipsilon*, the loss of DopEcR function resulted in inhibition of oriented flight of males towards female-emitted sex pheromone [11]. Because the identification of the 20E signaling pathways governing the expression of sexual behavior has been particularly focused on the characterization of receptors, little information is currently available for potential molecular targets of this endocrine regulation.

The vertebrate and invertebrate synaptotagmins constitute a large family of integral membrane proteins that participate in presynaptic vesicle trafficking and act as calcium sensors in neurotransmitter release [12,13]. Various isoforms of synaptotagmins, including the first identified and best-characterized, synaptotagmin I (sytI), are broadly expressed in the central nervous system [14,15]. In mammals, sytI appears to be a key regulator of synaptic and neuronal plasticity in different brain regions, and is required for specific behaviors and cognitive activities, including memory and learning ability [16,17]. In addition, steroid

hormones are suspected to regulate the expression of presynaptic proteins, and this modulation might coordinate the reorganization of neuronal networks [18]. In insects, especially in the moth *Manduca sexta*, the expression of sytI was associated with the formation of specialized synaptic contacts in the olfactory glomeruli of ALs at the time of metamorphosis [14].

In male *A. ipsilon* moths, the behavioral and central nervous system responses to female-produced sex pheromone are age-dependent and hormone-dependent [19–21]. Thus, newly emerged males are sexually immature and do not respond behaviorally to sex pheromone. Three to five days after emergence, males become sexually mature and are highly attracted to sex pheromone [21]. This increase in behavioral responsiveness is paralleled by an increase in the sensitivity of AL neurons to pheromone signals [19,20]. In males, 20E is known to be present in hemolymph and reproductive tissues throughout adult life [22]. Recent data showed that the administration of exogenous 20E triggered the behavioral and neuronal responses to sex pheromone in young immature males, and that this effect was associated with the 20E inducibility of EcR and USP in ALs [23]. In contrast, treatment with cucurbitacin B, an antagonist of 20E that is able to interact with the EcR–USP complex, led to a decrease in these sex pheromone responses in old mature males [23,24].

On the basis of the above data, we hypothesized that sytI could be a molecular target for the genomic 20E signaling pathway implicated in the olfactory plasticity related to the maturation of male sexual behavior in *A. ipsilon*. To address this hypothesis, we first cloned *A. ipsilon sytI* (*AisytI*), determined its tissue-related and age-related expression profiles, and examined the impact of its knockdown on the behavioral response of old mature males to sex pheromone in a wind tunnel. We then evaluated the effects of exogenous 20E injection in young immature males on the expression level of *AisytI* in the AL, as a function of both the injected dose and the duration of hormonal treatment. Finally, we blocked the genomic 20E signaling pathway through *EcR* silencing, and observed the effects of this disruption on both the amount of AL *AisytI* and the behavioral responsiveness to sex pheromone in old mature males.

Results

The primary structure of *AisytI* shows all the structural hallmarks of an I-type synaptotagmin

On the basis of the amino acid sequences of sytI identified in the lepidoptera *Bombyx mori* and *M. sexta* and the hemipteran *Apis mellifera*, we designed a pair of

degenerate DNA primers from the highly conserved C2B domain, and succeeded, via an RT-PCR reaction, in amplifying a partial fragment of 400 bp from total RNAs extracted from *A. ipsilon* brains. The remaining 5' and 3' ends of the fragment were then obtained with a rapid amplification of cDNA ends (RACE)/PCR-based strategy. 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 *AisytI*. This cDNA of 1758 bp contains a 164-bp 5'-UTR, a 1293-bp ORF encoding a 431-residue protein, and a 301-bp 3'-UTR, with a polyadenylation signal upstream of the poly(A) tail (Fig. S1). As in all synaptotagmin family members, the deduced amino acid sequence of AisytI consists of an intravesicular N-terminal domain (IN), a single-pass transmembrane domain (TR) followed by a cytosolic region containing two calcium/phospholipid-binding motifs termed C2A and C2B, and a short C-terminal domain containing a WHXL motif (Fig. S1).

The full protein sequence of *AisytI* shows a high percentage of amino acid identity, ranging from 76% to 90%, with sytI proteins identified in other insect species (Table 1). The *AisytI* sequence also has significant amino acid similarity with sytI cloned in humans (63%) and in the mouse *Mus musculus* (64%). On comparison of the sequences of C2A and C2B, high conservation between *AisytI* is observed in all studied species, with at least 76% identity (Table 1).

***AisytI* is highly expressed in the brain and especially in the ALs**

To gain some preliminary information on the function of *AisytI*, we determined its tissue distribution by

Table 1. Identity of amino acid sequences of the entire protein, C2A and C2B between *AisytI* and sytI identified in other species, expressed as the percentage of identical amino acids.

	Entire protein (%)	C2A (%)	C2B (%)
<i>AisytI</i>	100	100	100
<i>BmsytI</i>	90	97	97
<i>MssytI</i>	89	97	99
<i>AmsytI</i>	85	91	96
<i>TcsytI</i>	81	93	95
<i>DmsytI</i>	76	91	90
<i>ApsytI</i>	76	88	96
<i>MmsytI</i>	64	76	83
<i>Hsytl</i>	63	76	83

AmsytI, *Ap. mellifera* sytI; *ApsytI*, *Ac. pisum* sytI; *BmsytI*, *B. mori* sytI; *DmsytI*, *D. melanogaster* sytI; *Hsytl*, human sytI; *MmsytI*, *Mus musculus* sytI; *MssytI*, *M. sexta* sytI; *TcsytI*, *T. castaneum* sytI. For accession numbers of sytI sequences, see Experimental procedures.

quantifying its expression level by real-time quantitative PCR (qPCR) in the brain, AL, antennae, eyes, proboscises, thoracic muscles, wings, legs and abdomens of 5-day-old males. *AisytI* was expressed at higher levels in brains and ALs than in eyes, proboscises, muscles, or legs (Fig. 1A). No amplification of *AisytI* was detected in abdomens, wings, or antennae (data not shown). With a DIG-labeled cDNA probe located in the N-terminal part of *AisytI*, northern hybridization on brain total RNAs revealed only one transcript of ~1.7 kb, in agreement with the length of the corresponding cDNA (Fig. 1B).

Expression of *AisytI* in the brain and the AL is upregulated throughout adult life

In *A. ipsilon* males, the behavioral responsiveness and sensitivity of AL neurons to the female sex pheromone increase concomitantly with age [19–21]. To assess the

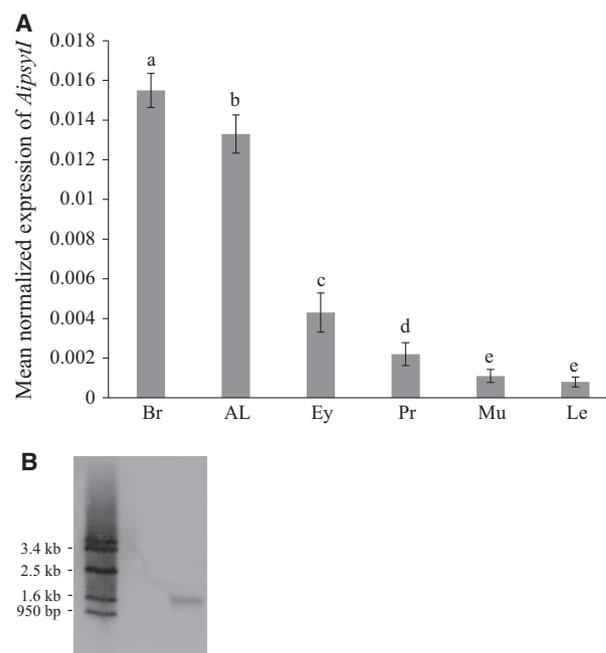


Fig. 1. Tissue-related *AisytI* expression in 5-day-old *Agrotis ipsilon* males. (A) RNA templates were extracted from the brain (Br), AL, eye (Ey), proboscis (Pr), thoracic muscle (Mu), leg (Le), wing, antenna, and abdomen. The *A. ipsilon* *RpL8* gene encoding a ribosomal protein was used as a reference gene for real-time qPCR. *AisytI* was highly expressed in the brain, and especially in the AL. Bars represent means \pm standard deviations, and those with the same letters are not significantly different (ANOVA; Tukey test; $\alpha = 0.05$). (B) Fifteen micrograms of brain total RNA was separated on agarose (1%) formaldehyde gel, transferred to nylon membranes, and hybridized with *AisytI* probe. Only one transcript, of ~1.7 kb, was detected in the brain.

possible involvement of *AisytI* in this age-related olfactory plasticity, we established its transcriptional pattern in the brains and ALs of 1–5-day-old males. Real-time qPCR analysis revealed that the transcriptional activity of *AisytI* was subject to age-dependent regulation that was similar in both investigated tissues (Fig. 2A,B). The amount of *AisytI* mRNA was low during the first 2 days after emergence, increased significantly from day 3 to day 4, and remained practically stable at day 5 (Fig. 2A,B). The expression of AL and brain *AisytI* in 4-day-old or 5-day-old sexually mature males was approximately twice as high as in 1-day-old or 2-day-old sexually immature males. Con-

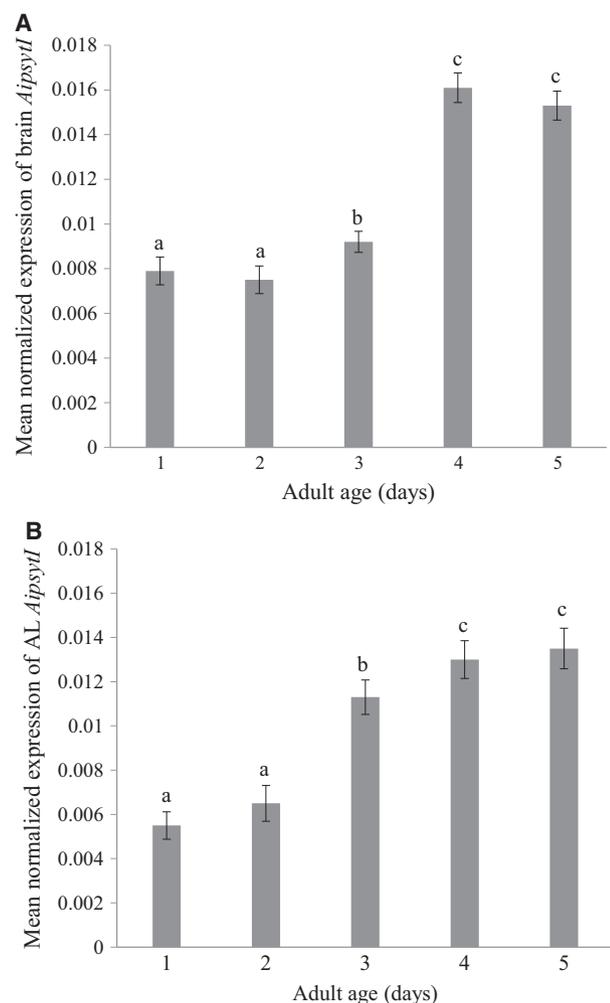


Fig. 2. Age-related *AisytI* expression in brains and ALs for 1–5-day-old *A. ipsilon* males. Transcriptional patterns of *AisytI* in brains (A) and ALs (B) are shown. The ribosomal gene *Rpl8* was used as a reference gene for real-time qPCR. *AisytI* expression increased with age. The bars represent means \pm standard deviations, and those with the same letters are not significantly different (ANOVA; Tukey test; $P < 0.05$).

versely, we noticed that the expression of *AisytI* in other tissues (eye, proboscis, muscle, and leg) was age-independent (data not shown).

***AisytI* knockdown results in reduced oriented flight towards sex pheromone**

By using RNA interference (RNAi) technology, we then evaluated the effects of *AisytI* silencing on the behavioral response of males to sex pheromone in the wind tunnel. Administration of *AisytI*-dsRNA to 1-day-old sexually immature males induced a significant decline in the amount of *AisytI* mRNA in the AL 4 days after the injection as compared with the three control groups (saline-injected, bacterial *LacZ*-dsRNA-injected and noninjected males) (Fig. 3A). This transcriptional silencing was accompanied by a sharp decrease in the oriented behavioral response of *AisytI*-dsRNA-injected males as compared with that of control groups [*G*-test: $G = 22.13$, $G = 14.28$, $G = 14.80$, degrees of freedom (d.f.) = 1, $P = 0.0000025$, $P = 0.00015$, $P = 0.00011$, between *AisytI*-dsRNA-injected males and noninjected, saline-injected and bacterial *LacZ*-dsRNA-injected males, respectively] (Fig. 3B). Moreover, the *AisytI*-dsRNA-injected males were unable to land at the pheromone source. There was no significant difference in the oriented behavioral response (*G*-test: $G = 0.87$, d.f. = 2, $P = 0.647$) or in the proportions of the three different behavioral sequences (partial flight, complete flight, and landing) (*G*-test: $G = 3.62$, d.f. = 3, $P = 0.3$) between the three control groups (Fig. 3B). General flight activity was high (> 92%) and statistically identical for all tested experimental groups (*G*-test: $G = 0.25$, d.f. = 3, $P = 0.97$), showing that the injections did not disturb the flight performance of the moths (Fig. 3C).

20E treatment induces an increase in the expression of AL *AisytI*

The age-dependent olfactory plasticity observed in *A. ipsilon* males was under the influence of the steroid-ergic system. Thus, the administration of exogenous 20E at 10^{-6} M was reported to highly increase the behavioral responsiveness to pheromone cues in young immature males [23]. To demonstrate a link between *AisytI* and 20E action on the olfactory system, we manipulated the circulating 20E titer by injecting exogenous 20E into young males, and examining its effects on the expression level of *AisytI* in the AL as a function of both the injected dose and the duration of the hormonal treatment. The administration of 20E at 10^{-6} M into 0-day-old sexually immature males induced a sharp increase in the amount of *AisytI* transcript in

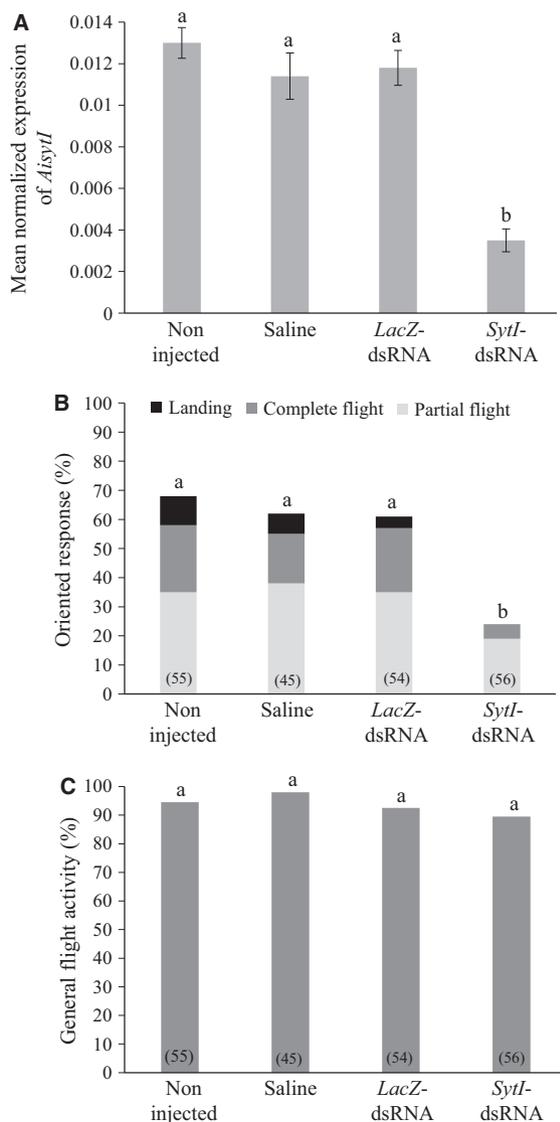


Fig. 3. Efficiency and effect of dsRNA-mediated *AisytI* silencing on the behavioral response to sex pheromone in *A. ipsilon* males. One-day-old sexually immature males received an injection of saline solution, bacterial *lacZ*-dsRNA, or *AisytI*-dsRNA, or no injection (noninjected). For each treatment, the amount of *AisytI* mRNA was quantified in the AL by real-time qPCR, and the percentages of oriented responses and of general flight activity were determined in the wind tunnel experiments 4 days after injection. (A) *AisytI* mRNA level in the ALs of *AisytI*-dsRNA-treated males. *AisytI*-dsRNA injection resulted in reduced mRNA amount. (B) Oriented responses (partial flight + complete flight + landing). *AisytI* knockdown induced inhibition of oriented upwind flight towards the sex pheromone in sexually mature males. (C) General flight activity (oriented responses + random flights). The flight behavior was not disturbed by any treatment. For *AisytI* expression, the bars represent means \pm standard deviations, and those with different letters are significantly different (ANOVA; Tukey test; $P < 0.05$). For behavioral tests, numbers in parentheses indicate numbers of tested males, and columns with the same letters are not significantly different (*G*-test; $P < 0.05$).

the AL 24 h after injection, and this overexpression reached a maximum level after 34 h, which was maintained by 48 h as compared with untreated males (Fig. 4A). Injection of saline solution had no effect on the transcriptional activity of *AisytI*. To define the minimal concentration of 20E required for induction of *AisytI* mRNA, 0-day-old sexually immature males were treated with 20E at various concentrations for 48 h. As shown in Fig. 4B, significant induction of *AisytI* in the AL was first observed at 10^{-9} M 20E, with

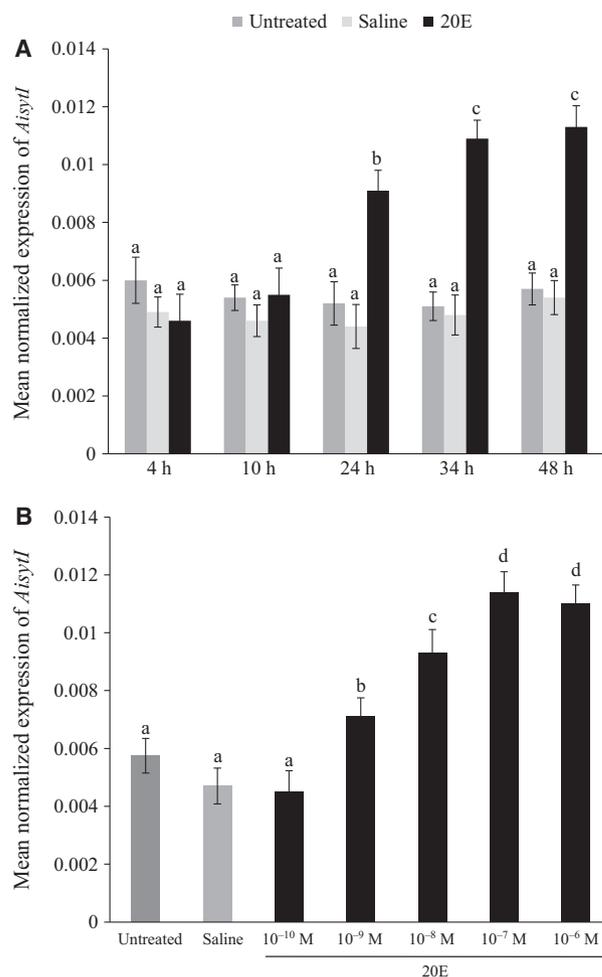


Fig. 4. Effects of 20E on AL *AisytI* expression in *A. ipsilon* males. Zero-day-old sexually immature males received an injection of 20E, and its effect on the expression level of *AisytI* in the AL was evaluated by real-time qPCR as a function of the injected dose and the duration of treatment. (A) Time course of the effect of 20E at 10^{-6} M on *AisytI* expression. (B) Dose-dependent effect of 48 h of 20E treatment on *AisytI* expression. 20E induced the transcriptional activity of AL *AisytI* in a dose-dependent and duration-dependent manner. The bars represent means \pm standard deviations, and those with the same letters are not significantly different (ANOVA; Tukey test; $P < 0.05$).

an increase up to 10^{-7} M in comparison with 2-day-old untreated males.

***Agrotis ipsilon* EcR (AiEcR) knockdown causes a decrease in the expression of AL *Aisytl*, and consequently in the behavioral response to sex pheromone**

In *A. ipsilon*, the neuronal and behavioral responses to sex pheromone are controlled by 20E through the activation of EcR/USP-dependent signaling pathway in the AL [23,24]. To test for potential involvement of *Aisytl* in this 20E regulatory pathway, we examined the impact of *AiEcR* silencing on both the expression level of *Aisytl* in the AL and the behavioral response of males to sex pheromone in the wind tunnel. The injection of *AiEcR*-dsRNA into 1-day-old sexually immature males resulted in a decreased amount of *AiEcR* mRNA in the AL 4 days after the injection as compared with the controls (saline-injected, bacterial *LacZ*-dsRNA-injected and noninjected males) (Fig. 5A). This *AiEcR* knockdown was associated with declines in *Aisytl* transcriptional activity in the AL (Fig. 5B) and in the oriented behavioral response to sex pheromone of *AiEcR*-dsRNA-injected males as compared with controls (*G*-test: $G = 15.01$, $G = 8.03$, $G = 10.31$, d.f. = 1, $P = 0.0001$, $P = 0.00045$, $P = 0.0013$, between *AiEcR*-dsRNA-injected males and noninjected, saline-injected, and bacterial *LacZ*-dsRNA-injected males, respectively) (Fig. 5C). No significant difference was observed in either the oriented behavioral response (*G*-test: $G = 1.05$, d.f. = 2, $P = 0.59$) or the proportions of behavioral sequences (partial flight, complete flight, and landing) (*G*-test: $G = 5.30$, d.f. = 3, $P = 0.15$) among the control groups (Fig. 5C). The general flight activity was high (> 95%) and statistically identical for all tested experimental groups (*G*-test: $G = 0.29$, d.f. = 3, $P = 0.96$) (data not shown).

Discussion

The main aim of the current study was to investigate the function of sytI and its link with the steroid signaling system in the age-dependent responses of males to sex pheromone in *A. ipsilon*. To achieve our goal, we first isolated a 1758-bp cDNA from the nervous tissue of male *A. ipsilon* that appeared to be full length, in that it closely matched the size of the transcript seen on northern blotting. This clone encoded a predicted *Aisytl* protein that showed the typical structural organization of synaptotagmins, including the N-terminal IN and TR followed by C2A and C2B cytosolic domains that were homologous to the C2 regulatory

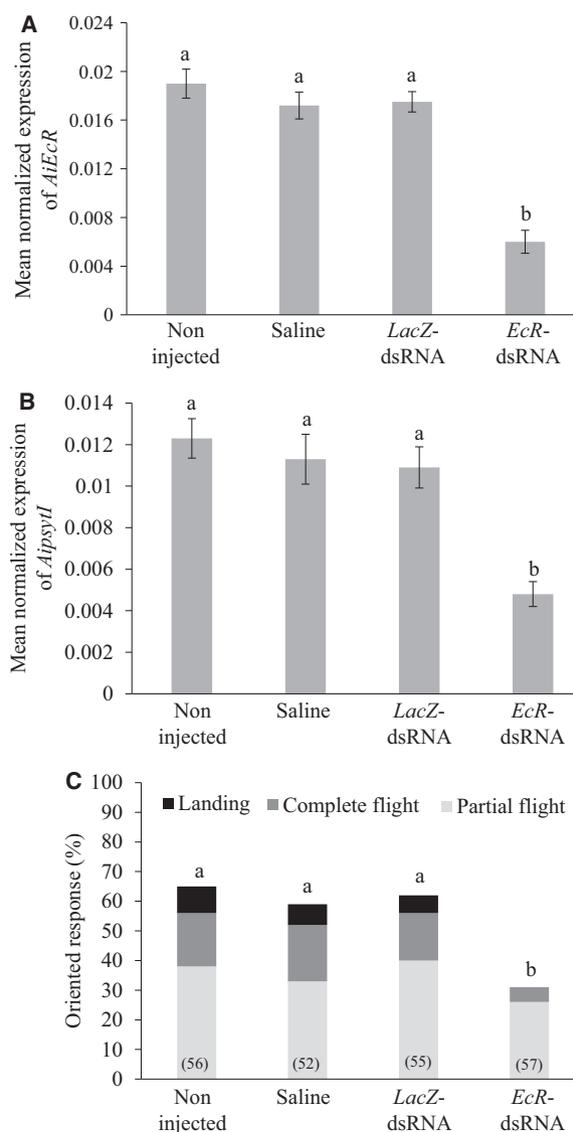


Fig. 5. Efficiency and effect of dsRNA-mediated *AiEcR* silencing on AL *Aisytl* expression and on the behavioral response to sex pheromone in *A. ipsilon* males. One-day-old sexually immature males received an injection of saline solution, bacterial *LacZ*-dsRNA, or *AiEcR*-dsRNA, or no injection. For each treatment, the amounts of *AiEcR* and *Aisytl* mRNA were quantified in the AL by real-time qPCR, and the percentages of oriented responses were determined in the wind tunnel experiments 4 days after injection. (A, B) The amounts of *AiEcR* (A) and *Aisytl* (B) mRNAs in the ALs of *AiEcR*-dsRNA-treated males. *AiEcR*-dsRNA injection led to decreases in the levels of *AiEcR* and *Aisytl* transcripts. (C) Oriented responses (partial flight + complete flight + landing). *AiEcR* silencing caused inhibition of oriented upwind flight towards the sex pheromone. For *AiEcR* and *Aisytl* expression, the bars represent means \pm standard deviations, and those with different letters are significantly different (ANOVA; Tukey test; $P < 0.05$). For behavioral tests, the numbers in parentheses indicate numbers of tested males, and columns with the same letters are not significantly different (*G*-test; $P < 0.05$).

domain originally described in protein kinase C [25], and the C-terminal WHXL motif. In vertebrates and invertebrates, several biochemical and genetic experiments have suggested that these regions may play distinct roles in the synaptic vesicle trafficking pathway through their interactions with other presynaptic membrane proteins [12,13,26]. The WHXL motif is thought to be involved in the docking of synaptic vesicles to active zones of synapse [27]. C2B has been proposed to act on the fusion step as a calcium sensor for neurotransmitter exocytosis [28,29], and it is speculated that C2A participates in both exocytosis and endocytosis for the recycling step of synaptic vesicles [26,29].

As in mammals, the insect synaptotagmins exist in several isoforms, owing to alternative splicing [30,31]. The overall protein sequence of *Aisytl* shares significant similarity with I-type synaptotagmins previously identified in other insect species but also in mammals, such as humans and the mouse *Mus musculus*. This sequence homology is especially pronounced in C2A and C2B, which show > 75% identity, whereas a strong divergence is apparent in both IN and TR, as reported for all other known sequences of synaptotagmins [14,32]. All of these compiled data provide evidence that *Aisytl* is a member of the sytI subfamily that shows selective evolutionary conservation of two C2 domain copies and may function as an essential component of the synaptic machinery in *A. ipsilon*.

Our study revealed tissue-dependent regulation of the transcriptional activity of *Aisytl*, which was characterized by predominant expression in the brain. This is consistent with previous findings in mammals and *Caenorhabditis elegans* showing that sytI is mainly localized in synaptic-rich regions within the adult nervous system [15,33]. *In situ* hybridization and immunocytochemical staining performed in *D. melanogaster* indicated that, in addition to its localization on synaptic vesicles, *D. melanogaster* sytI is transiently present in neuronal growth cones and is rapidly transported along axons to forming synapses during the embryonic and larval development of the nervous system [34]. We also found that *Aisytl* was highly expressed in olfaction-related brain areas, the ALs, that are known to be specialized for the primary integration of chemosensory cues. In concordance with these data, sytI has been detected in *M. sexta* ALs [14] and in rat olfactory bulbs [35], especially within spheroidal aggregates of neuropils called glomeruli.

Our results showed that the levels of *Aisytl* transcript were higher in both the brain and the ALs of sexually mature males than in those of sexually immature young males. This increased *Aisytl* transcriptional activity was positively correlated with the age-depend-

ent plasticity of AL neurons and behavioral responses to sex pheromone in *A. ipsilon* males. These data led us to hypothesize that *Aisytl* is required for the performance of the sex pheromone-oriented flight of the male by playing a role in the central processing of pheromone information through regulation of synaptic function in ALs. Indeed, the disruption of AL *Aisytl* function resulted in reduced behavioral response to sex pheromone in sexually mature males.

It may also be suggested that age-dependent changes in the expression of *Aisytl* contribute to the molecular basis of AL plasticity in relation to the maturation of male sexual behavior in *A. ipsilon*. This speculation is strengthened by other experimental data revealing a strong correlation between the expression level of sytI and the brain plasticity described in different physiological contexts. For instance, mouse sytI showed differential regulation of its expression with aging in association with neural and synaptic remodeling in the dorsal hippocampus, leading to deficiencies in cognitive activities [17]. In rats, stress exposure was shown to induce rapid fluctuations in the expression of brain sytI that were paralleled by multiple morphological and electrophysiological changes, thus causing behavioral alterations [36]. Moreover, upregulation of *M. sexta* sytI was observed within the glomeruli of ALs concomitantly with synaptic rearrangements that accompanied the metamorphic development of the olfactory system [14].

Studies on different moth species revealed that the expression of the sex pheromone-triggered flight behavior of male was not only dependent on age, but was also influenced by other intrinsic factors. For instance, brief exposure of naïve males to sex pheromone induces a long-term increase in the sensitivity of the behavioral response and AL neurons following pre-exposure for > 24 h [37]. In addition, it has been discovered that newly mated males become insensitive to sex pheromone, preventing them from remating until the next night. This transient inhibition of the behavioral response was paralleled by a decrease in the activity of the AL neurons [38]. sytI might also play a role in this experience and reproductive status-dependent plasticity of the central olfactory system in moths.

We have demonstrated that the administration of exogenous 20E to sexually immature males stimulates the expression of AL *Aisytl* in a dose-dependent and time-dependent manner. It has been previously reported that this hormonal treatment also induces an increase in the behavioral responsiveness to sex pheromone [23]. Thus, it seems reasonable to assume that the upregulation of AL *Aisytl* expression by 20E is

associated with its ability to enhance sex pheromone-guided flight in young males. Similarly, in adult female rats, it has been suggested that the ovarian steroids are capable of controlling the expression of sytI, and this molecular plasticity in the hippocampus might be critical for the reorganization of the neuronal networks involved in information processing throughout the estrous cycle [18]. Finally, we showed that RNAi-mediated silencing of *AiEcR* provoked a decline in the expression level of AL *AisytI* that correlated with inhibition of sex pheromone-oriented flight in old mature males. Taken together, these last findings provide direct evidence that *AisytI* is a 20E-upregulated gene involved in the EcR-dependent signaling pathway that is required for the expression of male sexual behavior, probably by acting on the central nervous system processing of sex pheromone. In *D. melanogaster* males, the processing of courtship memory is under the control of DopEcR-mediated and EcR-mediated 20E signaling pathways in the high-order sensory integration centers, the mushroom bodies, that regulate short-term and long-term memory, respectively [9]. In *A. ipsilon* males, we also succeeded in isolating a DopEcR that was exclusively expressed in the brain; like EcR, it was found to be involved in the age-dependent plasticity of the behavioral and neuronal responses to sex pheromone [11,24]. Future investigations on the functional interplay between these genomic and nongenomic hormonal signaling pathways are expected to highlight novel mechanisms through which steroid hormones regulate sexual behavior.

Experimental procedures

Insects and tissue collection

Agrotis ipsilon adults originated from a laboratory colony established in Bordeaux and transferred to Versailles. The colony is based on field catches in southern France, and wild insects are introduced each spring. Insects were reared on an artificial diet [39] in individual cups until pupation. Pupae were sexed, and males and females were kept separately under a light/dark cycle (16 h of light; 8 h of dark) 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 to be day 0.

All tissue dissections were performed in mid-scotophase (3–5 h after lights off), when males respond maximally to sex pheromone [40]. For *AisytI* cloning and tissue/age-related expression, brains, ALs, antennae, eyes, proboscises, thoracic muscles, wings, legs and abdomens of males were dissected in Ringer's solution, and then immediately

flash-frozen in Eppendorf 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 *AisytI*

Total RNAs were extracted with TRIzol reagent (TRI Reagent; Euromedex), according to the manufacturer's instructions, and were quantified by spectrophotometry at 260 nm. DNase treatment was performed (two units of TURBO DNase; Ambion) for 30 min at 37 °C, and this was followed by a 10-min inactivation at 75 °C. After DNase treatment, single-stranded cDNAs were synthesized from total RNAs (1 µg) with SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. The reaction contained a dNTP mix, RNase OUT, Oligo(dT) primer and sterile water in a final volume of 20 µL. The mixture was heated to 65 °C for 5 min before the enzyme was added, and then incubated for 1 h at 42 °C.

The degenerate DNA primers SytI_{dir1} and SytI_{rev1} were designed against the highly conserved amino acid sequences LGDICFS and FEVPFEQ of the C2B domains of *Ap. mellifera*, *B. mori* and *M. sexta* sytI (Table 2). PCRs were carried out with 200 ng of brain cDNA and 2.5 units of High Expand Fidelity DNA polymerase (Roche). SytI_{dir1} and SytI_{rev1} were then added at 0.4 µM, and each dNTP at 0.25 mM. Following an initial 5-min denaturation at 94 °C, the thermal amplification procedure included 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 45 s, and then final elongation at 72 °C for 10 min.

The 5' and 3' regions of the corresponding cDNA were obtained by 5'-RACE and 3'-RACE (SMART RACE cDNA Amplification Kit; Clontech), according to the manufacturer's instructions. For 5'-RACE, we used a specific reverse primer, SytI5'-RACE, and Universal Primer Mix (Clontech) as the forward anchor primer (Table 2). The 3'-RACE amplification was carried out with Universal Primer Mix as the reverse primer and a specific forward primer, SytI3'-RACE (Table 2). Touchdown PCR was performed with hot start as follows: 1 min at 94 °C; five cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C; five cycles of 30 s at 94 °C, 30 s at 66 °C, and 3 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 64 °C, and 3 min at 72 °C; and 10 min at 72 °C.

PCR products were purified by agarose gel electrophoresis (NucleoSpin Extract II; Macherey-Nagel, Düren, Germany) and cloned into PCRII-Topo plasmid (Invitrogen, Carlsbad, CA, USA). After colony isolation, DNA minipreps were prepared (NucleoSpin Plasmid DNA Purification; Macherey-Nagel), and the DNA clone containing the proper insert was then sequenced (GATC Biotech SARL, Marseille, France). By merging of the overlapping sequences obtained from the 5'-RACE and 3'-RACE, a

Table 2. List of the primers used in the study.

Primer name	Sequence (5'- to 3')
SytlDir1	TTRGGMGACATHTGTTTTYTC
SytlRev1	CTGTTTCRAAWGGYACYTCAAA
Sytl5'-RACE	AGCAGAAGCATATATCCGAGCACGA
Sytl3'-RACE	GAGCTTCGTCATTGGTCCGACATGCT
qSytlDir	TCGCCACTGCTGGAGACGCTG
qSytlRev	TGGTTCTATGTTTCATCTTGCG
qEcRDir1	GCATCACGTATAGCATGGCGCAAT
qEcRRev1	GCCGTTAATACTCGAAGCTGGTGA
AiRpL8dir	CCAGTTTGTCTACTGCGGCAA
AiRpL8rev	GCTTAACCCTAGTACGCTTGGA
DigSytlDir	TCGCCACTG CTGGAGACGCTG
DigSytlRev	GTCAGGTTTCCTCAGCGTTCTC
Aisytl T7 dir	taatacgactcactatagggACAAGGAGAAGGTTTCAGCCTGACA
Aisytl T7 rev	taatacgactcactatagggGTCAGTTTCACAGCCGTTGGTACA
AiEcR T7 dir	taatacgactcactatagggGCGACGGAAAACAGAAGTGAAGCC
AiEcR T7 rev	taatacgactcactatagggGCCGTTAATACTCGAAGCTGGTGA
LacZ T7 dir	taatacgactcactatagggATGACCATGATTACGCCAAGC
LacZ T7 rev	taatacgactcactatagggCCATTCGCCATTCAGGCTGCG

putative full-length cDNA of 1758 bp was generated, and named *Aisytl*.

Real-time qPCR

Real-time qPCR was performed on cDNA preparations from various tissues with the ICycler iQTM Real-Time PCR Detection System (Bio-Rad, Life Science, Marnes-la-Coquette, France), according to the manufacturer's instructions. Each 12- μ L reaction consisted of 6 μ L of Absolute Blue SYBR Green fluor (Thermo Scientific, Waltham, MA, USA), 4 μ L of cDNA (25 ng- μ L⁻¹), and 2 μ L of specific primer pairs at 10 μ M designed from IN of *Aisytl* (qSytlDir and qSytlRev) or the A/B domain of *AiEcR* (qEcRDir1 and qEcRRev1; the EcR-B1 isoform was used throughout the study) (Table 2). PCR conditions were 35 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. The lengths of amplified products were 207 bp and 199 bp for *Aisytl* and *AiEcR* respectively. 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 two primer pairs. Each run included a negative control (water) and a five-fold dilution series of pooled cDNA (from all conditions). The five-fold dilution series were used to construct a relative standard curve to determine the PCR efficiencies and for further quantification analysis. In all experiments, the primers gave amplification efficiencies of 90–100%. Three technical replicates were performed for each reaction, with at least five independent biological replicates, on the technical platform of the Integrative Biology Institute (Pierre et Marie Curie University, France). Expression levels were analyzed with ICYCLER IQ software and the GENORM VISUAL BASIC application for Microsoft

Excel, as described by Vandesompele [41]. The cycle threshold values (Ct values) were determined for the candidate gene and the reference gene, the *A. ipsilon* ribosomal protein RpL8 gene (accession number [JX975720.1](#)), which showed the most stable expression levels among other tested control genes, i.e. the RpL13, glyceraldehyde-3-phosphate dehydrogenase and β -actin genes. The average Ct value of each triplicate reaction was used to normalize the candidate gene expression level to the geometric mean of the *RpL8* level in Q-GENE software [42]. The sequences for specific primers of *A. ipsilon RpL8* are AiRpL8dir and AiRpL8rev (Table 2).

Northern blotting

Northern blot hybridization analysis was performed according to the manufacturer's instructions. RNA samples (15 μ g) were denatured with formamide (50%) and formaldehyde (2.2 M), separated on 1% denaturing agarose gel, and transferred to a Boehringer Mannheim positively charged nylon membrane. Blotted RNA was hybridized overnight at 55 °C with a digoxigenin (DIG)-labeled *Aisytl* probe. Immunological signal detection by chemiluminescence was performed as described in Roche's DIG system User's Guide for filter hybridization. A DIG-labeled molecular RNA marker ladder (Roche, Meylan, France) was run in parallel on northern blots to determine the molecular masses of hybridizing RNAs. The DIG-labeled probe was made by PCR from *Aisytl* cDNA with the PCR DIG probe synthesis kit (Roche, Meylan, France) with a pair of specific primers, i.e. DigSytlDir and DigSytlRev (Table 2). The DIG-labeled probe was used at a concentration of 25 ng-mL⁻¹ in hybridization solution.

Chemicals

20E was a gift from R. Lafont (Pierre et Marie Curie University, Paris, France), and the stock solution was made in ethanol at a concentration of 10^{-2} M, and then stored at -20 °C. The 20E stock solution was diluted with an NaCl (145 mM) solution to prepare a range of working solutions at concentrations ranging from 10^{-6} M to 10^{-10} M. For the wind tunnel experiments, we prepared an artificial pheromone blend diluted in hexane at 1 ng· μ L $^{-1}$, containing (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, and (*Z*)-11-hexadecenyl acetate (Sigma Aldrich, Saint-Quentin Fallavier, France) at a ratio 4 : 1 : 4, which has been used successfully in field trapping experiments [43,44].

Hormonal treatments

Day 0 males (0.2 ± 0.002 g body weight) were anesthetized with carbon dioxide, and received an injection of 2 μ L of 20E working solution in the abdomen with a Hamilton syringe before the onset of scotophase. After a postinjection delay, the expression level of *AisyI* in the AL was determined by real-time qPCR.

RNAi experiments

The dsRNA production and injection conditions, and the delay between injection and dissection/testing, were established by following the protocol used for the silencing of a G-protein-coupled dopamine/ecdyteroid receptor in *A. ipsilon* [11]. The dsRNAs designed against *AisyI* (544 bp), *AiEcR* (460 bp) or bacterial *LacZ* (372 bp) were produced with the MEGAscript T7 High Yield Transcription Kit (Ambion), according to the manufacturer's instructions. PCR was performed on 1 μ L of plasmid (50 ng· μ L $^{-1}$) with specific primers for each target gene (*AisyI* T7 dir/*AisyI* T7 rev, *AiEcR* T7 dir/*AiEcR* T7 rev, and *LacZ* T7 dir/*LacZ* T7 rev), with the following program: 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min (Table 2).

PCR products were purified with the Nucleospin Extract II kit (Macherey Nagel), and quantified by spectrometry. The transcription reaction was then performed overnight at 37 °C on 1 μ g of PCR product in a reaction mixture containing 2 μ L of each nucleotide, 2 μ L of T7 RNA polymerase enzyme and 2 μ L of T7 10 \times buffer in a final volume of 20 μ L. After spectrometric quantification and gel dock analysis, the reaction mix was incubated for 15 min at 37 °C with two units of Turbo DNase (Ambion). dsRNA precipitation was performed by addition of 30 μ L of diethylpyrocarbonate water and 20 μ L of LiCl, storage at -20 °C for 2 h, and centrifugation for 30 min at 16 000 *g*. The pellet was washed with 1 mL of 75% ethanol, centrifuged for 15 min at 12 000 *g* and dried before elution in 11 μ L of diethylpyrocarbonate water. Samples were dena-

tured at 95 °C for 5 min, and this was followed by a re-hybridization step of 1 h 30 min at room temperature. Then, dsRNA integrity was checked by loading on agarose gel. Before injection, dsRNA was diluted at 0.5 μ g μ L $^{-1}$ in saline solution (7.1 mM CaCl₂, 22 mM Na₂- β -glycerophosphate, 13.5 mM MgSO₄, 26.9 mM MgCl₂, 29.5 mM KCl, 23.9 mM glucose, pH 6.8).

Day 1 males were injected with 1 μ g of dsRNA in the abdomen. At day 5, their behavioral response to sex pheromone was tested in a wind tunnel, and the ALs were then dissected for cDNA synthesis and gene expression analysis by qPCR. Control groups consisted of bacterial *LacZ*-dsRNA-injected, saline-injected and noninjected males.

Wind tunnel experiments

Behavioral tests were performed in a 190-cm-long flight tunnel under red light illumination, as previously described [40]. Experiments were performed during the middle of the scotophase (4–7 h after lights off), when males respond maximally to sex pheromone [45]. Environmental conditions during the bioassay were held constant: 23 °C, $50\% \pm 10\%$ relative humidity, and wind speed of 0.3 ms $^{-1}$. 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 of the cage, was delivered for 3 min. During this period of time, the behavior of males (*AisyI*-dsRNA-injected, *AiEcR*-dsRNA-injected 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 oriented responses towards the sex pheromone. Both oriented and random flights were counted, in order to quantify the general flight activity of insects. All experiments were performed double-blind to avoid partial observations. On each day of experimentation, 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.

Pheromone stimulation was delivered with a programmable olfactometer adapted from Party *et al.* [46]. Air from the building was charcoal-filtered and rehumidified, and then divided into several equal flows (300 ± 10 mL·min $^{-1}$) with a Y-connector (model 1/8 inch P514; Upchurch Scientific) and a five-port manifold (model P-115; Upchurch Scientific). Each flow was connected to a miniature electrovalve (model LHDA1233115H; Lee Company, Westbrook, USA) driven by a Valve-Bank programmer (Auto-Mate Scientific, Berkeley, 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 composed of two hypodermic needles (18-g size) inserted through the septum and connected with poly(tetrafluoroethylene) tubing (internal

diameter, 1.32 mm; length, 20 cm). Ten microliters of the pheromone blend at 1 ng μ L was deposited on a filter paper and placed inside the vial. This dose (10 ng) was shown to give the best behavioral results with sexually mature virgin males [40]. Contaminated air was removed from the set-up by an exhaust fan after each behavioral test.

Bioinformatics and statistical analysis

For the bioinformatic study of the primary structure of insect and mammalian syts I, the protein sequences were aligned by the use of CLUSTALW in BIOEDIT 7.0. Accession numbers (IDs) for sytI protein sequences retrieved from GenBank are as follows: the lepidopteran *Ap. mellifera*, [NP_001139207.1](#); *B. mori*, [NP_001153672.1](#), *M. sexta*, [AAK01129.1](#); the hemipteran *Acyrtosiphon pisum*, [XP_003244566.1](#); the dipteran *D. melanogaster*, [NP_523460.2](#); the coleopteran *Tribolium castaneum*, [NP_001139384.1](#); human, [NP_001129278.1](#); and *Mus musculus*, [NP_033332.1](#).

AisytI and *AiEcR* expression level means were compared by the use of ANOVA followed by a Tukey multiple comparison *post hoc* test. For behavioral experiments, statistical differences between groups of insect experimental males were evaluated with an R \times C test of independence by means of a G-test and also by applying Williams's correction [47]. $P < 0.05$ was accepted as statistically significant.

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Author contributions

F. Bozzolan and S. Debernard designed research. F. Bozzolan, D. Limousin, E. Demondion, M.-A. Wycke and S. Debernard performed experiments. F. Bozzolan, L. Duportets, A. Abrieux, A. François and S. Debernard analyzed data. F. Bozzolan and S. Debernard wrote the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. Nucleotide and deduced amino acid sequences for *A. ipsilon* sytI (*AisytI*).