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Cloning of an octopamine/tyramine receptor and plasticity of its expression as a function of adult sexual maturation in the male moth *Agrotis ipsilon*

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Abstract

In the male moth *Agrotis ipsilon* behavioural response and antennal lobe (AL) neuron sensitivity to the female-produced sex pheromone increase with age and juvenile hormone (JH) level. We recently showed that the neuromodulator, octopamine (OA), interacts with JH in this age-dependent olfactory plasticity. To further elucidate its role, we cloned a full cDNA encoding a protein that presents biochemical features essential to OA/tyramine receptor (AipsOAR/TAR) function. The AipsOAR/TAR transcript was detected predominantly in the antennae, the brain and, more specifically, in ALs where its expression level varied concomitantly with age. This expression plasticity indicates that AipsOAR/TAR might be involved in central processing of the pheromone signal during maturation of sexual behaviour in *A. ipsilon*.

Keywords: antennal lobe, octopamine/tyramine receptor, olfactory processing, maturation, expression plasticity.

Introduction

In vertebrates and invertebrates, biogenic amines are neuroactive chemicals that play functional roles as neurotransmitter, neuromodulator and neurohomone through the activation of diverse signalling pathways in peripheral tissues, sense organs and the central nervous system (Libersat & Pflueger, 2004; Huber, 2005). Two monoamines found at relatively high levels in the invertebrate nervous system are octopamine (OA) and tyramine (TA). Until recently, TA was considered only to be a biosynthetic precursor of OA. However, it is now established that, like OA, TA is a true neuroactive substance (Downer *et al*., 1993; Kutsukake *et al*., 2000; Nagaya *et al*., 2002; Donini & Lange, 2004).

In insects, both amines have been linked to neuromodulation of a diverse range of behaviours. In the honeybee, TA has potential effects on both the behavioural responses to antennal stimulation with taste stimuli (Scheiner *et al*., 2002) and the transition to reproductive workers, influencing ovarian development (Sasaki & Harano, 2007). In the locust, TA is thought to influence the behavioural responses and behavioural state with regard to phase change (Rogers *et al*., 2004). In concert with TA, OA has been shown to play a role in the initiation and modulation of the central pattern generator controlling locomotion in *Drosophila* larvae (Fox *et al*., 2006). In the honeybee, OA is involved in nestmate recognition (Robinson *et al*., 1999), in division of labour within the community (Schulz *et al*., 2002), in responsiveness to foraging stimuli (Barron *et al*., 2002), and in memory reinforcement (Farooqui *et al*., 2003). In male moths, OA was also shown to enhance the sensitivity to sex pheromone during upwind flight behaviour (Linn & Roelofs, 1986, 1992; Linn *et al*., 1992, 1996).

In the noctuid moth *Agrotis ipsilon*, the behavioural response to sex pheromone is age dependent. Newly emerged males are sexually immature and do not respond behaviourally to the female-produced sex pheromone. Three to five days after emergence, males become sexually mature and are then highly attracted to sex pheromone (Gadenne *et al*., 1993). This age-based behavioural plasticity depends on the biosynthesis activity of juvenile hormone (JH), which controls the sensitivity of neurons in the primary olfactory centre, the antennal lobe (AL) (Anton

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& Gadenne, 1999; Gadenne & Anton, 2000). Our previous studies have revealed that the sensitivity of the peripheral pheromone detection system on the antennae of *A. ipsilon* males is age- and JH-independent (Gadenne *et al*., 1993). In honeybees, JH affects the initiation of foraging behaviour by increasing brain levels of OA, but OA can induce foraging also independently of JH (Schulz *et al*., 2002). In *A. ipsilon* males, we recently showed that although OA levels in ALs were similar in young immature and in sexually mature males, both OA and JH are necessary to elicit optimal neural and behavioral responses to sex pheromone (Jarriault *et al*., 2009). In particular, we showed that the sensitivity of AL neurons and the behavioural responses were optimal only when both JH and OA levels were high, and these two response parameters decreased when the level of any of the two modulators was artificially decreased (Jarriault *et al*., 2009), or were improved when OA was artificially increased (Barrozo *et al*., 2010).

As in vertebrates, the effects of insect biogenic amines are mainly mediated by interactions with G protein-coupled receptors (GPCRs) which are linked to different second messenger pathways (Roeder, 2005). OA receptors (OARs) have been identified in several species of insects, including fruit flies (Arakawa *et al*., 1990; Saudou *et al*., 1990), honeybees (Blenau *et al*., 2000; Grohmann *et al*., 2003), locusts (Vanden Broeck *et al*., 1995; Molaei *et al*., 2005) and moths (Ohta *et al*., 2003; Ohtani *et al*., 2006; Brigaud *et al*., 2009). These receptors have been classified according to their similarities in biochemical and pharmacological properties with vertebrate adrenergic receptors and constitute α-adrenergic-like OA receptors (αOARs), β-adrenergic-like OA receptors (βOARs) and OA/TA receptors (OAR/TARs) (Evans & Maqueira, 2005; Farooqui, 2007a). In appropriate heterologous expression systems, the functional characterization of cloned OAR/TARs demonstrated that they are stimulated by both TA and OA but exhibit a higher affinity for TA than OA and are, therefore, categorized as tyraminergic receptors (Saudou *et al*., 1990; Robb *et al*., 1994; Poels *et al*., 2001; Ohta *et al*., 2003).

In social insects, OARs and OARs/TARs are expressed throughout the central nervous system, notably in most regions of the brain where they are coupled to cAMP-signalling pathways controlling various behaviours (Farooqui, 2007a,b; Mustard *et al*., 2005). In honeybees, age-related changes in the expression level of a D-2 type dopamine receptor and a tyramine receptor within clusters of cell bodies in the mushroom bodies (MBs) were associated with shifts in the behaviour of adult workers (Humphries *et al*., 2003; Mustard *et al*., 2005).

To further elucidate the role of octopaminergic/tyraminergic systems in the age- and JH-dependent behavioural and central nervous responses to sex pheromone in *A. ipsilon* males, we searched for a putative OAR/TAR receptor and analysed its spatiotemporal expression profiles. Using a 5′/3′ rapid amplification of cDNA ends (RACE) PCR strategy based on the high degree of conservation of insect OAR/TAR proteins, we succeeded in cloning a cDNA encoding an aminergic receptor, which belongs to the cluster of the OA/TA receptor family and, thus, is referred to as AipsOAR/TAR. Reverse Transcription (RT)-PCR and real time qPCR experiments revealed that AipsOAR/TAR was expressed in various tissues including the antennae, the brain and more specifically the ALs. Moreover, our results show that AipsOAR/TAR expression levels varied concomitantly with age. These data suggest the existence of a correlation between age-based plasticity of OAR/TAR expression in the ALs and the maturation of sexual behaviour in the *A. ipsilon* male.

Results

Isolation and characterization of Agrotis ipsilon *octopamine/tyramine receptor*

Cloning of AipsOAR/TAR cDNA fragment. By taking advantage of the high degree of sequence conservation that exists in the transmembrane domains (TMs) of biogenic amine GPCRs, a partial fragment of 657 bp was first obtained via degenerate RT-PCR reaction of total RNA extracted from *A. ipsilon* brains. The remaining 5′ and 3′ ends of the fragment were then amplified by developing a RACE/PCR-based strategy. The nucleic acid sequences for the 5′- and 3′-RACE reaction products were assembled with the original fragment to generate a full-length cDNA named AipsOAR/TAR and deposited in the GenBank database under the accession number **FJ640850**. This cDNA of 2602 bp contains a putative coding region of 1431 pb, a 487 bp 5′-untranslated region (5′-UTR) and a 684 bp 3′-UTR, with a polyadenylation signal upstream of the poly(A) tail (Fig. 1). The AipsOAR/TAR cDNA was translated into a predicted amino acid sequence using the biosciences software. The open reading frame, which starts from ATG, encodes 477 amino acids (Fig. 1), predicting a 61 kDa protein with a calculated isoelectric point of 9.41 as determined using MWCALC (Infobiogen, France).

Sequence comparison and phylogenetic analysis. Hydrophobicity analysis of the AipsOAR/TAR protein revealed the presence of seven alpha-helical transmembrane domains (TM I–TM VII) connected by extra- and intracellular loops with the N-terminal oriented towards the outside of the membrane and the C-terminal within the cytosol in agreement with the typical GPCR conformation (data not shown). Analysis of the deduced amino acid sequence of AipsOAR/TAR showed the presence of three potential N-linked glycosylation sites (N_{11}, N_{16}, N_{345}) located in the extracellular N-terminal and the third

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ACGCGGGGAGTTGAGAGTCGGTCGCTACGGAGTACGCTCGCTACGGCCGCTGACGTATGTGCTACGCGAGAGTGC
TACGAAACTATATCTTTAGAACTTTGAACTAAGTTGGATAGAAGTGAATGTGAATATAATAGTAGAACTATAGCA
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CACCGAATATTAAATAGATTTGTTTACGCATCAAAAAATGAACAAGTCACACAAATGGGACTTCCTAGTAAAGAT
TTCCAGCAGGAGAAAATCGGAATATGAAAGGATGAATAAAGGAAATTCGCGACTCACGGTCCTTCCCTACAGTAC
AACCTGAAATGTAGCCAAGTCATACAACCACTTCAAG
1     ATG GGG CAA GCA GCT ACA CAC GCT GAT GCT AAC TAC ACA TTA ATA   45
1      M   G   Q   A   A   T   H   A   D   A   N   Y   T   L   I   15
46    AAT TAC ACT GAT GAG GTC ATC TTA GAC GAT AGA GAC GCT TGC GCT   90
16     N   Y   T   D   E   V   I   L   D   D   R   D   A   C   A   30
91    GTC GCC GAT GAT CCT AAA TAT CCG AGT AGC TTT GGG ATA ACA CTA   135
31     V   A   D   D   P   K   Y   P   S   S   F   G   I   T   L   45
136   GCG GTG CCA GAA TGG GAG GCA ATT TGT ACA GCA ATA GTA CTT ACT   180
46     A   V   P   E   W   E   A   I   C   T   A   I   V   L   T   60
                                           I
181   CTG ATA ATA ATC TCA ACG ATA GTT GGA AAC ATC TTG GTA ATC TTA   225
61     L   I   I   I   S   T   I   V   G   N   I   L   V   I   L   75
226   AGC GTG TTC ACA TAC AAG CCG CTC CGG ATC GTC CAA AAC TTC TTC   270
76     S   V   F   T   Y   K   P   L   R   I   V   Q   N   F   F   90
271   ATA GTA TCA CTC GCC GTG GCG GAT TTG ACA GTA GCA ATA CTG GTG   315
91     I   V   S   L   A   V   A   D   L   T   V   A   I   L   V   105
                                  II
316   CTA CCA CTG AAT GTG GCA TAC TCC ATT CTC GGA CAA TGG GTA TTC   360
106    L   P   L   N   V   A   Y   S   I   L   G   Q   W   V   F   120
361   GGA ATA TAT GTG TGC AAG ATG TGG CTG ACA TGC GAC ATA ATG TGC   405
121    G   I   Y   V   C   K   M   W   L   T   C   D   I   M   C   135
                                                     III
406   TGC ACT TCG TCC ATT TTA AAC TTA TGT GCA ATC GCA TTA GAC AGG   450
136    C   T   S   S   I   L   N   L   C   A   I   A   L   D   R   150
451   TAC TGG GCG ATT ACG GAT CCT ATA AAC TAC GCA CAA AAA AGA ACC   495
151    Y   W   A   I   T   D   P   I   N   Y   A   Q   K   R   T   165
496   TTA GAA AGA GTT CTC GTA ATG ATC GGA GTA GTT TGG ATT CTC TCA   540
166    L   E   R   V   L   V   M   I   G   V   V   W   I   L   S   180
                      IV
541   TTA ATT ATA AGC TCT CCT CCA TTA CTA GGT TGG AAC GAC TGG CCT   585
181    L   I   I   S   S   P   P   L   L   G   W   N   D   W   P   195
586   GAA GTG TTT GAA CCG GAC ACG CCC TGT CGT TTA ACT TCA CAA CCT   630
196    E   V   F   E   P   D   T   P   C   R   L   T   S   Q   P   210
631   GGT TTC GTC ATA TTC TCT TCA TCC GGA TCC TTT TAC ATA CCG CTA   675
211    G   F   V   I   F   S   S   S   G   S   F   Y   I   P   L   225
                                           V
676   GTT ATA ATG ACT GTA GTT TAT TTT GAA ATC TAT CTG GCC ACT AAG   720
226    V   I   M   T   V   V   Y   F   E   I   Y   L   A   T   K   240
721   AAA AGA CTT AGA GAT CGC GCT AAG GCC ACT AAA ATC AGC ACT ATC   765
241    K   R   L   R   D   R   A   K   A   T   K   I   S   T   I   255
766   TCT AGC GGT CAA AAT AAA TAC ACA AAT AAG GAC GAC CAA AAT GAT   810
256    S   S   G   Q   N   K   Y   T   T   K   D   D   Q   N   D   270
811   CAA GAC TCC GTG AGC TCC GAG GCA AAT CAC AAT GAA CAC CAA GGT   855
271    Q   D   S   V   S   S   E   A   N   H   N   E   H   Q   G   285
856   GTT ACG CGT TTA GTT TCA GAC AAT GAG AAG AAA AAA CGC ACA AGA   900
286    V   T   R   L   V   S   D   N   E   K   K   K   R   T   R   300
901   AAA CTG ACA CCA AAA AAG AAA CCA AAG AAA CGA TAC TGG AGC AAA   945
301    K   L   T   P   K   K   K   P   K   K   R   Y   W   S   K   315
946   GAT GAT AAA TCA CAA AAC AAA TTG ATC ATA CCC ATA TTG TCT AAC   990
316    D   D   K   S   Q   N   K   L   I   I   P   I   L   S   N   330
991   GAG AAT TCT GTA ACT GAT ATG GGT GAT AAT TTA GAA AAC AGG AAT   1035
331    E   N   S   V   T   D   M   G   D   N   L   E   N   R   N   345
1036  ACA TCT TCG GAG AGT AAC TCG AAG GAA ACC CAT GAA GAT GAT TTG   1080
346    T   S   S   E   S   N   S   K   E   T   H   E   D   D   L   360
1081  ATT GAA GTT AGC GAG ACT GTG CCA GTC AAG TCC AGT CAT AAG CGA   1125
361    I   E   V   S   E   T   V   P   V   K   S   S   H   K   R   375
1126  CCG AAG CCT AAC CAA CAA AGC GCT GTT TAC CAA TTC ATA GAA GAA   1170
376    P   K   P   N   Q   Q   S   S   V   Y   Q   F   I   E   E   390
1171  AAG CAG CGT ATA TCA TTG ACA CGT GAA CGT CGC GCG GCG CGG ACT   1215
391    K   Q   R   I   S   L   T   R   E   R   R   A   A   R   T   405
1216  CTT GGC ATA ATA ATG GGT GTG TTC GTG GTC TGT TGG TTG CCC TTC   1260
406    L   G   I   I   M   G   V   F   V   V   C   W   L   P   F   420
                      VI
1261  TTC GTC ATT TAC CTC GTC ATA CCG TTC TGT GCG AGT TGC TGT TTG   1305
421    F   V   I   Y   L   V   I   P   F   C   A   S   C   C   L   435
1306  TCT AAC AAG TTC ATT AAC TTT ATA ACT TGG CTT GGG TAC TGC AAT   1350
436    S   N   K   F   I   N   F   I   T   W   L   G   Y   C   N   450
                                  VII
1351  TCT GCC CTC AAT CCT CTT ATT TAC ACC ATA TTT AAT ATG GAC TTC   1395
451    S   A   L   N   P   L   I   Y   T   I   F   N   M   D   F   465

1396  AGG AGA GCG TTC AAG AAA TTA CTT TGT ATG AAA CCG   1431
466    R   R   A   F   K   K   L   L   C   M   K   P
TGATCGTGCCAAAGACAATTTGTGTAGGGTTTTATGCTTATACTGAATGATGCTGATTTGCTCAAATGTTTTAAC
TCATTCGTAAGTAGAGTACTAGCAATGGTCTTAAAATAATGAAAATTCGAGTTTATAAAAGTAAGTGACTACGAA
TCTCGATGTACACTTCTTGTATACAGAAATGTTATAATGATATTGCATAAATAGTATAATATCTTTCAAATATTG
TAGAGTAGTTTCATGTACATATTTAAAGGTTGAATAGTGCATAACACTTTTAGATTTCTATATGTATCTGTAAAT
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AATATGTACATATCTTGGTCCGTTTGTATATAATTATTTGTAAATTACAATATCTGTAACTCATACTTAAATACT
ACTTTTATTTAATAGAAATAAAGGTGACCTAAGATAAAGGTTCTTTCAGACATTAGATTATGATTGTTTTCACGA
TTGTATATCCCTTTGAAATAATTTGAGCCCTTATTTAAAGGGGATATTAGACTTGCAACAGTTTGATTAATTTAT
TCCTGGATTGTAATAAAATTTGAATTTCACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATATAAAAAAAAAAA
AAAAAAAAA
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Figure 1. Nucleotide and deduced amino acid sequences of *Agrotis ipsilon* octopamine/tyramine receptor (AipsOAR/TAR). Nucleotide (upper line) and amino acid (lower line) numbers are given on the left and on the right. Putative transmembrane domains (TM) are underlined and numbered from I to VII. Potential sites for N-linked glycosylation are shown in open boxes. Potential sites for phosphorylation by Protein kinase C and Protein Kinase A are indicated by shaded boxes. A polyadenylation signal (AATAAA) in the 3'UTR is designed in bold italic types.

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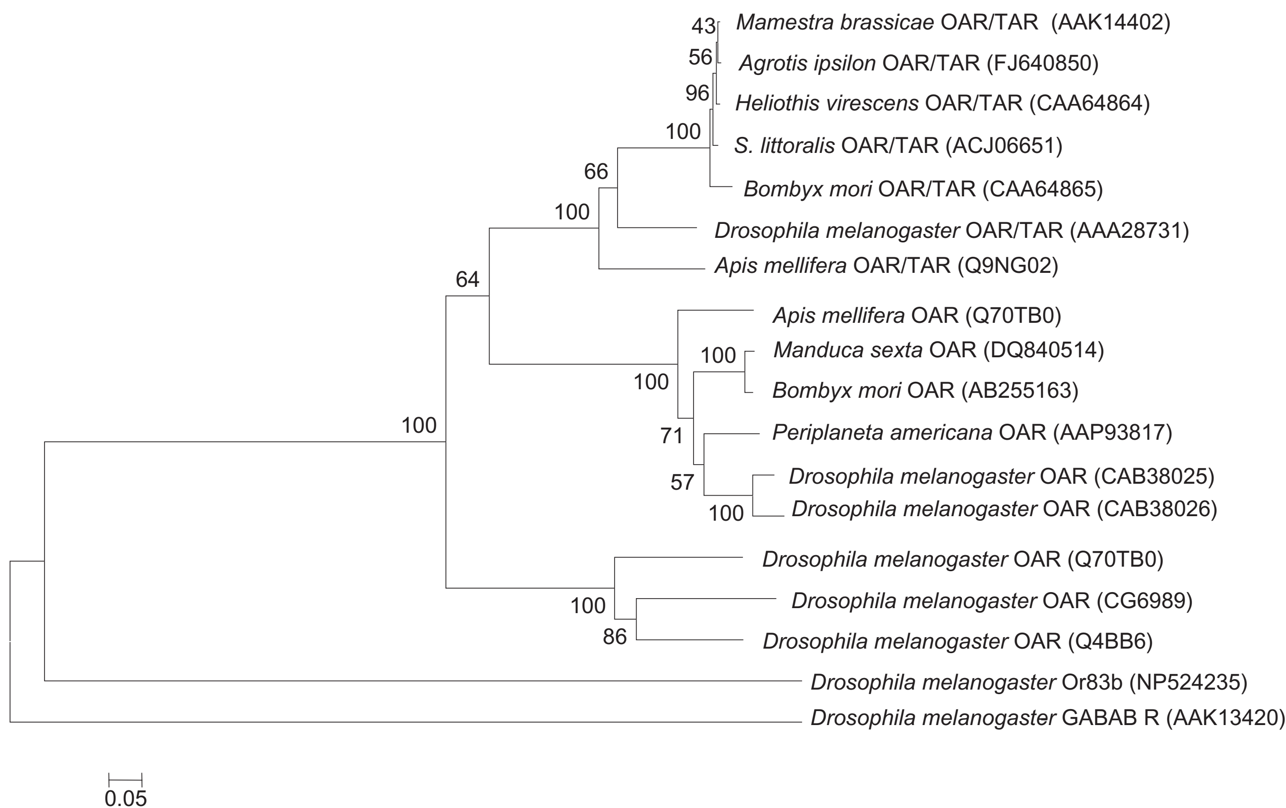

Figure 2. Phylogenetic tree comparing insect biogenic amine receptor proteins belonging to αOAR, βOAR and OAR/TAR classes, with respect to the classification proposed by Evans & Maqueira (2005). The amino acid sequences were aligned using Multalin program (Corpet, 1988) and the phylogenetic tree was inferred using MEGA version 4 (Tamura *et al*., 2007). Branch support was assessed by bootstrap analysis based on 1000 replications. The metabotropic GABA-B receptor (AAK13420) and the olfactory receptor Or83b (NP524235) of *Drosophila melanogaster*, both belonging to the 7 TM domain receptor family, were used as outgroups to root the tree. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Bioinformatic analysis comparing *Agrotis ipsilon* OAR/TAR to other biogenic amine receptor proteins was performed using MEGA version 4 (Tamura *et al*., 2007). The Accession numbers of each amino acid sequence is given in brackets.

extracellular loop (Fig. 1). In addition, several serine and threonine residues positioned in the intracellular loops were identified as sites for phosphorylation by cAMP-dependent Protein kinase A (PKA) and Protein Kinase C (Fig. 1). The phosphorylation of these residues could be involved in receptor desensitization during longer exposure to ligands. The AipsOAR/TAR protein contains also several amino acid residues that are shared between members of the biogenic amine receptor family. Two conserved cysteine residues (C_{125}, C_{204}) with two sequence motifs of D_{149}RY immediately downstream of TM III and N_{454}-X-X-X-Y at the C-terminal of TM VII are characteristic for catecholaminergic receptors. Three serine residues (S_{216},S_{217},S_{220}) in TM V with known spacing patterns in a signature sequence motif S_{216}-S-X-X-S are present in AipsOAR/TAR (Fig. 1). Finally, a conserved sequence of F_{413}-X-X-X-W-L-P followed by two phenylalanine residues (F_{420}, F_{421}) at the middle of TM VI are also commonly found in biogenic amine receptors (Fig. 1).

In order to determine to which subfamily the AipsOAR/TAR is most similar, a phylogenetic family tree was created by comparing the amino acid sequence of AipsOAR/TAR with those of known biogenic amine receptors from different insect species. As revealed in Fig. 2, the AipsOAR/TAR protein belongs to the cluster of the OA/TA receptor family. This clustering is further supported by the observation that AipsOAR/TAR, like other OAR/TARs, has a third intracellular loop much longer than the first two loops (data not shown). In addition, the amino acid sequence of AipsOAR/TAR showed 98%, 93%, 89%, 50% and 54% identity with *Mamestra brassicae* OAR/TAR (AAK14402), *Spodoptera littoralis* (ACJ006651), *Heliothis virescens* OAR/TAR (CAA64864), *Bombyx mori* OAR/TAR (CAA64865), *D. melanogaster* OAR/TAR (AAA28731) and *Apis mellifera* OAR/TAR (Q9NG02), respectively (Fig. 2). From the obtained values, AipsOAR/TAR is clearly assigned as a homologue of *M. brassicae* OAR/TAR.

Expression studies of the octopamine/tyramine receptor

Tissue-related expression. To determine the tissue distribution of AipsOAR/TAR, its expression level was deter-

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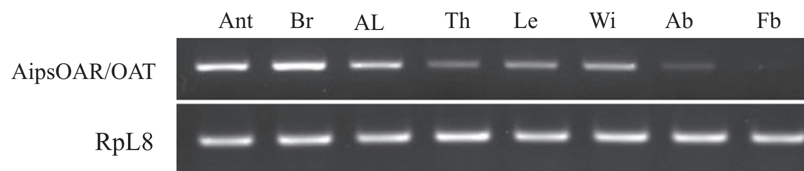

Figure 3. Tissue-related AipsOAR/TAR expression. Two hundred nanograms of total RNAs from various tissues of 5-day-old males were analysed by Reverse Transcription (RT)-PCR using a couple of AipsOAR/TAR specific primers. RNA templates were extracted from antenna (Ant), brain (Br), antennal lobe (AL), thorax (Th), leg (Le), wing (Wi), abdomen (Ab) and fat body (Fb). For Reverse Transcription (RT)-PCR controls, specific primers for *A.ipsilon* RpL8 gene encoding a ribosomal protein were used.

mined by RT-PCR of total RNA samples extracted from tissues of 5-day-old males by using a pair of specific DNA primers (qOARdir, qOARrev) designed from TM III of the receptor. The ubiquitous ribosomal gene RpL8 was used as a positive PCR control. RT-PCR analysis revealed the amplification of only one PCR product of expected size (199 pb) in all tested tissues except for the abdominal fat body (Fig. 3). The expression level of the AipsOAR/TAR gene was higher in the antenna, the brain and more specifically within the AL than in the legs, wings, abdomen and thorax (Fig. 3).

Age-related expression. To comprehend the role of the AipsOAR/TAR receptor in the age-related olfactory plasticity of *A. ipsilon* males, the expression of the corresponding gene was quantified by real-time PCR in various tissues of adult males of different ages. The real-time PCR results revealed that the expression of the AipsOAR/TAR gene is age-regulated only in the brain and more specifically in the ALs. The AipsOAR/TAR transcript was detected at day-1 after emergence and its expression level statistically increased until day-4, then decreased slightly at day-5 (Fig. 4). In contrast, the transcriptional activity of the AipsOAR/TAR gene was age-independent in the antenna (Fig. 4).

Discussion

Structural properties of the octopamine/tyramine receptor

The insect biogenic amines act through GPCRs coupled to different second messenger pathways. The insect OARs were originally classified according to their pharmacological properties and to their intracellular signalling pathways that differ in tissue preparations (Roeder, 1999). Such a classification system is problematic if more than one receptor subtype is present in the same tissue preparation. Evans & Maqueira (2005) proposed a new classification system for insect OARs, which emphasizes their structural and signalling similarities to vertebrate adrenergic receptors subtypes. Three classes of OARs emerged from this work with the αOARs, βOARs and OAR/TARs (Evans & Maqueira, 2005). Using a 5′-3′-RACE PCR-based strategy, we succeeded in cloning the full cDNA encoding the AipsOAR/TAR protein that shows characteristic features of the GPCR superfamily (Strader *et al.*, 1995; Valdenaire & Vernier, 1997) with the presence of seven potential membrane spanning segments. A conserved cysteine residue located at the C-terminal (C_{474}) is thought to be a putative target for palmitoylation that frequently occurs in other GPCRs. This posttranslation modification provides a lipophilic membrane anchor to create a fourth intracellular loop that participates in receptor-G protein interaction (O'Dowd *et al.*, 1989). The AipsOAR/TAR protein shares most of the highly invariant residues of biogenic amines receptors (Baldwin *et al.*, 1997). The two conserved cysteine residues (C_{125}, C_{204}) are predicted to form a disulphide bond, which is essential for the stability of the receptor protein (Dixon *et al.*, 1987). The two sequence motifs of D_{149}RY and N_{454}-X-X-X-Y are characteristics for catecholaminergic receptors and presumed to be required for binding to and activation of G proteins (Fraser *et al.*, 1988; Oliveira *et al.* 1999).

The three serine residues (S_{216},S_{217},S_{220}) in a signature sequence motif S_{216}-S-X-X-S might be involved in maintaining a hydrogen bond between the serine residue and hydroxyl groups of the benzoyl ring of catecholaminergic ligands (Strader *et al.*, 1989, 1995). Finally, a phylogenetic analysis of the AipsOAR/TAR receptor revealed that it belongs to the subfamily of OAR/TARs.

The exact nature of the ligand as well as the terminology of OAR/TARs are still subject to debate since some functional and pharmacological data proved that TA is more potent than OA at activating the receptor *in vitro*. For instance, the best pharmacologically characterized OAR/TAR from the Lepidoptera is the *Bombyx mori* B96Bom, which displays an affinity at least two orders of magnitude higher for TA compared to OA (Ohta *et al.*, 2003). This led to the hypothesis that members of this clade could rather function as specific tyraminergic receptors (Saudou *et al.*, 1990; Robb *et al.*, 1994; Vanden Broeck *et al.*, 1995; Blenau *et al.*, 2000; Kutsukake *et al.*, 2000; Nagaya *et al.*, 2002; Ohta *et al.*, 2003; Roeder *et al.*, 2003; Mustard *et al.*, 2005). However, other *in vitro* experiments demonstrated that OA and TA activated different signalling pathways through the *D. melanogaster* OAR/TAR receptor,

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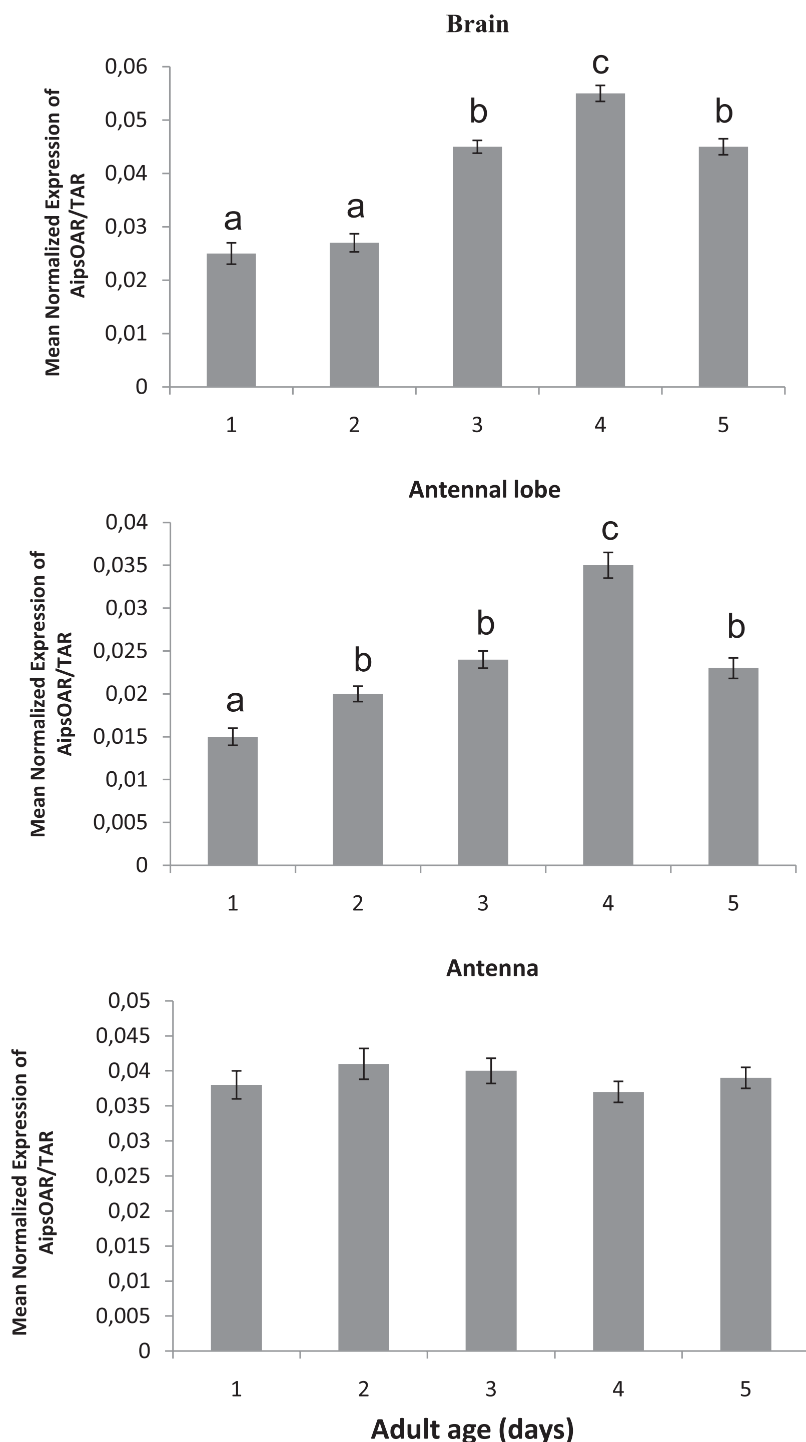

Figure 4. Age-related AipsOAR/TAR expression. Tissues of 1- to 5-day-old males were dissected and synthesized cDNAs were analysed by real-time qPCR using a couple of AipsOAR/TAR specific primers. The control used was the *Agrotis ipsilon* RpL8 gene whose expression was previously analysed to be invariant considering the age of males. Bars represent means ± SD (n = 3 repetitions). Bars with same letters are not significantly different (ANOVA; Tukey test; $\alpha = 0.05$).

which exhibited 'agonist-specific coupling' (Robb *et al.*, 1994). This suggests that agonists differing by a single hydroxyl group in their side chain are able to bias the interactions of a single GPCR with multiple second messenger systems by inducing specific conformational changes, which enable it to couple preferentially to separate G-proteins (Evans *et al.*, 1995; Kenakin, 1995). Over the last two decades, numerous GPCRs that can couple to more than one second messenger system have been identified both in vertebrates and invertebrates, indicating that this property is conserved in the animal kingdom. With regard to OAR/TARs, it is proposed that they might function *in vivo* as multifunctional proteins, being activated by OA and/or TA, depending on the identity of the biogenic amine released presynaptically at the level of target tissues (Lange, 2009).

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Tissue expression pattern and functional implications of the AipsOAR/TAR receptor

OA and TA are known to regulate a plethora of physiological responses as well as sensory and behavioral functions (Roeder, 2005; Farooqui, 2007a,b; Lange, 2009). In order to ensure their modulatory effects, these biogenic monoamines act on various peripheral tissues, sense organs and numerous areas within the central nervous system by functioning as a neurohormone, a neuromodulator and a neurotransmitter (Evans, 1980; David & Coulon, 1985; Roeder *et al*., 2003; Roeder, 2005; Lange, 2009). To gain some information about the functional role of the AipsOAR/TAR gene, its expression level was determined in different tissues from mature *A. ipsilon* males. The expression of the AipsOAR/TAR gene was detected in the whole body including the cephalic, thoracic, abdominal regions and extended to the locomotor appendages and the antennae. Thus, the AipsOAR/TAR gene exhibited a wide tissue expression in accordance with the pleiotropic action of OA and TA described in insects.

Importantly, the AipsOAR/TAR gene was predominantly expressed in the antennae, chemosensory organs that bear olfactory sensilla, as for MbraOAR/TAR, BmorOAR/TAR, and HvirOAR/TAR in the male moths *M. brassicae* (Brigaud *et al*., 2009), *B. mori* and *H. virescens* (Von Nickisch-Rosenegk *et al*., 1996), respectively, and in the AL, as for AmOAR/TAR in the honeybee *A. mellifera* (Farooqui *et al*., 2003; Mustard *et al*., 2005). Therefore, it is likely that AipsOAR/TAR plays a role in mediating TA- and/or OA-induced modulatory effects within the peripheral and central olfactory system in the male moth *A. ipsilon*. This interpretation is supported by our recent findings showing that the injection of mianserin, an antagonist of OARs, decreased the behavioral response and the sensitivity of AL neurons to sex pheromone in mature males (Jarriault *et al*., 2009). Moreover, in *M. brassicae* males, electrophysiological recordings of pheromone-sensitive olfactory sensilla demonstrated that the administration of chlorpromazine, a blocker of OARs, suppressed the firing activity of ORNs in response to pheromone stimulation (Grosmaitre *et al*., 2001). Over recent years, there has been an accumulation of evidence to show that OAR/TARs are also involved in the processing of olfactory information. For instance, in *D. melanogaster*, the *hono* adult mutant expresses reduced levels of an OAR/TAR in the antennae as well as in the MBs and is described to exhibit impaired avoidance behaviour towards repellant odours in T-maze experiments (Kutsukake *et al*., 2000).

Age-related plasticity of AipsOAR/TAR expression

In *A. ipsilon* males, the behavioural and central AL neuron responses to sex pheromone increase with age and JH biosynthetic activity, whereas the sensitivity of the antennae is age independent (Gadenne *et al*., 1993; Duportets *et al*., 1998; Anton & Gadenne, 1999). Our real-time PCR experiments demonstrated that the expression of the AipsOAR/TAR gene in the antennae remained stable as a function of age. On the contrary, the transcriptional activity of AipsOAR/TAR increased with age in the brain and more specifically in the ALs.

The functional significance of the changes in the AipsOAR/TAR expression depends on both OA and TA availability in the central nervous system. Several histological studies revealed that the major parts of the adult insect brain, such as the optic lobes, the central body, the ALs or the MBs, considered as one of the main sensory integration centers, are supplied with these neuroactive substances (Bräunig, 1991; Homberg, 1994; Kreissl *et al*., 1994; Stern *et al*., 1995; Kononenko *et al*., 2009). In the male moth *Manduca sexta*, ALs and MBs show a dense innervation with TA- and OA-immunoreactive neurons (Dacks *et al*., 2005; Iwano & Kanzaki, 2005). In *A. ipsilon* males, OA is also present in ALs but the amounts are age independent (Jarriault *et al*., 2009). Thus, the correlation between the age-based plasticity of AipsOAR/TAR expression in the ALs with that observed in the sex pheromone responses suggests that the AipsOAR/TAR receptor might be a contributor to the neuronal plasticity that takes place in the ALs during the maturation of sexual behaviour of male *A. ipsilon*. The increased AipsOAR/TAR expression might be associated with a high-level protein synthesis, which would be required to mediate age-dependent octopaminergic and tyraminergic effects in the AL network. In contrast, we cannot explain the observed decrease of expression occurring at day 5. It is possible that this decrease is not functionally important, if the protein level is already high.

A correlation between the expression level of aminergic receptors and neuronal changes in the adult brain has been previously reported during behavioural development in social insects. For instance, in the adult worker honeybee, a dopamine receptor (Amdop2) and a tyramine receptor (AmTyr1) exhibited age-based plasticity in their expression level within the MBs (Humphries *et al*., 2003; Mustard *et al*., 2005).

Mechanisms underlying the AipsOAR/TAR action in olfactory plasticity

The functional expression studies have shown that OAR/TARs and OARs are coupled to second messenger pathways to mediate either increases or decreases in intracellular cyclic AMP levels and/or the generation of intracellular calcium signals (Evans & Maqueira, 2005). It is known that the cAMP signalling pathways are associated with neuronal plasticity within the ALs and MBs in

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olfactory learning and memory processes (Menzel & Muller, 1996; Connolly *et al.*, 1996; Menzel, 2001; Roman & Davis, 2001; Farooqui *et al.*, 2003). In *A. ipsilon*, the activation of AipsOAR/TAR in the AL probably induces a cAMP pathway which might alter the activity and/or abundance of proteins responsible for neuronal signalling leading to changes in the intrinsic properties of neurons and ultimately in the responsiveness of males to the sex pheromone.

Most OARs are positively coupled to adenylyl cyclase via G_i-protein and have a high affinity for OA with no cross reactivity for TA (Arakawa *et al.*, 1990; Blenau & Baumann, 2001; Ohtani *et al.*, 2006). In contrast, the OAR/TARs appear to be coupled negatively via Gs-protein to adenylyl cyclase and exhibit a preference for TA over OA (Saudou *et al.*, 1990; Robb *et al.*, 1994; Vanden Broeck *et al.*, 1995; Blenau *et al.*, 2000; Poels *et al.*, 2001; Ohta *et al.*, 2003). Thus, it is speculated that TA, as a neuroactive substance, has functions that differ from those of OA (Ohta *et al.*, 2003; Roeder *et al.*, 2003; Roeder, 2005). In the male *A. ipsilon*, OA influences the central processing of sex pheromone by increasing the sensitivity of AL neurons during the maturation of sexual behaviour (Jarriault *et al.*, 2009) and also by affecting the coding properties of AL neurons (Barrozo *et al.*, 2010). TA might exert, via the activation of AipsOAR/TAR, an antagonist action to that of OA in the AL network. The resulting behavioural changes would be dependent on the balance between the opposite effects of both compounds linked to ratios of octopamine: tyramine receptors and OA : TA levels in the ALs.

Experimental procedures

Insects and tissue collection

Adults of *A. ipsilon* originated from a laboratory colony in Bordeaux. The colony was based on field catches in southern France and wild insects are introduced each spring. The animals were reared on an artificial diet (Poitout & Bues, 1974) in individual cups until pupation. Pupae were sexed and males and females were kept separately in an inversed light/dark cycle (16 h light: 8 h dark photoperiod) 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.

For OAR/TAR cloning and tissue expression, legs, wings, brains, ALs, antennae, thorax, abdomen and fat body of sexually mature 5-day-old males were dissected under saline solution and immediately deep frozen in liquid nitrogen, then stored at −80°C until treatment. To study the expression levels of OAR/TAR as a function of age, tissues of 1- to 5-day-old males were dissected in Ringer's solution and immediately transferred into an Eppendorf vial kept in liquid nitrogen, then stored at −80°C. For the collection of ALs, brains were first dissected then ALs were cut from the protocerebrum with a pair of fine scissors under saline solution, and immediately dipped in Eppendorf vials kept on liquid nitrogen, then stored at −80°C (Jarriault *et al.*, 2009).

RNA isolation and cDNA synthesis

Total RNAs were extracted with TRIzol reagent (Gibco BRL, Paisley, UK) according to the manufacturer's instructions, and were quantified by spectrophotometry at 260 nm. Single-stranded cDNAs were synthesized from total RNAs (1 μg) with 200 U of M-MLV reverse transcriptase using the Advantage™RT-for-PCR Kit (Clontech, Mountain View, CA, USA). The reaction contained a dNTP mix, Rnasin, Oligo(dT) primer and sterile water to a final volume of 25 μl. The mix was heated at 70 °C for 2 min before adding the enzyme and then incubated for 1 h at 42 °C.

For 5′ and 3′ RACE PCR, cDNAs were synthesized from 1 μg of 5-day-old male brain RNA at 42 °C for 1.5 h using the SMART™RACE cDNA Amplification Kit (Clontech) with 200 U of superscriptII (Invitrogen, Carlsbad, CA, USA), 5′- or 3′-CDS-primer and SMART II oligonucleotide.

Cloning of Agrotis ipsilon *octopamine/tyramine receptor*

Two degenerate DNA primers (OARdir1 and OARrev1) were designed on the basis of conserved amino acid sequences on the third transmembrane domain and the third intracellular loop of *H. virescens, B. mori, M. brassicae* OAR/TARs (CAA64864, CAA64865, AAK14402) and *D. melanogaster, A. mellifera, Periplaneta americana* αOARs (CAB38026, Q70TB0, AAP93817). PCRs were carried out with 200 ng of brain cDNA with 2.5 units of High Expand Fidelity DNA polymerase (Boerhinger Mannheim, Mannheim, Germany). The degenerate primers OARdir1 (5′-TAYGTNTGYAARATGTGG-3′) and OARrev1 (5′-TTYTCNCCCATRTCNGTNAC-3′) were added thereafter at 1 μM and each dNTP at 0.8 mM. Following an initial 5 min denaturation at 94 °C, the thermal amplification procedure included 35 cycles of denaturation for 30 s at 94 °C, annealing at 50 °C for 30 s, elongation at 72°C for 1 min 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) following the manufacturer's instructions. For 5′-RACE, we used 2 μl 5′-RACE-ready cDNA with a specific reverse primer OAR5′-RACE (5′-TCTGTTCTGACCGCTAGAGATAGTGCT-3′) and Universal Primer Mix (UPM, Clontech) as the forward anchor primer. The 3′-RACE amplification was carried out with UPM as the reverse primer and a specific forward primer OAR3′-RACE (5′-TGCGACATAATGTGCTGCACTTCGTCC-3′). Touchdown PCR was performed using hot start as follows: after 1 min at 94 °C, five cycles of 30 s at 94 °C and 3 min at 69 °C, then five cycles of 30 s at 94 °C, 30 s at 67 °C and 3 min at 72 °C, then 25 cycles of 30 s at 94 °C, 30 s at 65°C and 3 min at 72 °C, then 10 min at 72 °C.

The PCR products were purified by agarose gel electrophoresis (NucleoSpin® Extract II, Macherey-Nagel, Düren, Germany) and cloned into pCRII-Topo plasmid (Invitrogen). After colony isolation, DNA minipreps were prepared (NucleoSpin® Plasmid DNA Purification, Macherey-Nagel) and correct insertion was determined by restriction enzyme analysis. The DNA clone containing the proper insert was sequenced by GATC Biotech SARL (Marseille, France). By merging the overlapping sequences obtained from the 5′- and 3′-RACE, a putative full-length cDNA of 2705 bp was generated and named AipsOAR/TAR.

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PCR and qPCR

PCR was performed on 200 ng of cDNA preparations from various tissues with 1.25 units of High Expand Fidelity DNA polymerase (Boerhinger Mannheim). The specific primers qOARdir (5′-CAAGTCCAGTCATAAGCGACCGAA-3′) and qOARrev (5′-ACAAACAGCAACTCGCACAGAACG-3′) were added at 0.4 μM and each dNTP at 0.8 mM. Following an initial 5 min denaturation at 94 °C, the thermal amplification procedure included 30 cycles of denaturation for 30 s at 94 °C, annealing at 65 °C for 30 s, elongation at 72°C for 30 s and then final elongation at 72 °C for 10 min. Amplification products (199 bp) were loaded on 1.5% agarose gels and visualized with SYBR Safe.

Real-time qPCR was performed on cDNA preparations using the SYBR green detection system and ICycler iQ™ Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA) according to manufacturer's instructions. Tissues were isolated from male *A. ipsilon* at day 1 to day 5 after emergence. RNA was extracted with TRIzol reagent (Gibco) according to the manufacturer's instructions, DNAse treated (TurboDNase, Ambion, Foster City, CA, USA) and cDNAs synthesized with Superscript II reverse transcriptase (Invitrogen) using oligodT primers. Three independent RNA preparations were made for each sample. PCR conditions were 35 cycles of (95°C 30 s, 65°C 30 s, 72°C 30 s). qPCR was performed in triplicate. The standard curve was analysed for all primers and gave amplification efficiencies of 90–100%. Data were analysed with ICycler iQ software and results with geNORM Visual Basic application for Microsoft Excel as described by Vandesompele *et al*. (2002). The control used was the *A. ipsilon* ribosomal protein rpL8 (Ai-rpL8dir: CCAGTTTGTCTACTGCG-GCAA Ai-rpl8rev: GCTTAACCCTAGTACGCTTGGCA) whose expression was previously analysed to be invariant considering the age of the moth.

Bioinformatics

Protein alignments were carried out with Multalin (Corpet, 1988). Transmembrane domain positions and protein conformation were estimated by using the LOCALIZOME server (Sunghoon *et al*., 2006). The protein sequence was integrated in an alignment matrix by gathering selected insect OAR/TARs, αOARs and βOARs (Clustal W program) to construct a phylogenetic tree by using the neighbour-joining algorithm, with 1000 bootstraps (MEGA.4 program; Tamura *et al*., 2007) in *D. melanogaster* locomotion control (Saraswati *et al*., 2004).

Statistical analysis

To analyse the statistical differences of AipsOAR/TAR expression levels as a function of age, means were compared via one-way ANOVA followed by the post-hoc Tukey test if results were significant with the significance level $\alpha = 0.05$.

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