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RESEARCH ARTICLE

Characterization of the 5-HT_{1A} receptor of the honeybee (*Apis mellifera*) and involvement of serotonin in phototactic behavior

Markus Thamm · Sabine Balfanz · Ricarda Scheiner · Arnd Baumann · Wolfgang Blenau

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Abstract Serotonin plays a key role in modulating various physiological and behavioral processes in both protostomes and deuterostomes. The vast majority of serotonin receptors belong to the superfamily of G-protein-coupled receptors. We report the cloning of a cDNA from the honeybee (*Am5-ht1A*) sharing high similarity with members of the 5-HT_1 receptor class. Activation of Am5-HT_{1A} by serotonin inhibited the production of cAMP in a dose-dependent manner (EC_{50} = 16.9 nM). Am5-HT_{1A} was highly expressed in brain regions known to be involved in visual information processing. Using in vivo pharmacology, we could demonstrate that Am5-HT_{1A} receptor ligands had a strong impact on the phototactic behavior of individual bees. The data presented here mark the first comprehensive study—from gene to behavior—of a 5-HT_{1A} receptor in the honeybee, paving the way for the eventual elucidation of additional roles of this receptor subtype in the physiology and behavior of this social insect.

Keywords 5-HT · Biogenic amine · Cyclic AMP · GPCR · Phototaxis · Signal transduction

Abbreviations

5-CT	5-Carboxamidotryptamine
5-HT	5-Hydroxytryptamine
5-MT	5-Methoxytryptamine
AD	Antibody diluent
Am5-HT_{1A}	*Apis mellifera* 5-HT_{1A} receptor
Am5-ht1A	Gene or cDNA encoding *Apis mellifera* 5-HT_{1A} receptor
$[cAMP]_i$	Intracellular cAMP level
CNS	Central nervous system
CPL3	Third cytoplasmic loop
HA	Hemagglutinin A
HEK 293	Human embryonic kidney cells
PBS	Phosphate-buffered saline
TBS-T	Tris-buffered saline containing Tween 20
TM	Transmembrane domain

M. Thamm · R. Scheiner · W. Blenau (✉)
Institute of Biochemistry and Biology, Animal Physiology, University of Potsdam, Karl-Liebknecht-Str. 24-25, Haus 26, 14476 Potsdam, Germany
e-mail: blenau@uni-potsdam.de

S. Balfanz · A. Baumann
Institute of Structural Biology and Biophysics 1, Forschungszentrum Jülich, 52425 Jülich, Germany

Introduction

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT) acts as a messenger substance in most animal phyla. It controls and modulates a great variety of important physiological and behavioral processes [1]. Disruption of the serotonergic system has been linked to several human disease states, such as schizophrenia, migraine, depression, suicidal behavior, infantile autism, eating disorders, and obsessive-compulsive disorder (for a review, see: [2]). In insects, serotonin signaling is involved in the modulation of heart rate, secretory processes [3], development [4], circadian rhythms [5], aggression [6], behavioral gregarization in locusts [7], and learning and memory [8]. Serotonin is present in large quantities in the central nervous system (CNS) of the honeybee, *Apis mellifera* [9–11]. The distribution of serotonergic neurons has been intensively studied in the brain of adult and pupal worker honeybees [12, 13]. Serotonin levels in the head increase during the transition from the larval to the pupal stage [9].

In adults, serotonin levels in the brain increase with age, with the highest concentrations being found in foragers [9, 10]. Significant differences in serotonin levels have been observed in the optic lobes of nectar versus pollen foragers in normal age-structured colonies [9] and in the antennal lobes of forager versus nurse bees in single-cohort colonies, which consist of same-aged bees [11]. These results indicate that some differences in serotonin level occur independently of age, but instead are related to the specific task that the bee performs. Injection of serotonin into the bee brain impairs the acquisition and retrieval of learned behavioral patterns [14, 15].

Current knowledge regarding the 5-HT receptor subtype(s) mediating the effects of serotonin in the honeybee is limited. In vertebrates, six main classes of G-protein-coupled 5-HT receptors have been classified on the basis of sequence homology, gene organization, coupling to second-messenger pathways, and pharmacological properties (for recent reviews, see: [16, 17]). The 5-HT_1 and 5-HT_5 receptors couple preferentially to $G_{i/o}$ proteins and inhibit cAMP synthesis. The 5-HT_2 receptors couple preferentially to $G_{q/11}$ proteins, which mediate the hydrolysis of inositol phosphates and a subsequent increase in cytosolic Ca^{2+} levels. The 5-HT_4, 5-HT_6, and 5-HT_7 receptors couple preferentially to G_s proteins and promote cAMP formation.

The invertebrate serotonergic system might be similarly complex [18, 19]. For example, *Drosophila melanogaster* is known to express at least four 5-HT receptor subtypes that are predicted to be orthologs of the mammalian 5-HT_{1A} [20], 5-HT_2 [4], and 5-HT_7 [21] receptors. Although the molecular and functional properties of dopamine and phenolamine receptors have been characterized in the honeybee [22–27], little is known about 5-HT receptors in this insect. At the molecular level, only a 5-HT_7 receptor has been characterized in *A. mellifera* so far [28]. No molecular data on other 5-HT receptor classes of the honeybee are as yet available.

In the present study, we have characterized an *A. mellifera* 5-HT_{1A} receptor (Am5-HT_{1A}) that is widely expressed in the CNS. High expression levels have been found in brain areas known to be involved in visual information processing. Using in vivo pharmacology and behavioral testing, we provide evidence that serotonin is involved in the regulation of honeybee phototactic behavior and that the Am5-HT_{1A} receptor is a likely mediator of this effect. In a cell line stably expressing the Am5-HT_{1A} receptor, application of serotonin inhibits NKH 477-stimulated cAMP synthesis with an EC_{50} of $\sim$16.9 nM (NKH 477 is a water-soluble forskolin analog). This effect can be mimicked by 5-carboxamido tryptamine (5-CT) and 5-methoxytryptamine (5-MT) and blocked by the antagonists methiothepin, prazosin, and WAY 100635. This study has therefore elucidated unique molecular and pharmacological details of an insect 5-HT_{1A} receptor and advances our knowledge concerning the complexity of the serotonergic system in insects.

Materials and methods

Cloning of *Am5-ht1A* cDNA

Total RNA was isolated from *Apis mellifera* brains with TRIZOL reagent (Invitrogen, Karlsruhe, Germany). Poly(A)$^+$ RNA was isolated by using the Micro-FastTrack^{TM} 2.0 Kit (Invitrogen). Synthesis of cDNA was performed by use of the Superscript^{TM} First-strand cDNA Synthesis System for RT-PCR (Invitrogen). Specific primers (sense 5′-TTGAATTCATGGAGGAACACGTG AACCAG-3′; antisense 5′-TTTAAATCCACTGTCAGC G-3′) were designed to amplify the entire coding region of the receptor. The polymerase chain reaction (PCR) was carried out for 2.5 min at 94°C (1 cycle), followed by 35 cycles of 40 s at 94°C, 40 s at 58°C, 30 s at 72°C, and a final extension of 10 min at 72°C. The PCR product was cloned into pGEM-T vector (Promega, Mannheim, Germany) and subsequently analyzed by DNA sequencing (AGOWA, Berlin, Germany).

Multiple sequence alignment and phylogenetic analysis

Amino acid sequences used for phylogenetic analyses were identified by protein-protein BLAST searches of the NCBI database with the deduced amino acid sequence of *Am5-ht1A* (Am5-HT_{1A}) as "bait." Multiple sequence alignments of the complete amino acid sequences were performed with ClustalW. Values for identity (ID) and similarity (S) were calculated by using the BLOSUM62 substitution matrix in BioEdit 7.0.5. MEGA 4 was used to calculate the genetic distances between the core sequences and to construct neighbor-joining trees with 10,000-fold bootstrap re-sampling. The *D. melanogaster* ninaE-encoded rhodopsin 1 and the *D. melanogaster* FMRFamide receptor were used as out-groups.

Antibody production and purification

The anti-Am5-HT_{1A} antiserum was raised against a fusion protein containing part of the third cytoplasmic loop (CPL3) of Am5-HT_{1A} (Fig. 1). The cDNA fragment was amplified by PCR with specific primers (sense 5′-TTT GAATTCGGAACCATTGTGCAGCC-3′; antisense 5′-T TTAAGCTTTTAGGTGACCGTGGTCGATTG-3′). The fragment was cloned into pMAL-c2X vector (New England Biolabs), and the construct was called pMAL-Am5-HT_{1A}-CPL3. The fusion protein (MBP-Am5-HT_{1A}-CPL3) was over-expressed in *E. coli* BL21 CP and purified by

amylose affinity-chromatography (New England Biolabs, Frankfurt, Germany). The polyclonal rabbit antiserum was raised commercially (Pineda-Antikörper-Service, Berlin, Germany).

For purification of the anti-Am5-HT$_{1A}$ antibodies, the CPL3 of Am5-HT$_{1A}$ was cloned into pET-30a vector (Novagen, Darmstadt, Germany) and over-expressed (HIS-Am5-HT$_{1A}$-CPL3). Approximately 1.6 mg of purified HIS-Am5-HT$_{1A}$-CPL3 was coupled to a HiTrap NHS-activated high performance (HP) column (Amersham Biosciences, Freiburg, Germany). Antibodies from 50 ml antiserum were affinity-purified by standard protocols.

Western blot analysis

Entire honeybee brains and peripheral tissues/organs were homogenized, and membrane proteins were isolated. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10 or 12% gels; 5–10 μg protein, as determined by a modified Bradford assay, was run per lane. Proteins were transferred to polyvinylidene difluoride membranes (Roth, Karlsruhe, Germany). Membranes were blocked with 5% dry milk in Tris-buffered saline containing Tween 20 (TBS-T 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% Tween 20) for 30 min at room temperature. Membranes were probed with affinity-purified anti-Am5-HT$_{1A}$ antibodies (dilution 1:1,000 in TBS-T). For controls, antibodies were pre-absorbed to MBP-Am5-HT$_{1A}$-CPL3 (15 μg/ml). Membranes were washed with TBS-T, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (1:10,000; anti-rabbit-HRP, American Qualex, La Mirada, CA). Signals were visualized with an enhanced chemiluminescence detection system (Super Signal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL).

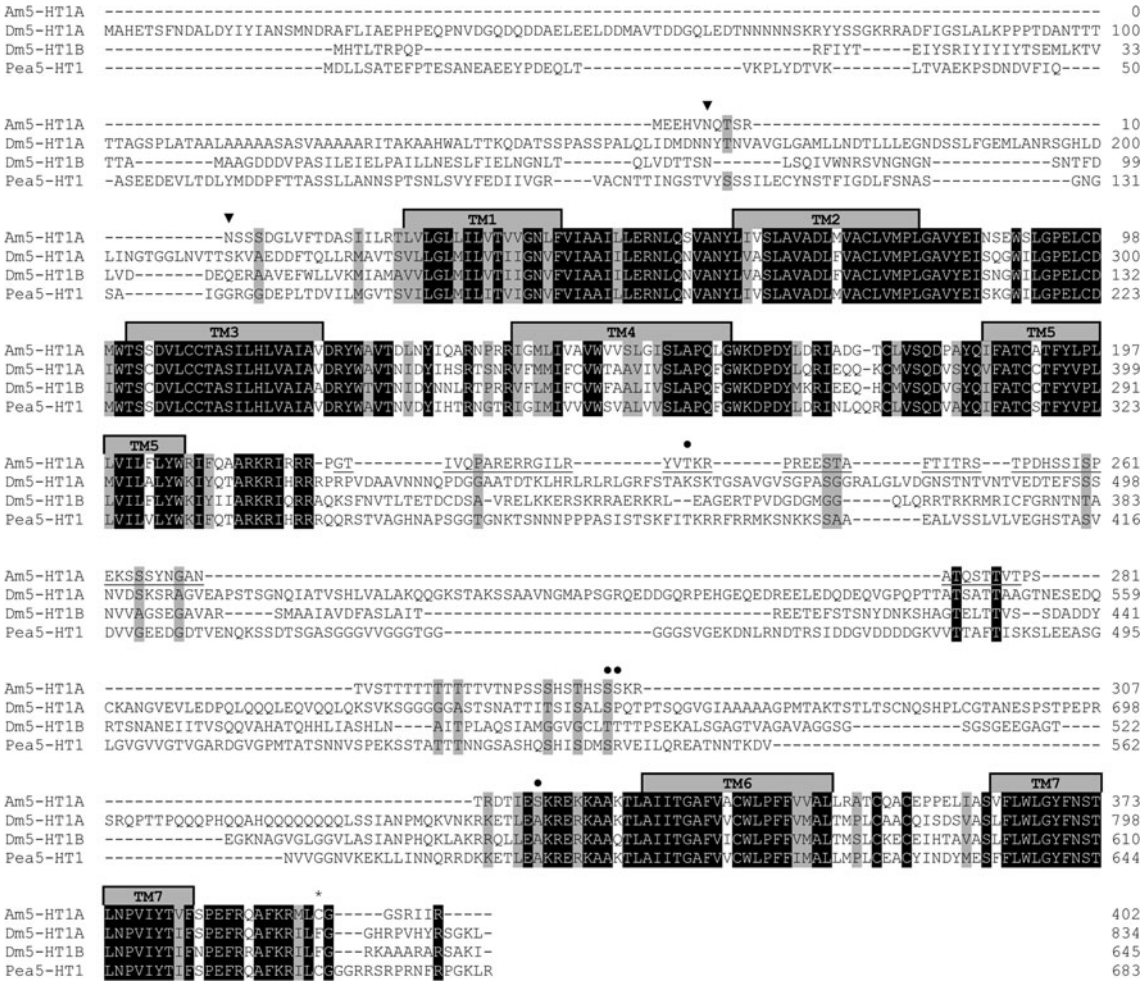

Fig. 1 Amino acid sequence alignment of Am5-HT$_{1A}$ and orthologous receptors from *Drosophila melanogaster* (Dm5-HT$_{1A}$; accession no. CAA77570), Dm5-HT$_{1B}$ (no. CAA77571), and *Periplaneta americana* (Pea5-HT$_1$, no. FN298392). Identical residues (≥80%) between the receptors are shown as *white letters* against *black*, whereas conservatively substituted residues are shaded. Putative transmembrane domains (TM1-7) are indicated by *gray bars*. Potential *N*-glycosylation sites (*inverted filled triangle*), phosphorylation sites via PKC (*filled circle*), and putative palmitoylation sites (*asterisk*) of Am5-HT$_{1A}$ are labeled. Underlined letters represent the region within the CPL3 used to raise Am5-HT$_{1A}$-specific antibodies. The amino acid position is indicated on the *right*

Western blots with membrane proteins (10 µg protein per lane) of human embryonic kidney cells (HEK 293) expressing Am5-HT_{1A}-HA receptors (see below) were incubated with specific anti-hemagglutinin A (HA) antibodies (1:5,000; Anti-HA High Affinity; Roche, Penzberg, Germany) in TBS-T. Membranes were washed with TBS-T, followed by incubation with secondary antibodies (1:5,000, anti-rat-HRP; American Qualex, La Mirada, CA) for 1 h. Signals were visualized by enhanced chemiluminescence.

Immunofluorescence staining of brain sections

Honeybee brains were dissected, fixed for 2 h at 4°C in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4, PBS), and washed (4 × 15 min) with PBS. Brains were then incubated in 10% sucrose in PBS for 1 h at 4°C and in 25% sucrose in PBS at 4°C overnight. After cryofixation at −155°C, brains were cut into 20–25-µm-thick sections, which were mounted on coverslips coated with poly-L-lysine.

The sections were subsequently exposed to the following steps: (1) rinsing in PBS for 5 min; (2) incubation in 0.1% Na-borohydride in PBS for 20 min; (3) 4 × 5 min washing with PBS; (4) incubation in 0.01% Tween 20 in PBS for 5 min; (5) washing in PBS for 5 min; (6) incubation for 90 min in a blocking solution (PBS containing 1% normal goat serum, 0.8% bovine serum albumin, 0.5% Triton X-100), which was also used as antibody diluent (AD); (7) overnight incubation at 4°C in primary antisera (rabbit anti-Am5-HT_{1A} 1:1,000 and rat anti-serotonin 1:100; Chemicon, Temecula, CA) in AD); (8) washing in PBS for 4 × 5 min; (9) incubation in Alexa Fluor 568 goat anti-rabbit IgG 1:100 (Molecular Probes, Eugene, USA) or in Alexa Fluor 488 goat anti-rabbit IgG 1:100 (Molecular Probes, Eugene, OR) and Cy3-conjugated AffiniPure goat anti-rat IgG 1:400 (Jackson Immunoresearch, West Grove, PA) diluted in AD, for 1.5 h at room temperature; (10) washing in PBS 4 × 5 min; (11) coverslip mounting with Mowiol 4.88 (Farbwerke Hoechst, Frankfurt, Germany). Sections from five individual brains were treated with the Am5-HT_{1A} antiserum and sections from two brains with pre-absorbed antiserum. Fluorescence images were recorded with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Construction of pc*Am5-ht1A*-HA expression vector

An expression-ready construct of *Am5-ht1A* cDNA was generated by PCR, which was performed with specific primers (sense 5′-TTTAAGCTTCC-ACCATGGAGGAA CACGTGAACC-3′; antisense 5′-TTTGAATTCGCGAA TTATCCTG-GAACCACC-3′). Additionally, a double-stranded HA-encoding oligonucleotide (HA-oligo) was generated by annealing two complementary oligonucletides (sense 5′-AAAGAATTCTACCCATACGACGTCCC AGACTACGCTTAAGCGGCCGCTTTTT-3′; antisense 5′-AAAAAGCGGCCGCTTAAGCGTAGTCTGGGACGT CGTATGGGTAGAATTCTTT-3′). The oligo was ligated to the 3' end of *Am5-ht1A* cDNA to monitor the transfection efficiency and receptor protein expression. The PCR product was digested with *Hin*dIII and *Eco*RI, and the HA-oligo was digested by *Eco*RI and *Not*I. The digested DNA fragments were then sub-cloned into pcDNA3.1(+) vector (Invitrogen) yielding pc*Am5-ht1A*-HA. The correct insertion was confirmed by DNA sequencing.

Functional expression of the Am5-HT_{1A}-HA receptor

Approximately 8 µg pc*Am5-ht1A*-HA vector was introduced into exponentially growing HEK 293 cells ($\sim 4\times10^5$ cells per 5-cm Petri dish) by a modified calcium phosphate method. Stably transfected cells were selected in the presence of the antibiotic G418 at 0.8 mg/ml. Isolated foci were propagated and analyzed for the expression of Am5-HT_{1A}-HA by immunocytochemistry and Western blotting with anti-HA antibodies (Roche).

Functional characterization of Am5-HT_{1A} receptors

Assays to determine the ability of Am5-HT_{1A}-HA receptors to attenuate adenylyl cyclase activity were performed as described earlier [28, 29]. Am5-HT_{1A}-expressing cells were grown in minimal essential medium with GlutaMAX, 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, and antibiotics (all from Invitrogen). Cells were incubated with ligands for 30 min at 37°C in PBS containing the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; final concentration 100 µM). Cells were lysed by adding 0.5 ml ice-cold ethanol. After 1 h at 4°C, the lysate was transferred to a reaction tube and lyophilized. The amount of cAMP produced was determined with the TRK 432 cyclic AMP assay kit (GE Healthcare, Freiburg, Germany). Data were analyzed and displayed by using PRISM 4.01 software (GraphPad, San Diego, CA).

Responsiveness to light

Having emerged from their brood cells, adult worker bees were kept in cages (60 bees per cage) and fed with 50% sucrose for 5 days. Subsequently, they were fed with various receptor ligands (each at a concentration of 10 mM in 50% sucrose) for 2 days ad libitum. The phototactic behavior of each bee was measured in an arena with a diameter of 35 cm as described earlier [30]. In short, the dark arena could be illuminated with light sources (green

LEDs, 520 nm) of various relative intensities (3.125; 6.25; 12.5%), which were adjusted by neutral density filters. Two light sources of identical intensity were always placed opposite each other. When a light source was switched on in the dark arena, a bee usually started moving towards it (Fig. 8). Once the bee reached the light source, it was switched off, and the identical light source on the opposite part of the arena was switched on. This procedure was repeated four times for each light intensity. The walking time of the bee was measured, and the mean time of four runs for each light intensity was calculated. The light intensities were applied in ascending order. The order of light intensities has previously been shown to have no effect on walking speed [30].

Differences in walking speed for bees treated with the different compounds could have been caused by differences in visual sensitivity, by differences in locomotor activity, or by both. In order to test for effects on locomotor activity, we measured the total length of the walking path that each bee covered within 1 min in total darkness, prior to illuminating the arena. Afterward, the bee was left in the dark arena for adaptation for another 4 min.

Walking times and walking distances in the dark of the different treatment groups were distributed normally ($P > 0.05$; Kolmogorov-Smirnov Z test for all groups, SPSS 12). Figure 9 shows mean walking times ± SD. The effects of different treatments were analyzed by using ANOVA with repeated measurements (SPSS 12).

Results

A cDNA fragment encoding a putative 5-HT receptor of the honeybee (*Am5-ht1A*) was amplified by using a polymerase chain reaction (PCR)-based strategy. The longest open reading frame (1,209 bp) encodes a protein of 402 amino acids with a calculated molecular weight of 44.5 kDa. The deduced amino acid sequence of *Am5-ht1A* (Am5-HT$_{1A}$) shows the hallmarks of G-protein-coupled receptors. The hydropathy profile and topology predictor *Phobius* [31] propose seven transmembrane domains (TM1-7, data not shown) that are flanked by an extracellular N-terminus and an intracellular C-terminus.

Sequence motifs, which are essential for the three-dimensional structure, ligand binding, and signal transduction of the receptor, are well conserved in Am5-HT$_{1A}$ (Fig. 1). Among these are the tripeptide D-R-Y (Asp$_{121}$-Arg$_{122}$-Tyr$_{123}$) required for G protein coupling [32], and the N-P-x-x-Y motif (Asn$_{375}$-Pro$_{376}$-Tyr$_{379}$) for receptor desensitization and internalization [33] (Fig. 1). Four consensus sites for phosphorylation by protein kinase C (PKC) ([S/T]-x-[R/K]) are found within the third cytoplasmic loop. Two consensus motifs for potential N-glycosylation (N-x-[S/T]) are located in the extracellular N-terminus. A cysteine residue in the C-terminus (C$_{395}$) is a possible site for palmitoylation. This post-translational modification is thought to stabilize the structure of the receptor by creating a fourth cytoplasmic loop [18].

Comparison of the amino acid sequence of Am5-HT$_{1A}$ with NCBI databases identified several protostomian and deuterostomian 5-HT$_{1A}$ receptor orthologs. The highest amino acid identity/similarity (ID/S) existed to the 5-HT$_{1A}$ receptors of the hemimetabolous insect *Periplaneta americana* (Pea5-HT$_1$; ID 39%, S 48%), the holometabolous insects *Papilio xuthus* (Px5-HT$_1$; ID 51%, S 63%) and *D. melanogaster* (Dm5-HT$_{1A}$; ID 30%, S 38%; Dm5-HT$_{1B}$; ID 37%, S 46%), and the crustaceaen *Penaeus monodon* (Pem5-HT$_1$; ID 38%, S 48%) (Fig. 1). A multiple amino acid sequence alignment of Am5-HT$_{1A}$ with arthropod, molluscan, and human 5-HT receptors was used to calculate a phylogenetic tree. The Am5-HT$_{1A}$ receptor was incorporated into the comprehensive branch of the 5-HT$_1$ receptor class and was robustly placed in a clade of protostomian 5-HT$_{1A}$ receptors (Fig. 2).

Functional analyses of Am5-HT$_{1A}$ in HEK 293 cells

A HEK 293 cell line stably expressing Am5-HT$_{1A}$ was generated in order to examine second messenger coupling and the pharmacological properties of the receptor. Expression of Am5-HT$_{1A}$ was confirmed by Western blotting and immunohistochemistry (Fig. 3). The ligand specificity of the Am5-HT$_{1A}$ receptor was tested by the application of various biogenic amines (serotonin, dopamine, tyramine, octopamine, and histamine; final concentration 10 μM). Only serotonin significantly inhibited NKH 477-induced cAMP production in Am5-HT$_{1A}$-expressing cells, but this did not occur in non-transfected control cells (Fig. 4a). The dose-response relationship of 5-HT on the intracellular cAMP level ([cAMP]$_i$) was examined with serotonin concentrations ranging from 1 nM to 30 μM (Fig. 4b). In Am5-HT$_{1A}$-expressing cells, the 5-HT effect was concentration-dependent and saturable, resulting in a sigmoidal dose–response curve (Fig. 4b). Half-maximal reduction of cAMP production (EC$_{50}$) was achieved at a serotonin concentration of 16.9 nM (log EC$_{50}$ = −7.77 ± 0.08, mean ± SEM). Maximal attenuation of cAMP synthesis (by ~60%) was observed at serotonin concentrations of ≥3 μM.

In order to characterize the pharmacological profile of Am5-HT$_{1A}$, we examined the effects of various 5-HT receptor agonists and antagonists. The non-selective 5-HT receptor agonists 5-methoxytryptamine (5-MT) and 5-carboxamidotryptamine (5-CT), the latter of which is a selective agonist for mammalian 5-HT$_1$ and 5-HT$_7$

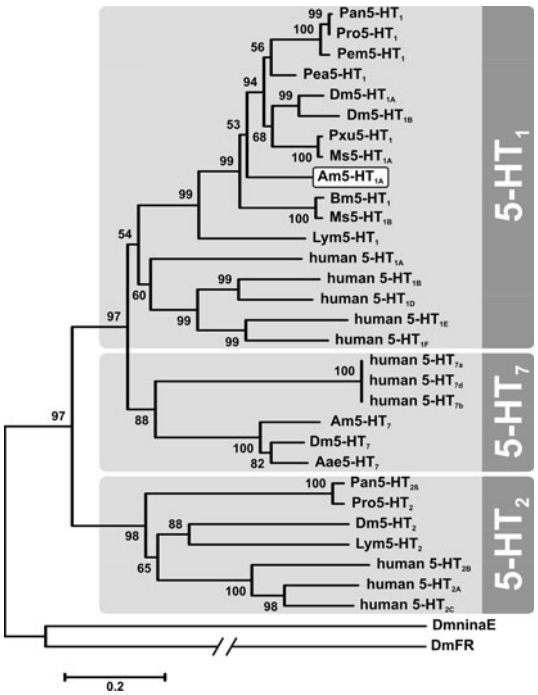

Fig. 2 Phylogenetic analysis of Am5-HT_{1A} and various 5-HT receptors. Alignments were performed with BioEdit (version 7.0.5) by using the core amino acid sequences lacking the variable regions of the N- and C-terminus and the CPL3. The genetic distance was calculated with MEGA4. The receptor sequences followed by their accession numbers are listed in the order illustrated: *Panulirus interruptus* (Pan5-HT_1, no. AY528822); *Procambarus clarkii* (Pro5-HT_1, no. ABX10973), *Penaeus monodon* (Pem5-HT_1, no. AAV48573), *Periplaneta americana* (Pea5-HT_1, no. FN298392), *Drosophila melanogaster* (Dm5-HT_{1A}, no. CAA77570), Dm5-HT_{1B} (no. CAA77571), *Papilio xuthus* (Pxu5-HT_1, no: BAD72868), *Manduca sexta* (Ms5-HT_{1A}, no. DQ840515), *Apis mellifera* (Am5-HT_{1A}, no. FN645449), *Bombyx mori* (Bm5-HT_1, no. CAA64862), Ms5-HT_{1B} (no. DQ840516), *Lymnaea stagnalis* (Lym5-HT_1, no. L06803), human 5-HT_{1A} (no. NP_000515), human 5-HT_{1B} (no. NP_000854), human 5-HT_{1D} (no. NP_000855), human 5-HT_{1E} (no. NP_000856), human 5-HT_{1F} (no. NP_000857), human 5-HT_7 isoform a (no. NP_000863), human 5-HT_7 isoform d (no. NP_062873), human 5-HT_7 isoform b (no. NP_062874), *Apis mellifera* (Am5-HT7, no. AM076717), Dm5-HT_7 (no. A38271), *Aedes aegypti* (Aae5-HT_7, no. AAG49292), Pan5-HT_2 (no. AY550910), Pro5-HT_2 (no. ABX10972), Dm5-HT_2 (no. CAA57429), Lym5-HT_2 (no. U50080), human 5-HT_{2B} (no. NP_000858), human 5-HT_{2A} (no. NP_000612), human 5-HT_{2C} (no. NP_000859), *D. melanogaster* ninaE-encoded rhodopsin 1 (DmninaE, no. NM_079683), and *D. melanogaster* FMRFamide receptor (DmFR, no. AAF47700). The *numbers* at the nodes of the branches represent the percentage bootstrap support for each branch. The *scale bar* allows conversion of branch lengths in the dendrogram to genetic distance between clades

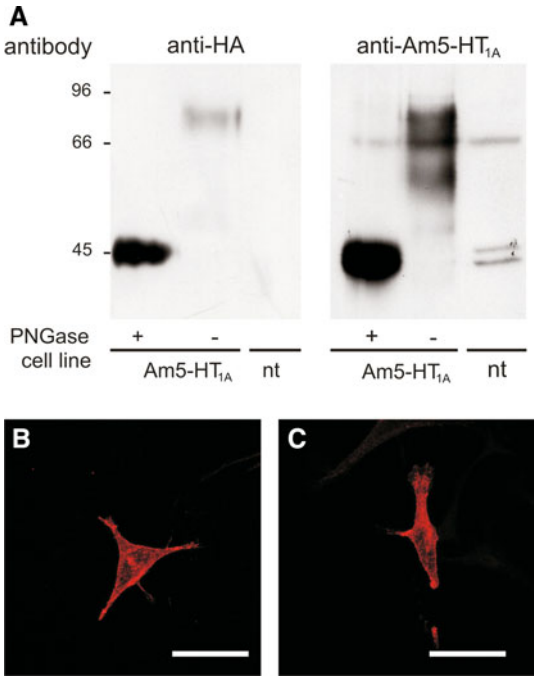

Fig. 3 Expression of Am5-HT_{1A} in HEK 293 cells. **a** Western blot of membrane proteins (10 µg per lane) from HEK 293 cells treated without (−) or with (+) PNGase F. Blots were incubated with anti-HA-antibody (*left*) and anti-Am5-HT_{1A}-antibody (*right*). Deglycosylation resulted in a shift of the protein band from ~80 to ~44 kDa. **b** Immunocytochemical analysis of Am5-HT_{1A}-expressing HEK 293 cells. Cells were incubated with anti-HA-antibody. **c** Immunocytochemical analysis of Am5-HT_{1A}-expressing HEK 293 cells. Cells were incubated with anti-Am5-HT_{1A} antibody

receptors, reduced NKH 477-mediated cAMP production by ~40 and ~50%, respectively (Fig. 4c). These agonistic effects were concentration-dependent. The EC_{50} values for 5-MT and 5-CT were 3.63 µM (log EC_{50} = −5.44 ± 0.22, mean ± SEM) and 700.5 nM (log EC_{50} = -6.15 ± 0.15, mean ± SEM), respectively. Of the antagonists tested, the non-selective mammalian 5-HT receptor antagonist methiothepin was the most effective one (Fig. 4d). Prazosin (Fig. 4d) and WAY 100635 (data not shown) were only able partially to block the serotonin effect.

Generation of anti-Am5-HT_{1A} antibodies and immunohistochemical localization of Am5-HT_{1A}

A polyclonal antiserum directed against a part of the third cytoplasmic loop of Am5-HT_{1A} was generated as described (see "Materials and methods"). The antibodies recognized a single band of ~50 kDa in Western blots of *A. mellifera* brain proteins (Fig. 5a). The signal was completely lost when the antiserum was pre-absorbed with the fusion protein (15 µg/ml) used for immunization, indicating that the antibody specifically recognized the Am5-HT_{1A} receptor protein. Furthermore, the Am5-HT_{1A} receptor protein was expressed in all parts of the honeybee brain and in the ventral nerve cord (Fig. 5c). In contrast, no Am5-HT_{1A} expression could be detected in additional tested tissues and organs (i.e., wing muscles, thoracic salivary glands, crop, ventriculus, rectum, Malpighian tubules, sting apparatus/sting poison gland; data not shown).

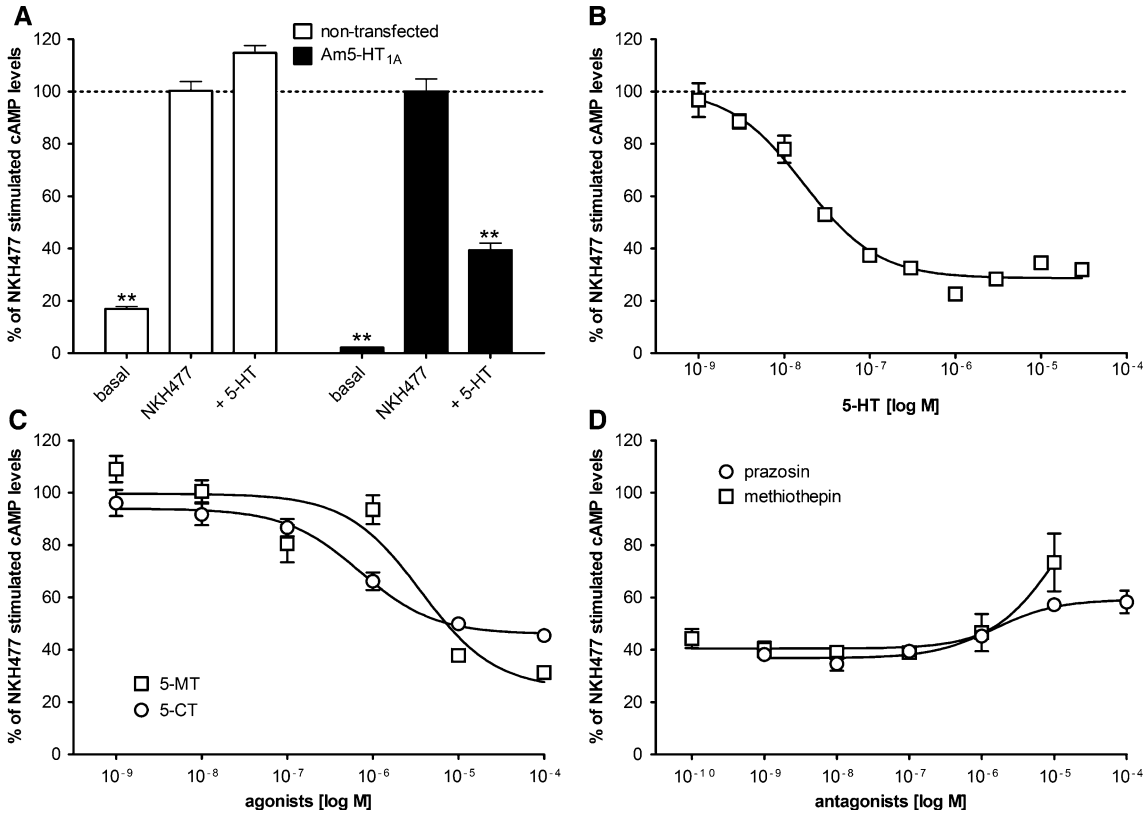

Fig. 4 Modulation of intracellular cAMP levels in HEK 293 cells constitutively expressing the Am5-HT$_{1A}$ receptor and in non-transfected cells. The amount of cAMP is given as the percentage of the value obtained with 10 µM NKH 477 (100%), a water-soluble forskolin analog. Error bars indicate SEM and are, in some cases, too small to be seen. The statistical analysis is based on a one-way ANOVA followed by Dunnett's multiple comparison test; $^{**}P < 0.01$. **a** Effect of NKH 477 and 5-HT (10 µM) on cAMP levels in non-transfected cells and in Am5-HT$_{1A}$-expressing cells. To determine the basal [cAMP]$_i$, cells were incubated with 100 µM IBMX only (basal). Data represent the mean ± SEM of four values. Asterisks indicate statistically significant differences for drug versus NKH 477 (100%) for a given cell line. **b** Dose-dependent effect of 5-HT (10^{-9} to 3×10^{-5} M) on [cAMP]$_i$. Data represent the mean ± SEM of four values. **c** Dose-dependent effects of Am5-HT$_{1A}$ receptor agonists (10^{-9} to 10^{-4} M) on NKH 477-stimulated cAMP production in Am5-HT$_{1A}$-expressing cells. Data represent the mean ± SEM of four values. **d** Dose-dependent effects of Am5-HT$_{1A}$ receptor agonists (10^{-10} to 10^{-4} M) on 5-HT-mediated (500 nM) inhibition of NKH 477-stimulated cAMP production in Am5-HT$_{1A}$-expressing cells. Data represent the mean ± SEM of four values

The cellular distribution of Am5-HT$_{1A}$ was investigated by the immunohistochemical analysis of cryosections of the honeybee brain (Fig. 6). The highest labeling intensity was found in brain regions known to be involved in visual information processing: the proximal lamina of the optic lobes and ocellar tracts. In addition, Am5-HT$_{1A}$-like immunoreactivity was detected in the medulla and the lobula of the optic lobes, the lip and basal ring of the mushroom body calyces, and the pedunculus and α- and β-lobes of the mushroom bodies (Fig. 6). Preabsorption of the antibody (see above) completely abolished labeling. Double-staining with anti-serotonin and anti-Am5-HT$_{1A}$ antibodies revealed a close spatial relationship, although no co-localization, of the amine and the receptor. Some fine serotonergic processes were detected in the strongly Am5-HT$_{1A}$-immunoreactive ocellar nerves (Fig. 7a–c). In the lamina, serotonergic fibers were localized in the proximal half of layer C (see also [12, 34]), which was devoid of receptor labeling (Fig. 7d, f). However, serotonergic fibers sent fine processes distally toward the fenestrated layer (arrowheads in Fig. 7d; [34]), which also contained Am5-HT$_{1A}$ immunoreactivity (Fig. 7b, f). In the medulla, serotonergic fibers were present only in the upper two-thirds of the neuropil and were confined mainly to the serpentine layer and adjacent neuropil (Fig. 7d; [12, 35]). In contrast, Am5-HT$_{1A}$ was distributed uniformly throughout the medulla (Fig. 7e, f). Lobula layers 2, 5, and 6, which were characterized by a high Am5-HT$_{1A}$ receptor density (Fig. 7e, f), also exhibited a network of serotonin-immunoreactive fibers (Fig. 7d, f; [12]).

Influence of serotonin and Am5-HT$_{1A}$ receptor ligands on the responsiveness of individual worker bees to light

The phototactic behavior of bees was studied after feeding animals with various Am5-HT$_{1A}$ receptor ligands (see

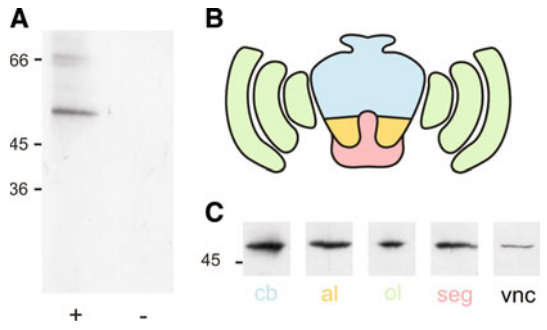

Fig. 5 **a** Western blot analysis with the anti-Am5-HT_{1A}-antibody. The specificity of the anti-Am5-HT_{1A}-antibody was tested on *A. mellifera* brain proteins. The anti-Am5-HT_{1A}-antibody (1:1,000) recognized a single band of ~50 kDa on Western blots (+). Western blot analysis with anti-Am5-HT_{1A}-antibody (1:20,000) pre-absorbed with 15 µg/ml of the peptide used for immunization resulted in no detectable bands (−). **b** Schematic drawing showing various regions of the honeybee brain. **c** Western blot analysis of membrane proteins (5 µg per lane) from various regions of the honeybee brain. Expression of Am5-HT_{1A} could be detected in samples from central brain (cb), antennal lobes (al), optic lobes (ol), subesophageal ganglion (seg) and ventral nerve cord (vnc)

Materials and methods). Compared with untreated controls, the behavior of young worker bees fed with serotonin (10 mM over a 2-day period) was significantly changed. Whereas the bees of the control group went straight to the light source, serotonin-fed bees often stopped and left the direct route to the light source (Fig. 8). This effect was reflected by the increasing time the bees needed to cover the distance between two light sources of equal intensity. The phototaxis assay is most sensitive at very low light intensities [30]. Under these conditions, serotonin-fed bees were significantly slower (14.3 ± 8.8 s, mean ± SD) than untreated controls (8.7 ± 3.4 s, mean ± SD) (Fig. 9a). The effect of serotonin could be mimicked by the 5-HT receptor agonist 5-CT fed to the bees at 10 mM over a 2-day period (untreated controls 11.4 ± 3.3 s, mean ± SD; 5-CT-fed bees 16.8 ± 9.2 s, mean ± SD) (Fig. 9c) but not by 5-MT (data not shown). The strongly reducing effect of serotonin on phototactic behavior (controls 9.2 ± 2.4 s, mean ± SD; 5-HT-fed bees 16.1 ± 7.5 s, mean ± SD) could be prevented by feeding the animals a mixture of serotonin (10 mM) and the Am5-HT_{1A} receptor antagonist prazosin (10 mM) over a 2-day period (11.7 ± 5.8 s, mean ± SD) (Fig. 9e). Feeding of prazosin alone had no effect on phototactic behavior (12 ± 7.7 s, mean ± SD) (Fig. 9e). None of these experiments affected general locomotor activity, i.e., walking behavior measured in the dark. The distance covered by the bees in 1 min of complete darkness did not significantly differ between treatment groups (Figs. 8, 9b, d, f).

Discussion

Serotonin modulates a variety of behaviors and physiological states in the honeybee [14, 15, 36–40]. In the present study, we have characterized a 5-HT receptor cDNA of the honeybee, *Apis mellifera*. The amino acid sequence of this receptor shares pronounced sequence and functional similarity with mammalian [17, 41] and protostomian [20, 29, 42–45] 5-HT_1 receptors. Sequence motifs essential for ligand binding, receptor activation, and G-protein coupling typical for the 5-HT_1 receptor subclass are well conserved in Am5-HT_{1A} [17, 41].

To test the hypothesis that Am5-HT_{1A} couples to $G_{i/o}$ proteins, we have established a HEK 293 cell line stably expressing the receptor. In response to serotonin, Am5-HT_{1A}-expressing cells show a decrease in NKH 477-

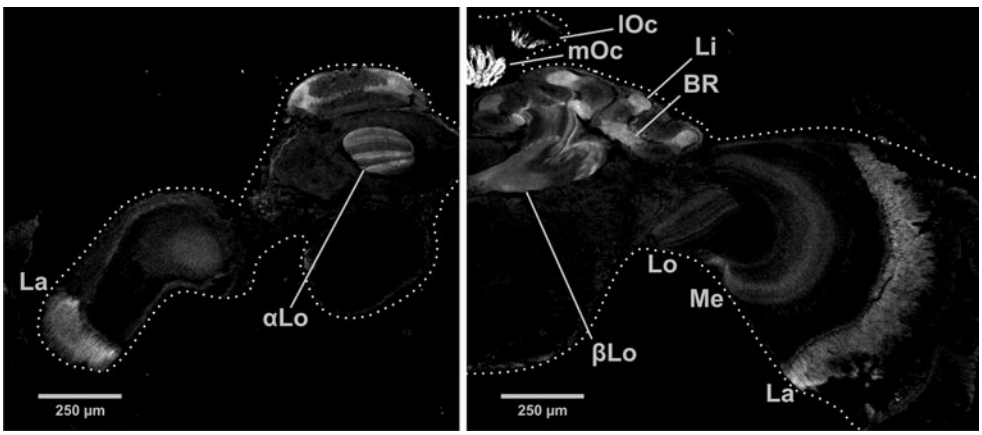

Fig. 6 Immunohistochemical analysis of brain sections with the anti-Am5-HT_{1A}-antibody. Frontal sections of anterior (**a**) and posterior (**b**) regions of the honeybee brain are shown. Specific labeling is seen in the basal ring (br), lip (li), peduncle (pd), and α- and β-lobes of the mushroom bodies. Further labeling was found in the lobula (lo), medulla (me), and lamina (la) of the optic lobes and in the median (moc) and lateral ocellar (loc) tract. *Scale bar* 250 µm

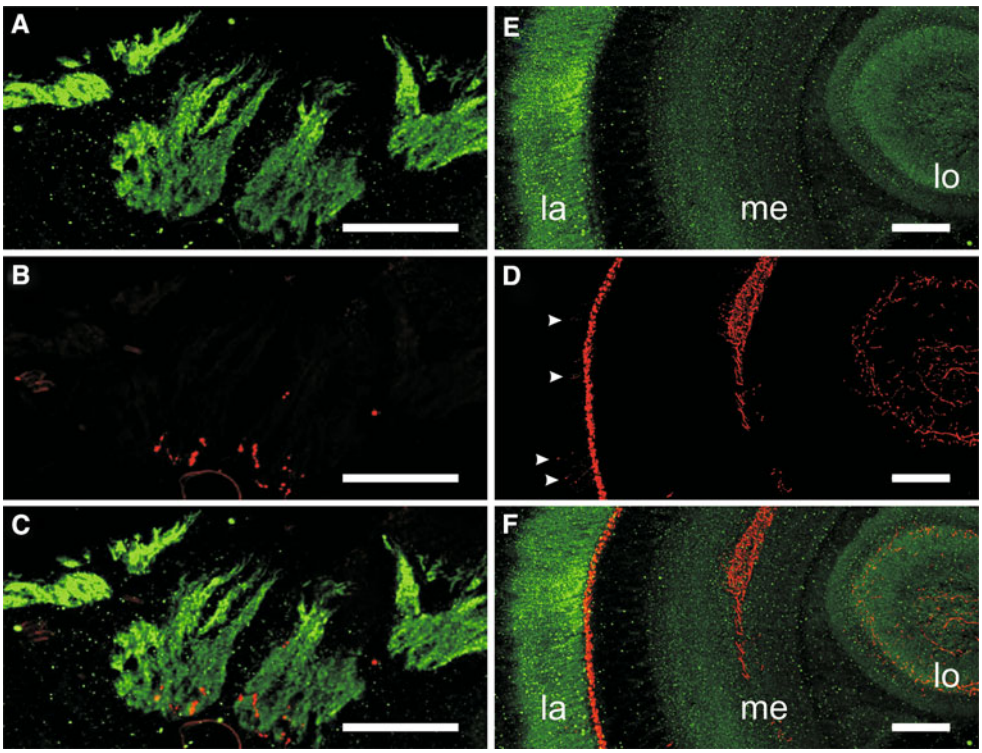

Fig. 7 Immunohistochemical analyses of brain sections with the anti-Am5-HT$_{1A}$-antibody (**a**, **e**) and an antibody against serotonin (**b**, **d**). **c**, **f** The composite images. Details of the ocellar tracts (**a**–**c**) and the optic lobes (**e**–**f**) are shown. For further explanation, see text. *Scale bar* 100 µm. *lo* lobula, *me* medulla, *la* lamina

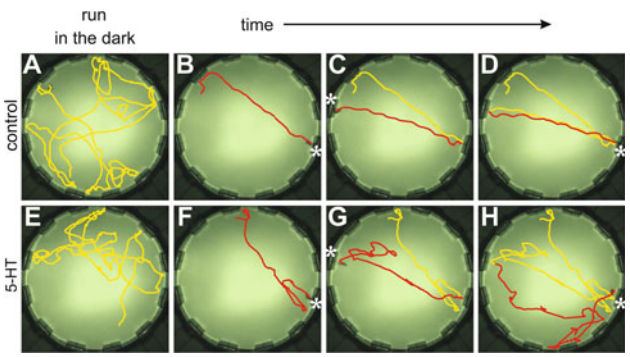

Fig. 8 Examples of walking paths of bees in the dark (**a**, **e**) and at illumination with light sources of lowest intensity (**b**–**d**, **f**–**h**). The *upper panel* shows the walking paths of a control bee fed with 50% sucrose. The *lower panel* shows the walking paths of a bee treated with 5-HT. The position of the light source is indicated by an asterisk. **b**, **f** Display the walking path of the first run to light of a specific intensity (*red*). **c**/**g** and **d**/**h** Display the walking paths of the second and the third runs to a light source of the same intensity (*red*). In **c**, **g**, **d**, and **h** the previous runs are displayed in *yellow*

stimulated cAMP-synthesis by up to 60% compared with non-transfected cells. Adenylyl cyclase activity is also inhibited by 5-HT$_1$ receptors of *D. melanogaster* (Dm5-HT$_{1A}$ and Dm5-HT$_{1B}$), *P. americana* (Pea-HT$_1$), and the crustaceans *Panulirus interruptus* (5-HT$_{1\alpha pan}$) and *Procambarus clarkii* (5-HT$_{1\alpha pro}$) in a comparable manner [20, 29, 45]. The EC$_{50}$ of serotonin for Am5-HT$_{1A}$ is 16.9 nM and is thus similar to the efficacy of serotonin at orthologous receptors of other arthropod species (30 and 18 nM for Dm5-HT$_{1A}$ and Dm5-HT$_{1B}$, respectively, [20]; 31 nM for 5-HT$_{1\alpha pro}$, [45]). In contrast, the 5-HT$_1$ receptors of *P. americana* (150 nM, [29]) and *Boophilus microplus* (83 nM; [44]) exhibit higher EC$_{50}$ values.

Of all the potential 5-HT receptor agonists tested, only 5-CT and 5-MT inhibit cAMP production, although at higher concentrations than serotonin. We have identified three substances that block the action of serotonin, either completely or partially. The full antagonist methiothepin is also a potent antagonist at the Am5-HT$_7$ receptor [28] and must therefore be considered as a non-selective 5-HT receptor antagonist in *A. mellifera*. Prazosin and WAY 100635 represent partial antagonists. Prazosin also displays high-affinity binding to *D. melanogaster* 5-HT$_1$ receptors and is able to inhibit the action of serotonin via these receptors [20]. WAY 100635 is an inverse agonist at the Pea5-HT$_1$ receptor [29]. In *D. melanogaster*, WAY 100635 has been demonstrated to block some of the behavioral effects of the mixed 5-HT receptor agonist LSD [46] and is considered to act on 5-HT$_{1A}$-like receptors to reduce aggressive behavior [47].

Little is known about the distribution of 5-HT$_1$ receptors in insects. In *D. melanogaster*, Dm5-HT$_{1A}$ and Dm5-HT$_{1B}$

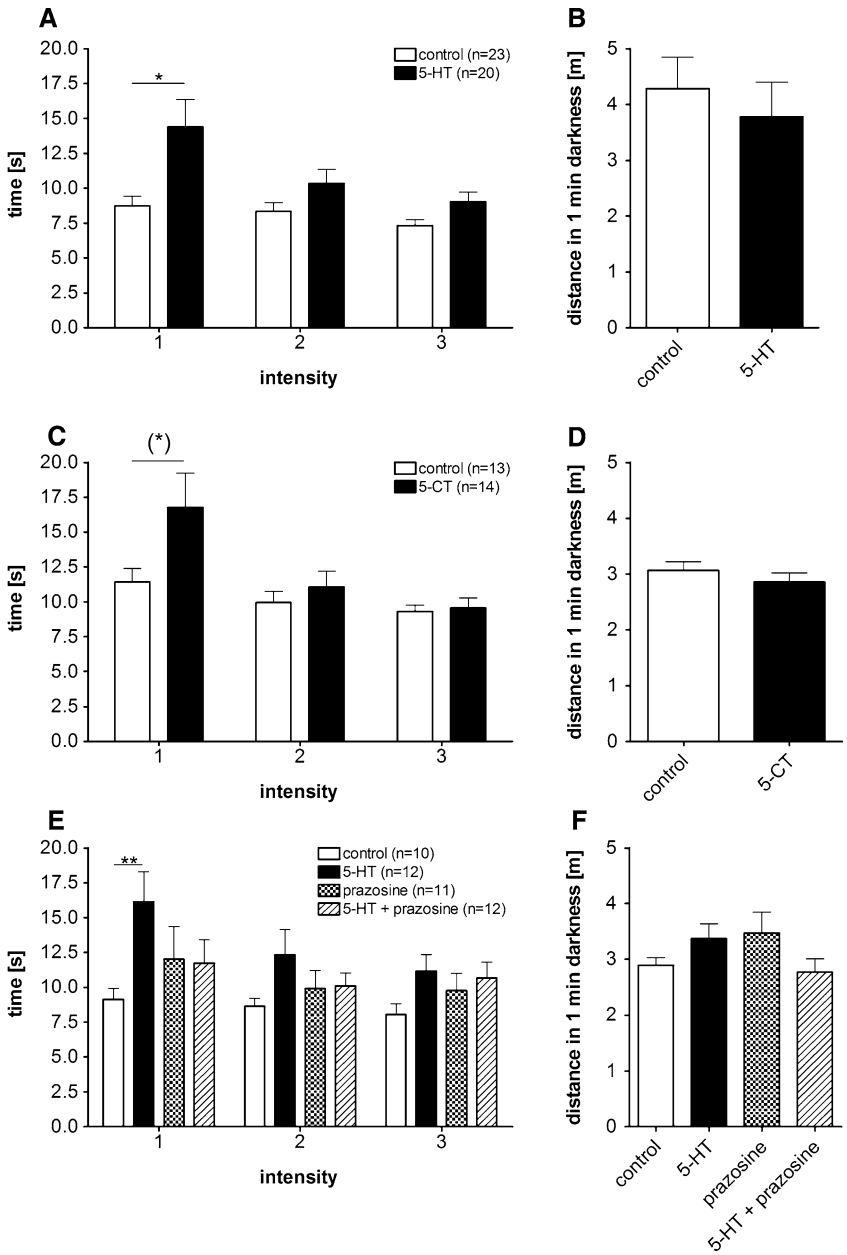

Fig. 9 **a**, **c**, **e** Mean walking times of bees fed with sucrose ("control") or bees fed with various ligands of the 5-HT receptor toward three light sources of increasing intensity. The means of four runs ± SD are shown. **b**, **d**, **f** Mean lengths (±SD) of walking paths in the dark before illumination of the arena of controls and groups fed with various Am5-HT_{1A} receptor ligands. Significant differences between groups are shown by asterisks (*$P \leq 0.05$; **$P \leq 0.01$, one-way ANOVA followed by Tukey's multiple comparison test). The number of bees tested is given in *brackets*

receptors are predominantly expressed in the mushroom bodies [5, 48]. Dm5-HT_{1B} is also expressed in certain clock neurons [5]. Interestingly, both Dm5-HT_{1B} and Pea5-HT_1 are expressed in neurosecretory cells of the *pars intercerebralis* [5, 29]. With an antibody directed against Am5-HT_{1A}, we have been able to identify regions in the honeybee brain expressing this receptor. A high density of Am5-HT_{1A} has been found in the mushroom bodies, structures of the insect brain that are involved in learning and memory (for recent reviews, see: [49–54]). Injection of serotonin into the bee brain has been shown to impair the acquisition and retrieval of learned behavioral patterns [14, 15]. Memory formation in the honeybee depends upon the activity of cAMP-dependent protein kinase [55], which is concentrated in the mushroom bodies [39]. Since activation of Am5-HT_{1A} by serotonin leads to a decrease in $[cAMP]_i$, and as Am5-HT_{1A} is expressed in the mushroom bodies, Am5-HT_{1A} is a prime candidate for mediating the impairing effects of serotonin on honeybee learning.

Particularly strong anti-Am5-HT_{1A} immunofluorescence signals have been observed in the optic lobes (lamina, medulla, and lobula) and the ocellar tract. This agrees well with earlier findings of radioligand-binding studies that have revealed a relatively uniform distribution of [^{3}H]5-HT binding sites in each of the three optic ganglia [56, 57]. In addition, double-staining with anti-serotonin and anti-Am5-HT_{1A} antibodies has revealed a close spatial relationship of the neurotransmitter and the receptor in these

brain regions. Consequently, we reason that serotonin acting via Am5-HT$_{1A}$ is involved in the modulation of visual information processing and visually guided behaviors. A possible participation of serotonin in these processes has been discussed since the time of early histochemical [58] and immunohistochemical [12] studies showing that serotonin is present in the optic lobes. Furthermore, injection of serotonin into the optic lobe inhibits the response to moving stripe patterns [37] and decreases the amplitude of field potentials [38].

We have also investigated the effects of serotonin and Am5-HT$_{1A}$ receptor ligands on the phototactic behavior of individual bees in behavioral experiments. Serotonin reduces the positive phototactic behavior of bees toward a light source of low intensity (Fig. 9). The effects of Am5-HT$_{1A}$ agonists indicate that the observed behavioral change is most likely mediated by Am5-HT$_{1A}$. The strongest effect is observed with the natural agonist serotonin. The less efficacious Am5-HT$_{1A}$ agonist 5-CT (EC_{50} = 700.5 nM) also reduces phototactic behavior. In contrast, 5-MT, which is a non-selective 5-HT receptor agonist but a poor Am5-HT$_{1A}$ agonist (EC_{50} = 3.63 μM), does not affect phototaxis at all. Most convincingly, the effect of serotonin on phototactic behavior could be prevented by the co-application of the Am5-HT$_{1A}$ receptor antagonist prazosin. However, we cannot exclude that additional 5-HT receptor subtypes might have contributed to the observed effects for the following reasons: (1) The molecular identities, the tissue distribution, and the pharmacological properties of all members of the honeybee 5-HT receptor family have so far not been unraveled. (2) In situ hybridization to tissue sections has shown that *Am5-ht7* mRNA is also present in the optic lobes [28]. Physiologically, the Am5-HT$_7$ receptor could potentially antagonize the Am5-HT$_{1A}$ effect. In contrast to Am5-HT$_{1A}$, activation of Am5-HT$_7$ with serotonin increases $[cAMP]_i$ in HEK 293 cells. The same effect was observed after 5-CT application. Whether Am5-HT$_7$ activity is blocked by prazosin, however, has not been investigated [28]. (3) The mRNA of a third receptor type, Am5-HT$_{2\alpha}$, is also expressed in the optic lobes (J. Schlenstedt, unpublished observation). When heterologously expressed, activation of Am5-HT$_{2\alpha}$ (also known as Am16 [19]) by serotonin increases $[Ca^{2+}]_i$ (M. Thamm, unpublished observation). Whether 5-CT activates Am5-HT$_{2\alpha}$ could not be tested, because 5-CT increases $[Ca^{2+}]_i$ even in non-transfected HEK 293 cells.

The modulation of photosensitivity by serotonin has also been shown in other arthropods. Recently, Rodriguez Moncalvo and Campos reported that serotonergic neurons modulate the *D. melanogaster* larval response to light and that this effect may be mediated by Dm5-HT$_{1A}$ receptors [59]. In the crayfish *P. clarkii*, a photosensitive neuron is modulated via a 5-HT$_{1A}$ receptor. This neuron is involved in the regulation of circadian rhythmicity [60]. The abundance of this 5-HT$_{1A}$ receptor in the crayfish eyestalk oscillates in a circadian manner [61]. Interestingly, the *D. melanogaster* 5-*ht1A* mRNA has also been found to oscillate with a phase of *Zeitgeber* time 18 [62], whereas no circadian variation has been seen in the mRNA or protein levels of Dm5-HT$_{1B}$ [5]. Yuan et al. [5] have further shown that Dm5-HT$_{1B}$ affects the circadian light sensitivity of flies by decreasing the activity of the protein kinase SHAGGY, which, in turn, produces increased stability of the transcription factor TIMELESS. In contrast, the Dm5-HT$_{1A}$ receptor seems to have a sleep-regulating role, since flies mutant for Dm5-HT$_{1A}$ have short and fragmented sleep patterns [48].

In order to verify a general role of serotonin and 5-HT$_1$ receptors in the circadian rhythmicity of arthropods, further studies are necessary to analyze whether brain mRNA levels of *Am5-ht1A* also oscillate in honeybees. This analysis is interesting from a functional aspect, since the age-related division of labor in honeybees has been shown to be associated with changes in activity rhythms; young adult bees perform hive tasks with no daily rhythms, whereas older bees forage with strong daily rhythms [63, 64].

The honeybee is a well-established model organism for studying various behaviors including learning and memory, division of labor, defense behavior, and circadian rhythmicity [51, 54, 56, 57, 65, 66]. Many of these behaviors are modulated by the biogenic amine serotonin. The detailed characterization of the molecular and pharmacological features and of the expression pattern of Am5-HT$_{1A}$ provides the basis for forthcoming studies on the functional contribution of this receptor subtype to honeybee behavior and physiology. To date, we have been able not only to establish a new role for serotonin in the modulation of honeybee phototaxis, but also to provide first evidence for Am5-HT$_{1A}$ being a likely mediator of this serotonin effect. The involvement of Am5-HT$_{1A}$ in the control and modulation of additional behaviors, such as circadian rhythmicity or learning and memory, can now be analyzed by interfering with Am5-HT$_{1A}$ expression (injection of dsRNAs or morpholinos) or receptor activation (application of identified Am5-HT$_{1A}$ ligands).

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