



## Isolation and functional characterization of an allatotropin receptor from *Manduca sexta*

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### ABSTRACT

*Manduca sexta* allatotropin (Manse-AT) is a multifunctional neuropeptide whose actions include the stimulation of juvenile hormone biosynthesis, myotropic stimulation, cardioacceleratory functions, and inhibition of active ion transport. Manse-AT is a member of a structurally related peptide family that is widely found in insects and also in other invertebrates. Its precise role depends on the insect species and developmental stage. In some lepidopteran insects including *M. sexta*, structurally-related AT-like (ATL) peptides can be derived from alternatively spliced mRNAs transcribed from the AT gene. We have isolated a cDNA for an AT receptor (ATR) from *M. sexta* by a PCR-based approach using the sequence of the ATR from *Bombyx mori*. The sequence of the *M. sexta* ATR is similar to several G protein-coupled receptors from other insect species and to the mammalian orexin receptor. We demonstrate that the *M. sexta* ATR expressed in vertebrate cell lines is activated in a dose-responsive manner by Manse-AT and each Manse-ATL peptide in the rank order ATL-I > ATL-II > ATL-III > AT, and functional analysis in multiple cell lines suggest that the receptor is coupled through elevated levels of Ca<sup>2+</sup> and cAMP. In feeding larvae, Manse-ATR mRNA is present at highest levels in the Malpighian tubules, followed by the midgut, hindgut, testes, and corpora allata, consistent with its action on multiple target tissues. In the adult corpora cardiaca–corpora allata complex, Manse-ATR mRNA is present at relatively low levels in both sexes.

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### 1. Introduction

Juvenile hormone (JH) is a sesquiterpenoid that possesses numerous developmental and physiological functions in insects, including the regulation of molting and metamorphosis in immature insects (Riddiford, 1994) and the control of reproductive events and egg maturation in adult insects (Wyatt and Davey, 1996). The control of JH levels in the insect hemolymph is achieved, in part, by neuropeptides that stimulate or inhibit its biosynthesis by the corpora allata (CA), allatotropin (AT) and allatostatin (AST), respectively (Weaver and Audsley, 2009). A great deal of progress has been made in the characterization of these neuropeptides using biochemical methods and physiological assays performed *in vitro*. More recently, major advances in studying the full complement of neuropeptides in insects came with the utilization of bioinformatic tools to predict the genes encoding neuropeptides from genome

sequences and EST libraries. Neuropeptides can occur as unique components or as members of families based on their sequence similarity whose members often have similar functions.

Insect neuropeptides were first characterized by their biochemical isolation, structural elucidation, and activity in a number of bioassays; and were usually named based on their first known biological activity. AT was first characterized in the tobacco hornworm, *Manduca sexta*, (and designated Manse-AT) based on its ability to stimulate JH biosynthesis in the CA of adult, female lepidopteran insects *in vitro* (Kataoka et al., 1989). Subsequently in *M. sexta*, it was shown that Manse-AT possesses cardioexcitatory activity in pharate adults (Veenstra et al., 1994) and inhibits active ion transport across the larval midgut epithelium *in vitro* (Lee et al., 1998). Additional functions for Manse-AT in other lepidopterans include the stimulation of JH biosynthesis by the larval CA in some, but not all, lepidopteran insects (Audsley et al., 2000; Li et al., 2002), stimulation of foregut contractions in *Helicoverpa armigera* and *Lacanobia oleracea* (Duve et al., 1999, 2000), and the stimulation of ventral diaphragm oscillation in *Pseudaletia unipuncta* (Koladich et al., 2002). Additional activities were described for

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Manse-AT, or the endogenous homolog, in other insects including the stimulation of JH biosynthesis by CA of insects from the orders Diptera (Tu et al., 2001; Veenstra and Costes, 1999), Hymenoptera (Rachinsky and Feldlaufer, 2000), Dermaptera (Rankin et al., 2005) and Coleoptera (Abdel-latif and Hoffmann, 2010); the stimulation of myotropic activity in insects from the orders Orthoptera (Paemen et al., 1991), Dictyoptera (Rudwall et al., 2000), Coleoptera (Spittaels et al., 1996), and Hemiptera (Santini and Ronderos, 2007); and the photic entrainment of the circadian clock in an insect from the order Dictyoptera (Petri et al., 2002). Like many neuropeptides, Manse-AT has multiple functions, and its primary role may differ depending on the developmental stage of the insect.

Manse-AT is a member of a peptide family that has been isolated from numerous insect species, deduced from the sequences of homologous genes, or identified *in silico* from nucleotide sequence databases (Elekovich and Horodyski, 2003; Weaver and Audsley, 2009). Related peptides have been isolated from mollusks (Harada et al., 1993; Li et al., 1993) and annelids (Ukena et al., 1995) based on their myotropic activity, and it has been suggested that the ancestral role for this peptide family is related to its myotropic role (Elekovich and Horodyski, 2003). Despite its widespread appearance in numerous insects, the AT sequence has not been identified in *Drosophila melanogaster* or other members of that genus (Hewes and Taghert, 2001; Vanden Broeck, 2001), or in the hymenopteran insects *Apis mellifera* (Hummon et al., 2006) or *Nasonia vitripennis* (Hauser et al., 2010). In some lepidopteran insects including *M. sexta*, multiple mRNAs that differ by alternative splicing are transcribed from the AT gene in a stage- and tissue-specific manner and predict the presence of peptides that are structurally related to Manse-AT [ATL peptides; (Abdel-latif et al., 2003; Horodyski et al., 2001; Sheng et al., 2007; Yin et al., 2005)], and whose biological activities overlap with those of Manse-AT (Lee et al., 2002).

Neuropeptides exert effects on their cellular targets by binding to high affinity receptors, the vast majority of which are members of a large family of structurally-related proteins that possess seven hydrophobic membrane-spanning regions, the G protein-coupled receptors (GPCRs). Ligand binding transduces a signal mediated by activation of a GTP-binding protein (G protein) that leads to alterations in the levels of second messengers that, in turn, exert functional changes in the target cell and the organism. The specificity of GPCRs is generally determined by expression of the GPCR in a heterologous cell line or in *Xenopus* oocytes, and the subsequent demonstration of ligand-dependent changes in second messengers that are monitored by the quantification of their levels, expression of reporter gene constructs, or the opening of channel proteins. The characterization of neuropeptide GPCRs has also been greatly advanced by the use of molecular tools and the determination of genome sequences to identify similar GPCRs in different insects which respond to the homologous neuropeptide.

The AT receptor (ATR) was first identified in the silkworm, *Bombyx mori*, (Bommo-ATR) by the systematic screening of GPCRs that were highly expressed in the corpora cardiaca–corpora allata (CC–CA) complex with neuropeptides in a heterologous expression system (Yamanaka et al., 2008). Transcript levels of Bommo-ATR were determined in multiple tissues at two larval stages by quantitative RT-PCR (qPCR), consistent with the multiple functions of the peptide. Bommo-ATR mRNA levels differed between the two larval stages, and levels were highest in the CC–CA, brain, epidermis, and testes. *In situ* hybridization demonstrated that Bommo-ATR mRNA was present in the CC, but not the CA.

We used the sequence of Bommo-ATR to design oligonucleotide primers to isolate the homologous GPCR from *M. sexta* (Manse-ATR). In order to determine if Manse-ATR reacted to Manse-AT or the Manse-ATL peptides, we expressed Manse-ATR in

a heterologous cell line and tested whether these peptides activate the Manse-ATR and then determined their relative potencies. We showed that Manse-AT and each Manse-ATL mediated peptide-dependent responses in a dose-responsive manner at high affinity. Each of the Manse-ATL peptides exhibited a higher potency to Manse-ATR than Manse-AT. Then, to determine which second messenger the receptor is coupled to, Manse-ATR was expressed in other cell lines to demonstrate that the receptor activation increases both cAMP and Ca<sup>2+</sup> levels suggesting that it may act through two distinct G proteins.

## 2. Materials and methods

### 2.1. Insects

Larvae of the tobacco hornworm, *M. sexta*, were reared individually on an artificial diet (BioServ) at 25 °C under a long-day (16L:8D) photoperiod (Bell and Joachim, 1976). The day of ecdysis to the fifth larval instar is designated day 0. Wandering larvae, characterized by the appearance of a prominent dorsal vessel, were collected and transferred to plastic vials with vermiculite. These larvae molted into pupae 5 days later. Pharate adults were selected when the pupal cuticle above the wing is dark and soft (Schwartz and Truman, 1983) and transferred to a wooden growth chamber containing a tobacco plant and 10% sucrose at 25 °C under a long-day (16L:8D) photoperiod where the adult moths emerged. Eggs were collected daily from the leaves of the tobacco plant and placed in a petri dish in the larval growth chamber overlaid with a metal mesh screen and artificial diet inside a covered plastic chamber, in which the larvae were allowed to hatch. For the collection of adult CC–CA complexes, pharate adults were separated by sex and placed in different chambers in which the moths enclosed.

### 2.2. Isolation of the ATR cDNA

Midguts from day 0, fifth instar larvae were dissected in *Manduca* saline buffer (Riddiford et al., 1979) and stored overnight at 4 °C in RNA later (Ambion) followed by removal of the solution and storage of the tissue at –80 °C. RNA was extracted using Trizol reagent (Gibco-BRL) and Poly A<sup>+</sup> RNA was selected using the PolyA-Tract mRNA Isolation System III (Promega). Then, cDNA was synthesized using Stratascript reverse transcriptase (Stratagene) with 0.15 µg poly A<sup>+</sup> RNA using a Random Primer (Promega). A 64-fold degenerate sense primer, 5'-GT(ACGT)GC(ACGT)GA(CT)TT(CT)ATGGT-3' (S-DEG) was designed from the sequence VADFMV present within transmembrane domain II (TM-II) of *B. mori* BNGR-A16 (Yamanaka et al., 2008). A 256-fold degenerate antisense primer, 5'-A(AG)(AG)TG(ACGT)AC(ACGT)GG(AG)AA(AG)TA-3' (AS-DEG) was designed from the amino acid sequence YFPVHL present in the TM-VI of *B. mori* BNGR-A16. We utilized a touchdown PCR protocol (Don et al., 1991) in which the annealing temperatures were gradually decreased to prevent mispriming and preferential amplification of undesirable alternate products. The PCR reaction was performed with approximately 1 ng of cDNA as a template in PCR buffer (Stratagene) containing 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, and 0.4 µM of each primer in 50 µl. After an initial denaturation step at 94 °C for 4 min, the mixtures were amplified in a thermal cycler (Perkin–Elmer 480) for 3 cycles (94 °C, 45 s; 60 °C, 45 s; 72 °C, 45 s), followed by 3 cycles (94 °C, 45 s; 55 °C, 45 s; 72 °C, 45 s), followed by 3 cycles (94 °C, 45 s; 50 °C, 45 s; 72 °C, 45 s), followed by 40 cycles (94 °C, 45 s; 47 °C, 45 s; 72 °C, 45 s), followed by a 5 min final extension at 72 °C. The PCR products were analyzed on a 3% NuSieve-GTG agarose (FMC Bioproducts) gel. The faint ~600 bp PCR product was excised from the gel, and reamplified for 40 cycles (94 °C, 45 s; 47 °C, 45 s; 72 °C, 90 s), preceded by an initial

denaturation step at 94 °C for 4 min, and followed by a 5 min final extension at 72 °C. Then 0.5 µl (2.5 units) of Taq2000 (Stratagene) was added to the PCR reaction and incubated at 72 °C for 9 min to insure the addition of the single 3'-A overhang. The PCR product was subcloned into pCRII-TOPO (Invitrogen), and plasmid minipreps were screened by *Eco RI* digestion. The sequence of one of these inserts was then determined using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and analyzed at the Ohio University Sequencing Facility.

### 2.3. Rapid amplification of cDNA ends (RACE)

RACE-ready cDNA was synthesized using the SMART RACE cDNA Amplification Kit (Clontech) and SMART MMLV reverse transcriptase (Clontech) with 0.15 µg day 0, fifth instar larval midgut RNA in 10 µl volume. The reactions were terminated by addition of 20 µl Tricene-EDTA buffer (Clontech) and reactions were stored at -20 °C.

The following gene-specific primers were designed based on the sequence of the RT-PCR product:

3'-RACE primer-1: 5'-GGTGTCCGTTCTGACGCTGACGTTCC-3'  
 3'-Nested RACE primer-1: 5'-TACGCGATCGTCGGCCCCGCTCAAGT-3'  
 3'-RACE primer-2: 5'-TCCATCGTCTCCAAATGCCCAAACC-3'  
 3'-Nested RACE primer-2: 5'-GGCGGACTGAGGTGTAGGTGGACGA-3'  
 5'-RACE primer-1: 5'-GAACGTGACGCTCAGAACGGACACC-3'  
 5'-Nested RACE primer-1: 5'-TTTTGCACAGCACGTCTCCA-3'  
 5'-Nested RACE primer-2: 5'-CGATGCAGACCAAGGCATTCCCTA-3'

All RACE reactions were conducted using 2.5 µl of RACE-ready cDNA using reaction conditions recommended in the SMART cDNA Amplification Kit. After an initial denaturation step at 94 °C for 4 min, the mixtures were amplified for 3 cycles (94 °C, 30 s; 70 °C, 30 s; 72 °C, 4 min), followed by 3 cycles (94 °C, 30 s; 68 °C, 30 s; 72 °C, 4 min), followed by 3 cycles (94 °C, 30 s; 65 °C, 30 s; 72 °C, 4 min), followed by 33 cycles (94 °C, 30 s; 60 °C, 30 s; 72 °C, 4 min), followed by a 5 min final extension at 72 °C. All nested RACE reactions were conducted using 5 µl of a 1:50 dilution of the RACE product as template. After an initial denaturation step at 94 °C for 4 min, the mixtures were amplified for 20 cycles (94 °C, 30 s; 68 °C, 30 s; 72 °C, 3 min), followed by a 5 min final extension at 72 °C.

To determine the 3'-sequence, 3'-RACE-ready cDNA was amplified with the Universal Primer A Mix (UPM, Clontech) and 3'-RACE primer-1 as the gene-specific primer. A nested RACE was then performed using the Nested Universal Primer A (NUP) and 3'-Nested RACE primer-1. The 2.1 kb RACE product was cloned into pCR2.1 (Invitrogen) and sequenced. Since this product did not contain the poly-A sequence, a second 3'-RACE reaction was performed with UPM and 3'-RACE primer-2, followed by a nested RACE using NUP and 3'-Nested RACE primer-2. The 600 bp product was cloned into pCR2.1 (Invitrogen) and sequenced.

### 2.4. Sequencing and phylogenetic analysis

Sequences were determined using the Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and analyzed at the Ohio University Sequencing Facility. Transmembrane domains were predicted using TMHMM v. 2.0 (Krogh et al., 2001). Sequences were aligned using CLUSTALW MEGA version 4 software was used to construct a Neighbor-joining tree. For node support, bootstrap analysis was performed using 500 replicates. The evolutionary distances were computed using the Poisson substitution method.

### 2.5. Expression and analysis of ATR

The full-length ATR coding region was amplified using approximately 3 ng of cDNA prepared from day 0 fifth instar larval midgut RNA with 0.4 µM of a sense primer 5'-CACCATGCTTAA-CAAAAGCATA-3' and antisense primer 5'-CTAAGCGTAA-TATCCACTCTTAAATCC-3' using the AccuPrime Taq DNA Polymerase System (Invitrogen) in a 25 µl volume. After an initial denaturation step at 95 °C for 5 min, the DNA was amplified for 35 cycles (95 °C, 30 s; 50 °C, 30 s; 68 °C, 2 min), followed by a 10 min final extension at 68 °C. The 1.3 kb PCR product was cloned into the pcDNA 3.1 Directional TOPO Expression Vector (pcDNA3.1D, Invitrogen) to obtain the ATR expression plasmid, pcDNA3.1D-Manse-ATR. The orientation of the insert was verified by diagnostic *Bgl II* digestion and sequencing using the T7 and BGH primers which flank the insert, and internal primers. Plasmid DNA was purified using the EndoFree Plasmid Maxi Kit (Qiagen).

### 2.6. Peptides

Manse-AT and Manse-AKH were obtained from Bachem. F7D and F7G were generously provided by Dr. Jane Witten, University of Wisconsin, Milwaukee. FMRamide and corazonin were obtained from Sigma. Manse-ATL peptides were custom synthesized by Research Genetics. Peptide stocks were prepared in 10% acetonitrile and diluted in DMEM/F12 without phenol red supplemented with 0.1% BSA. Peptide concentrations of the stocks were determined using the BCA Assay (Pierce), and Manse-AT and the Manse-ATL peptides were checked for correct mass by MALDI-TOF-MS.

### 2.7. Cell culture and transfections

Chinese hamster ovary (CHO) WTA11 and PAM28 cells stably expressing apoaquorin (Euroscreen, Belgium) and human embryonic kidney (HEK) 293 cells were cultured in monolayers in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12) (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen). For CHO-WTA11 cells, 250 mg/ml Zeocin (Invitrogen) was added to the medium; and for CHO-PAM28 cells, 5 µg/ml puromycin (Sigma) was added to the medium. The cells were cultured at 37 °C with a constant supply of 5% CO<sub>2</sub>.

Transfections with pcDNA3.1D-Manse-ATR or pcDNA3.1D (empty vector) were carried out in T75 flasks at 60–80% confluency. The transfection medium for CHO-WTA11 or CHO-PAM28 cells was prepared according to the manufacturer's protocol with 703 µl serum-free DMEM/F12, 46.8 µl FuGENE 6 (Roche), and 7.8 µg of pcDNA3.1D-Manse-ATR (or empty vector) and added dropwise to 3.5 ml of the cell culture following 30 min incubation at room temperature. The transfection medium for the HEK293 cells was prepared according to the manufacturer's protocol with 200 µl serum-free DMEM, 10 µl Dreamfect Gold (OZ Biosciences) and 2.5 µg of plasmid DNA. The plasmid DNA consisted of 2.0 µg of pcDNA3.1D-Manse-ATR (or pcDNA3.1D) and 0.5 µg of a reporter gene plasmid. The reporter gene is luciferase ORF downstream of a cyclic AMP (cAMP) response element (CRE). This transfection medium was then added dropwise to 2.5 ml of cell culture medium. The cells were incubated overnight, after which 10 ml of cell culture medium was added to allow an additional overnight growth period prior to the monitoring of Ca<sup>2+</sup> or cAMP.

### 2.8. Aequorin assay

Cells were detached using 1× Phosphate Buffered Saline (PBS) containing 0.2% EDTA (pH 8.0), and rinsed off the flask with DMEM/

F12. The number of viable and nonviable cells were determined using a NucleoCounter NC-100 (Chemometric). The cells were pelleted for 4 min at 150 g at room temperature and resuspended in DMEM/F12 without phenol red (Gibco), and with L-glutamine and 15 mM HEPES supplemented with 0.1% BSA (Sigma) (BSA medium) at a concentration of  $5 \times 10^6$  cells/ml. Coelenterazine h (Invitrogen) was added to a concentration of 5  $\mu$ M and cells were incubated in the dark at room temperature with gentle shaking for 4 h. The cells were then diluted 10-fold in BSA medium and incubated for an additional 30 min in the dark with gentle shaking. Peptides were dissolved in BSA medium and dispensed in 50  $\mu$ l aliquots into the wells of a white 96-well plate. After injection of 50  $\mu$ l of the cell suspension into each well, light emission was recorded using Mithras LB940 (Berthold Technologies) for 30 s. The cells were then lysed by injection of 50  $\mu$ l 0.3% Triton X-100 and light emission was monitored for an additional 8 s as a measure of the total cellular  $Ca^{2+}$  response. BSA media was used as a negative control in each row, and 1  $\mu$ M ATP, which activates an endogenous ATP receptor in CHO cells, was used to check the functional response. The total response (ligand + Triton X-100) is representative of the number of cells present in the well. The BSA media response (blank) was subtracted from the wells of the same row. Calculations were made using the output file using MikroWin software (Mikrotek) in Excel (Microsoft). Further analysis was done in GraphPad Prism 5.

### 2.9. cAMP reporter assay

Cells were detached with  $1 \times$  PBS containing 0.2% EDTA (pH 8.0), and rinsed off the flask with DMEM/F12. The cells were pelleted for 4 min at 150 g at room temperature and resuspended at a concentration of  $1 \times 10^6$  cells/ml in DMEM/F12 without phenol red, but containing 200  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX, Sigma) to prevent cAMP breakdown. Peptides were dissolved in DMEM/F12 media without phenol red containing 200  $\mu$ M IBMX in the presence or absence of 10  $\mu$ M forskolin (Sigma), and 50  $\mu$ l aliquots were dispensed into the wells of a white 96-well plate. Then 50  $\mu$ l of cells were added to the wells and the plate was incubated in a  $CO_2$  incubator at 37  $^\circ$ C for 3–4 h. Then, 100  $\mu$ l of SteadyLite Plus (Perkin-Elmer) was added to each well and the plate was incubated for 15 min in the dark. Light emission resulting from the luciferase enzymatic activity was recorded for 5 s/well using Mithras LB940. Results were analyzed using MikroWin and further processed by Excel and GraphPad Prism 5 software.

### 2.10. Quantitative RT-PCR

Tissues from individual day 1, fifth instar larvae or adults were dissected in *Manduca* saline buffer (Riddiford et al., 1979), transferred to an Eppendorf tube on dry ice and stored at  $-80^\circ$ C in 500  $\mu$ l of TriReagent (Sigma). Tissues were homogenized using a plastic pestle and RNA was extracted as recommended by the manufacturer (Sigma). DNA was then digested with 0.04 units/ $\mu$ l RNase-free DNase I (Ambion), and the absence of DNA was assessed by qPCR on randomly selected samples. Total RNA content was measured using RNA binding fluorescent dye and the Qubit fluorometer (Invitrogen). Then, 0.25  $\mu$ g of total RNA was used to synthesize cDNA with Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega) and Random Primers (Promega) in the presence of Riboblock RNase Inhibitor (Fermentas).

The qPCR reaction was performed using cDNA synthesized from 5 ng RNA using 7.5  $\mu$ l  $2 \times$  iTaq SYBR Green Supermix with ROX (Bio-Rad) and 7.5 pmol of each primer in a final volume of 15  $\mu$ l. The reaction was incubated for 2 min at 95  $^\circ$ C, followed by 40 cycles (95  $^\circ$ C, 15 s; 60  $^\circ$ C, 1 min) in an Mx3000P thermal cycler (Stratagene). After the amplification steps, the specificity of the PCR

products were assessed by generating a melting curve using the following conditions; 95  $^\circ$ C, 15 s; followed by 60  $^\circ$ C, 1 min; followed by an increase in temperature in 0.7  $^\circ$ C increments from 60  $^\circ$ C to 95  $^\circ$ C. Primers were designed using Primer3 software to have a  $T_m$  from 59–61  $^\circ$ C, a G + C content from 20 to 60%, and to yield a product from 90 to 130 bp. The absence of RNA hairpins at the site of primer binding was confirmed using Mfold software (Zuker, 2003). The sequences of the primers used for Manse-ATR qPCR were 5'-TTCCTTGAGACGTGCTGT-3' (forward – corresponding to nucleotides 558–575 of Fig. 1) and 5'-ACTTGAACCTGAGCGGG-3' (reverse – corresponding to nucleotides 688–672 of Fig. 1). The sequences of the primers used for the reference gene (rpS3) (Jiang et al., 1996) were 5'-CATGATCCACTCCGGTGAC-3' (forward) and 5'-GACCTTAATTCGAGCACTCC-3' (reverse). The amount of Manse-ATR mRNA relative to rpS3 mRNA was determined using the  $\Delta\Delta C_T$  quantification method (Livak and Schmittgen, 2001).

## 3. Results

### 3.1. Isolation of an allatotropin receptor (ATR) cDNA

The only insect in which an ATR has been characterized thus far is the silkworm, *B. mori* (Yamanaka et al., 2008). To identify an ATR from *M. sexta*, we designed degenerate oligonucleotide primers based on the sequence of the *B. mori* ATR cDNA (BNGR-A16) to amplify a portion of a putative *M. sexta* ATR cDNA using day 0, 5th instar larval cDNA. We used this tissue because the larval midgut is a known target of Manse-AT (Lee et al., 1998), and the large size of the larval midgut compared with the adult female CA facilitates the isolation of sufficient RNA as starting material. The amplification product of  $\sim$ 600 bp was sequenced and a TBLASTN search (Altschul et al., 1997) showed that the translated sequence exhibited the highest sequence identity to BNGR-A16, the ATR from *B. mori* (Yamanaka et al., 2008). Using the same poly A<sup>+</sup> RNA preparation, we conducted 5'- and 3'-RACE to yield a cDNA of 3006 bp (excluding the poly A sequence) (Fig. 1). An in-frame stop codon is present 27 bases upstream from the first ATG, and a consensus sequence directing polyadenylation is present 16 bases upstream from the poly A sequence.

The cDNA encodes a protein of 457 amino acids which contains seven transmembrane domains characteristic of a G protein-coupled receptor. A TBLASTN search showed that this sequence is most similar to BNGR-A16 (Yamanaka et al., 2008), exhibiting 82% sequence identity in the region between TM-I and TM-VII (Fig. 2) suggesting that this is a receptor for allatotropin in *M. sexta*. Other insect proteins that exhibit a high level of identity include an additional GPCR from *B. mori* (BNGR-A5), which has not yet been functionally characterized, and GPCRs from *Anopheles gambiae*, *Tribolium castaneum*, *N. vitripennis*, and *A. mellifera*. The most similar mammalian protein was the orexin receptor which exhibited 48% identity with Manse-ATR in the region between TM-I and TM-VII.

### 3.2. Functional characterization of ATR with Manse-AT and Manse-ATL-I, -II, and -III

We initially expressed Manse-ATR in CHO-WTA11 cells (Euroscreen, Belgium). This cell line expresses the  $Ca^{2+}$  reporter apoaequorin and  $G_{\alpha 16}$ , a promiscuous G protein that couples most GPCRs to phospholipase C $\beta$  activation and subsequent mobilization of intracellular  $Ca^{2+}$  (Stables et al., 1997). Receptor-expressing cells exhibited dose-dependent  $Ca^{2+}$  responses to Manse-AT and each Manse-ATL peptide (Fig. 3B). All other peptides tested at 10  $\mu$ M, including Manse-AKH (Ziegler et al., 1985), the FLRFamides F7G and F7D (Kingan et al., 1996), CCAP (Stangier et al., 1987), corazonin

(Veenstra, 1989) and FMRamide (Price and Greenberg, 1977), exhibited no response, demonstrating the specificity of the response of Manse-ATR to AT and the ATL peptides. The  $EC_{50}$  value for Manse-AT was 121.1 nM. Surprisingly, the ATL peptides showed significantly lower  $EC_{50}$  values. The  $EC_{50}$  values obtained were 0.54 nM for Manse-ATL-I; 7.0 nM for Manse-ATL-II; and 23.3 nM for Manse-ATL-III. Only Manse-ATL-I functioned as a full agonist in this cell line, the other peptides behaved as partial agonists. Untransfected CHO-WTA11 cells or CHO-WTA11 cells transfected with pcDNA3.1D (empty vector) did not show any responses to Manse-AT or Manse-ATL peptides.

To determine which second messenger pathway Manse-ATR couples with, we first expressed Manse-ATR in CHO-PAM28 cells. This cell line lacks  $G\alpha_{16}$ , thus causing the receptor to couple to its

original second messenger pathway, but expresses apoaquorin to allow a test of whether receptor activation results in increased levels of intracellular  $Ca^{2+}$ . Fig. 3C shows that Manse-AT and each Manse-ATL peptide specifically increases  $Ca^{2+}$  levels. The  $EC_{50}$  values obtained were 4.0 nM for Manse-AT, 8.5 pM for Manse-ATL-I, 37.2 pM, Manse-ATL-II, and 3.6 nM for Manse-ATL-III. In this cell line, the  $EC_{50}$  values were significantly lower than those obtained in CHO-WTA11 cells, but the relative order of ligand potency was maintained. Each of these peptides behaved as full agonists. Untransfected CHO-PAM28 cells or CHO-PAM28 cells transfected with pcDNA3.1D (empty vector) did not show any responses to Manse-AT or Manse-ATL peptides.

We then tested whether activation of Manse-ATR is coupled to changes in cAMP levels. We expressed this receptor in HEK293

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1           M L N K S I N I S I L L L I I V E S S T S E
1  ATGATTTGAAAGAAGAAAAAGACATTCAAATGCTTAACAAAAGCATAAACATATCAATCTACTCCTAATATCGTTGAATCTTCAACGTCGGAA

23  I I E D N I T R E P I K A T E L N R R I I R L I E I K E S N E D L
100 ATTATAGAAGACACATAACAGAGAACCAATTAAGCAACCGAATTAATAGAGAAATTAAGAAATTAAGAAATCAAGAAAGCAATGAAGATCCT

56  P N R Y K R S L P E N D K E P P E T K E N T T E E C V G A A E F C
199 CCAACAGATATAAAGAAGTTTACCAGAGAACGACAAAGAACCTCCAGAAACAAAAGAAAACACGACGGAAGAATGCGTAGGACGAGCGGAGTTTTC

TM-I
89  N M T K E A Y I A M L Q E Y I Y P Q T Y E W V L I A T H S I V F L
298 AATATGACCAAGAAGCATACTGGCATGCTTCAAGAATACATATACCCACAGACGATGAATGGTGTAAATAGCTACTCATTCCATAGTCTCTCTT

TM-II
122 T G L I G N A L V C I A V Y R N H S M R T V T N Y F I V N L A V A
397 ACTGGACTAATAGGGAATGCCTTGGTCTGCATCGCGCTATAGAAACCATCTATGAGGACCGTGACGAACTACTTCATCGTGAATTTGGCCGTGGCG

TM-III
155 D F M V I L F C L P A T V L W D V T E T W F L G D V L C K M L L L Y
496 GATTTTATGGTGATACTTTCTGCCTGCCAGCTACGGTGTCTGGGATGTTACTGAGACATGGTTCCTTGGAGACGTGTGTGCAAAAATGCTGCTTTAT
---S-DEG-->
188 F Q S V S V T V S V L T L T F I S V D R W Y A I C F P L K F K S T
595 TTTCACTGTGTCCGTGACGGTGTCCGTCTGACGCTGACGTTCTCGTGGACCGTGGTACGCGATCTGCTCCCGCTCAAGTTCAGTGCAGC

TM-IV
221 T S R A K T A I L I I W I L S L S F N S P D L V V L K T D K P V P
694 ACCAGCCGCGCAAGACTGCCATCTCATATGATCTTGTCTCTATCGTTCAATTCACCAGACCTGGTGGTGTGAAGACCGACAAGCCAGTTCCT

TM-V
254 L R F E L E Y L V Q C I A T W S S Q A D P V W H I L K V G T V T C A T A T A C G
793 CTAAGGTCGAGTTGGAGTACCTGGTGCAGTGCACCTGCTTCGACCGGCTGACCCGCGTGGCAGATATCTTGAAGAGTGGTGTTCATATACAG

TM-VI
287 I P L L L M T V A Y L Q I V R V L W H S D K I P G Q A E T I K L A
892 ATACCCTCTCGTGTACTGTGGCGTACCTTCAGATAGTGCGGTCTGTGGCATTGAGCAAGATTCCAGGCCAGGCCGAGACCAATCAAACTAGCC

TM-VII
320 P A E Q T Q L R S R R K A A K M L V A V V V M F A V V C Y F P V H L
991 CCGGCTGAACAGACTCACTGCGTCCCGCAGGAAGGCAGCAAGATGTTAGTAGCTGTGTGCTCATGTTCCGCGTGTGTTACTTCCCTGTTCACTC

AS-DEG-----
353 L S V L R Y T L D M E Q S D A I T F L A L V S H V M C Y A N S A V
1090 CTGTCTGTGTTGAGGTACACTTTAGACATGGAACAGAGCGACCCATTACTTTCTTAGCGCTAGTCTCTCAGCTCATGTGCTACGCGAAGTCCGCGCTC

N P L I Y N F M S G K F R R E F R R A F C C S S R P V H E N F T S
1189 AACCCACTCATTCAACTTTATGAGCGGTAAATTCGCGGTGAATTCGCGGTGCTTCTGCTGCTCGTCAAGGCGGTTACAGAGAAGTTCACCGAGC

L T R V T T S K K K E Q S C D K S L S Q R N V S N T T F I Q N G F
1288 CTCACGCGCTCACCCTTCAAGAAGAAGAACAGAGTTGCGACAAAAGCTTAAGTCAAAGGAACGTTAGCAATACCAGCTTTATTGAGATGGATTT

452 K S G Y Y A *
1387 AAGAGTGGATATTACGCTTAGAAAATTGTGATACAGTTAATTTTACATACATACATAGTATCGCGCTCTATCTTATAAGAGTATGCAGAGACTA
1486 TGGATTGCTAATTTTACGATCTTACGTACTTCTGTCGCTTCCCTCCACTTCATCAATCTTTTATGATTCCTGCGGTTCCGGTACTTTTACACT
1585 GACGTTTACTAGAGATCAGATATTCATTCGAAGGTTCCGTTGGGTCGAAACCTACCGGCTTCCATCCACATTCGCTTATAAACCTCTTTGTGA
1684 GTTCCCATCATCCATCGTCTCCAAATGCCAAACCACTTTAGCATACCCATTAATATGTAATAAGTAAACTGAATCATTACTCTGGTTTGTGATA

-----3R2----->
1783 AATTAAGGACTTCAAATACAAATGATTACATCATGAGACGGAACATGCTTGGTGGAAATGGGGTGTATCGGTTGACCTCTGCTACCCTTTCAGA
1882 GATAGATGCGTAATGTTTTCAAATACAAATATAGTAATTTATAGCAAAACGGTGTACAAATACCTTCAAAGAGTATTCTTAAAGTATTTAAAAATC
1981 TTGTTGGTCCAGGATTTAGCCGGGAAAAGTAGTGACACACTGATTTCCACGTTGGGCGAATCTCGGGCAGTGTGCTGAAAATATCAAGATATGTT
2080 ATCTTGAACACAGGCTCTTGTCTGGGGTGGCAACAGGTAACGCGCCGAGGCACTTACAGGAGCTCGCGTTGCTTGAAGACCTGTTTACAGAGAG
2179 AAGTTTCGAGTCCCTTGTCTGCTCGCCCGTACCTCGCATCGTCTTTATCTGTTGTTGCTCAGGCCTTGGCTTGAAGCCGCACTGTTTGA
2278 CGATGCTTTTATAACTCTTTAATGTTTATCTACTGCAATGTTTACTCTGAGCTATAAGTCTAATATCTCTGAGCTATATGATCAAAATGA
2377 ATAAGTCTTATGATTTATTTAGTGCAGCCCTCATGACCGCGGACTGAGGTGTAGGTGGAAGCAAAACGTCATCATCAATATCATACATA

-----3NR2----->
2476 TTTTACAATTTAAAAATAATTTACAGCTGTGATTTACGCAAGTGAACCTCACACTATTTTCTGACAAAATGAAAAAAGAAAAATGTAGGTAGAT
2575 ATTGTAAGTGGCTTTTTCGATGACTAACACGACGCTCCTCCCGTACTATGAATGGTGCAGTCTTTCGAAACATCGTGAAGAAAAATTTAAATATCAATAC
2674 TGCGATAGAATCCCAAACTAGTTTAAATTCGAAGAATAGGTGTTAAATATAAGTTATGTTATAAAATATACCTGTATCTATAGTCTCCACTGCTG
2773 GCTTGTACTACTGAGAGGGCTTCCGCCCTTAGTCCACACGAGATTTACTGTTGGGTGTCACCTGGTATTTTCAACTTTTACCTTCAAATTTTTCATG
2872 AAAAATTTTGGTGTGACGGCTTTTCCGGATCTACGATGATTTGTAAGGTTATTTCTGTAGAATCAATTAATTTGCTATAATACCGATGTGAT
2971 ATTTAATATGTGATTTTATAATAATATTTGG (A)n

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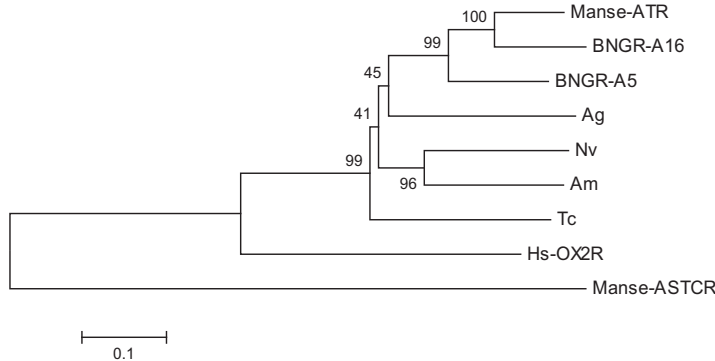
**Fig. 1.** The *M. sexta* allatotropin receptor (Manse-ATR) sequence. The nucleotide sequence of the Manse-ATR cDNA is shown below the deduced amino acid sequence (GenBank acc. no. HQ634154). Nucleotides are numbered from the 5' to the 3'-end and the amino acids are numbered from the putative initiation codon. The stop codon in the 5'-untranslated region and the polyadenylation signal are underlined. The translation stop codon is indicated by an asterisk. The transmembrane domains are indicated by boxes and labeled TM-I through TM-VII. The positions and polarities of the degenerate primers used from the initial PCR amplification and the RACE-PCR primers are shown below the nucleotide sequence.

**A**

Manse-ATR	--MLNKSINISILLII--VESSTSEIIEDNITREPIKATELNRRIRL-IEIKESNEEDLPNRYKRSIPENDKEPPEAKE---NTTEECVGA	85
BNGR-A16	-----MTTVEEDLVNPKKMKANKI-----ISEHDD-----RFK-----TDTNSSEFEE-----AENETCVGDP	48
BNGR-A5	MALRKEBLAITMIIICNYVLSNFDSIPESIRVRKSDVNTTSRSSLKNLNEMTKQSNNE--TEFGR-LLDATEMTTEYDN---FTEEP	88
Ag	--MTNASNVNPAEDLLVPFASNHSYTANGSAFWILKDLELATIQSPLEDN-HTTHSYPAVDLNDHNDVLCDDSDQMEYENNC	92
Trica-ATR	-----MLFFLLATILLSHAQAHDGLTSPHERANNSLFVSKPRP-----RNDTFIDDQFDY-----LVRDKRDWDE-----	69
Nv	-----MESLVVTLTFLATSASGETSEAE-----DSSANDS-----LDPASNCT	39
Am	-----MTWLGSMVFALIDATSDDDYFSLD-----YTDESD-----YNATNCT	37
Hs-OX2R	-----MSGTKLEDSPPCRNWSASELN-----ETQEPFL	29
	TM-I	
Manse-ATR	EFNMKTKEAYIAMLQ-EYIYPQTYEWWLIATHSIVLFTGLIGNALVCIAVYRNHSMRTVTNYFIVNLAVADFMVILFCLPATV	179
BNGR-A16	QYCNMTKEEVKMIQ-EYIYPNPYEWILIATHTFVFIITGLFGNALVCVAVYRNHSMRTVTNYFIVNLAVADFMVILFCLPATV	142
BNGR-A5	AFCNLTREYEMEMLN-DYVFPQPYEWWLIATHAVKSVFVIGLIGNALVCIAVYRNHSMRTVTNYFIVNLAVADFMVILI	182
Ag	DFCNLTYSYEQQLM-DYIYPSTGEWILIASHTVVFVLMGLVGNALVCIAVYTNHTMRTVTNYFIVNLAVADFMVILFCLP	186
Trica-ATR	GNVTFSEQEFIDSLW-ELIAPKSWITVLVILHSLVPIIGIIGNILVCVAVYRNHTMRTVTNYFIVNLAVADFMVILFCLP	163
Nv	NNLCISEDEYLDLDEM-AYIYPKSYEWWLIVLHCTVPIVGLVGNALVCIAVYRNHTMRTVTNYFIVNLAVADFMVILI	133
Am	NIYICISNEEYVDRMM-NYIYPKFWDWLIVLHSHSIVFVIGLIGNALVCIAVYRNHTMRTVTNYFIVNLAVADFMVILFCLP	131
Hs-OX2R	NPTDYDDEEFLRYLWREYVLPKEYEWWLIAGYIIVPVVALIGNVLCVAVWKNHMMRTVTNYFIVNLAVADFMVILFCLP	124
	TM-III	TM-IV
Manse-ATR	VLCKMLLYFQSVSVTVSGLTFTFISVDRWYACFPPLKFKSTTSRAKTAIILIIWLSLSF-----NSPDLVV	245
BNGR-A16	GLCKVLPYFQSVSVTVSGLTFTFISVDRWYACFPPLKFKSTTSRAKTAIILIIWLSLSF-----NIPELVV	208
BNGR-A5	AMCRIVLYFQSVSVTVSGLTFTFISVDRWYACFPPLKFKSTTSRAKTAIILIIWLSLSF-----NIPFVV	248
Ag	AMCKVVIYFQTVSVTVSGLTFTFISIDRWYACFPPLKFKSTTSRAKTAIILIIWLSLSF-----DLPEFLV	252
Trica-ATR	TMCKIVLYFQSVSVTVSGLTFTFISIDRWYACFPPLKFKSTTSRAKTAIIGIWIIVLALAC-----DIPEMIV	229
Nv	MPCKIVLYFQTVSVTVSGLTFTFISIDRWYACFPPLKFKSTTSRAKTAIILIIWVMALE-----DIPDLVV	199
Am	TLCKAVPYLQTVSVTVSGLTFTFISIDRWYACFPPLKFKSTTSRAKTAIIGIWIIVLALFSGSYMHIYCFSLNKQHYEKHAP	226
Hs-OX2R	SLCKVIVLYFQTVSVTVSGLTFTFISIDRWYACFPPLKFKSTTSRAKTAIILIIWVSCII-----MIPQAVI	190
	TM-V	
Manse-ATR	LKTDKPVV-LRFELEYLVQCIATWSSQAD-PVWHILKVVFIYTIPLLLMTVAYLQIVRVLWHSDKIPGQAEITK---LAPAEQT	324
BNGR-A16	LKLVRFVPLR-ELPYLLQCYGTWSPSSE-LVWHILKVLIIYTLPLVLMAYVAYHQIARVLSNGIPGQADTKK---LATAELT	286
BNGR-A5	LQVQTKMQ-LRFNVQYFMQCASTWSDSESLTWHIIKALFLYTFPLLLMTIAYCQIVRVLWRSNIPGHTESHK---LCSTQ	337
Ag	LTTRR-KK-LRFDIKLFQCVATWDNETE-KTFYIVKFLVLLYTLPLLFMTVAYFQIVRVLWRSNIPGHRESRNP	336
Trica-ATR	VTTIP-TV-DEVDTVLLTQCAPTWTETD-TIFFILKMLVLFYLIPLLFMSIAYLQIIRVLWKSQVNPHQIMDASGGGRQ	313
Nv	FYTHQDRK-LHGKTLILFTQCLPSWSRENQ-IAFNIIKILILLYTGPLMFMSFAYCQIVRVLWRND-IPGHNLSTR	280
Am	LHTVPPETH-IKIKTILFTQCNISWSKKNQ-VIFITVKLIIFLYTGPLIFMSVAYVQIVKVLWKSQ-IPGHNLSSR	306
Hs-OX2R	MECSTVPEGLANKTTLFTVCDERWGGEIYPKMYHICFPLVYMAPLCLMVLAYLQIFRKLWCRQIPGTSSVQKWKVLP	281
	TM-VI	TM-VII
Manse-ATR	-----QLRSRRAKMLVAVVVMFAVCFYPVHLLSVLRYTLDMEQS---DAITFLALVSHVMCYANSVAVNPLIYNFM	402
BNGR-A16	-----QLRSRRAKMLVAVVIMFAVCFYPVHLLSVLRYTIDMGOT---EFITIALVSHVMCYANSVAVNPLIYNFM	364
BNGR-A5	TTPSIHTNASTEGQLRSRRAKMLVAVVIMFAVCFYPVHLLSVLRYVAFDVQQT---DVMTICIALI	428
Ag	-----CVGNTSTMQLRARRKAAKMLVAVVIMFAVCFYPVHLLSVLRYTVDIGQS---DIVAVLSLFSHWL	423
Trica-ATR	-----MNASTEGQLRSRRAKMLVAVVIMFAVCFYPVHLLSVLRYTVDIGQS---DGNRAFSLSISHWL	398
Nv	-----SNVGNPEGQLKSRRAKMLVAVVIMFAVCFYPVHLLSVLRYTVDIGQS---DLVNITSLVHWLYANSVAVNPLI	366
Am	-----SGGGNEVQLRSRRAKMLVAVVIMFAVCFYPVHLLSVLRYTVDIGQS---KWINAISLSIAHGLCYFNSAVNPLI	392
Hs-OX2R	TKSRMSAVAAEIKQIRARRKTAARMLVAVVIMFAVCFYPVHLLSVLRYTVDIGQS---KWINAISLSIAHGLCYFNSAVNPLI	376
Manse-ATR	RAF-----CCSTRPVHE-----NFTSLTRVTTSSKKEQSCDKGLSQRVNSN-----TTFIQNGFKSGYYA-----	457
BNGR-A16	RAF-----CCSTSPGQQ-----DFTSMRVT--KSDSSIMASFKPGHTS-----TTFVHN-NKNGHMT-----	415
BNGR-A5	RSYFKCPCCHTTPAPEQNGASFEPIGSSRARTIRTTVRRHDSVSVYRLAHLSPSN-HNIHRDYIQNTNTP	523
Ag	NALEKRCCLRSSHAYGGR-----VGGYDDRSLSLCHTATRLN-----VSPSTRSNYHLASVRDARFKVISN	503
Trica-ATR	RAFEH-----CQORSQGHG-----FQFSAVYRKEKDSGIASRTHSRDLEIQR-----VNDPEPRHNRK	464
Nv	RTE-----CCPRGGGSHN-----RAVYRMAGRSTRHSITITIFNTAARKS-----VTIKRSNPFEDIQE-----	419
Am	LLL-----LANK-----	399
Hs-OX2R	AAFS-----CCLGVHHRQEDRLTRGRSTESRKSLLTQISNFDNISLSEQVVLTS-----ISTLPAANGA	444

Ag PSPRPAKL 511

**B**



**Fig. 2.** Alignment of Manse-ATR with related receptors. (A) Manse-ATR (GenBank acc. no. HQ634154) is compared with the *B. mori* AT receptor (BNGR-A16, GenBank acc. no. NM\_001134242) (Yamanaka et al., 2008), a receptor from *B. mori*, BNGR-A5 (GenBank acc. no. NM\_001134268) (Yamanaka et al., 2008), receptors deduced from *T. castaneum* (Tc) (GenBank acc. no. XM\_968645) and *N. vitripennis* (Nv) (GenBank acc. no. XM\_001604532) mRNA sequences; and *A. gambiae* (predicted from BLAST analysis and the presence of potential splice junctions) and *A. mellifera* (Am) (GenBank acc. no. XM\_001120335) genomic sequences; and the human orexin receptor-2 (Hs-OX2R) (GenBank acc. no. AF041245) (Sakurai et al., 1998). The *M. sexta* allatostatin C receptor (Manse-ASTCR) (GenBank acc. no. HQ634155) is used as an outgroup to root the tree. Dashes are used to optimize alignments. The transmembrane domains predicted for Manse-ATR are indicated above the sequences and labeled TM-I through TM-VII. Amino acids that are identical to those in Manse-ATR are highlighted in gray. (B) Phylogenetic analysis of ATR-like GPCR proteins. A phylogenetic tree was constructed using neighbor-joining method with MEGA version 4 software (Kumar et al., 2007). Bootstrap values, shown next to each branch, were calculated using 800 replicates. Evolutionary distances were calculated using Poisson substitution model.

cells. This cell line contains the luciferase gene under the control of a cyclic AMP response element (CRE). Cells were transfected with pcDNA3.1D-Manse-ATR or with pcDNA3.1D (empty vector) and cultured in the presence of the phosphodiesterase inhibitor IBMX. We assayed different concentrations of each peptide in the presence or absence of forskolin, which increases intracellular levels of cAMP. In the absence of forskolin, Manse-AT and each Manse-ATL resulted in a dose-dependent increase of cAMP concentration, measured by assaying luciferase activity (Fig. 3D). The EC<sub>50</sub> values obtained were 76.5 nM for Manse-AT, 46.2 fM for Manse-ATL-I, 5.1 pM Manse-ATL-II, and 0.37 nM for Manse-ATL-III. The EC<sub>50</sub> values measured in HEK293 cells were lower than in either of the other cell lines, but again, the relative order of ligand potency was maintained. When the peptides were tested in the presence of forskolin, there was no effect on cAMP levels. In this cell line, Manse-ATL-I and Manse-AT functioned as full agonists, but Manse-ATL-II and -III behaved as partial agonists. Untransfected HEK293 cells or HEK293 cells transfected with pcDNA3.1D (empty vector) did not show any responses to Manse-AT or Manse-ATL peptides, either in the presence or absence of forskolin.

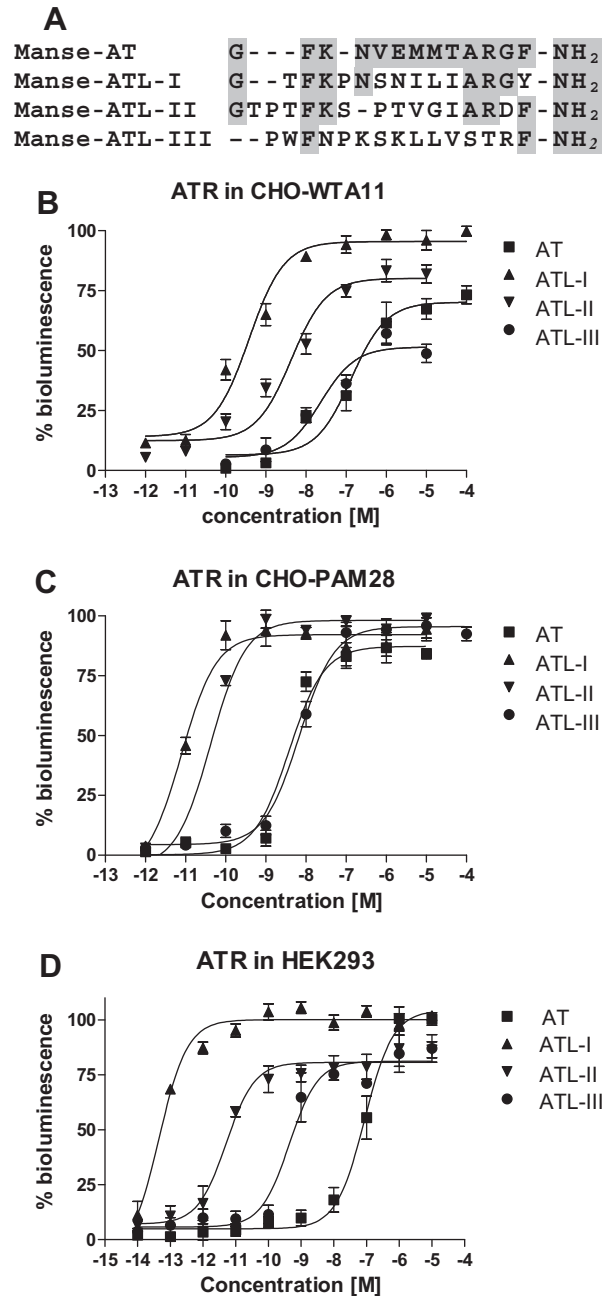
### 3.3. Expression of Manse-ATR

We conducted an initial screen of tissue localization of Manse-ATR mRNA in feeding larvae by quantitative RT-PCR (qPCR) (Fig. 4A). The mRNA is most abundant in the Malpighian tubules suggesting a role for Manse-AT or the Manse-ATL peptides in control of water balance. A high level of Manse-ATR mRNA was also detected in the midgut which is consistent with the role of each of the peptide ligands on the inhibition of ion transport across the epithelium (Lee et al., 1998). Other tissues which contain high levels of Manse-ATR mRNA include the hindgut and the testes. All other tissues tested showed either low or undetectable levels of Manse-ATR mRNA. We obtained similar results when we used actin as the reference gene (data not shown).

We then determined the levels of Manse-ATR mRNA in the CA and CC of feeding larvae by qPCR (Fig. 4B). The mRNA levels were higher in the CA than in the CC, in contrast to *B. mori* when expression was observed only in the CC (Yamanaka et al., 2008). In adult insects, Manse-ATR mRNA was detected in the CC–CA complex of both females and males (Fig. 4B), although Manse-AT stimulates JH biosynthesis exclusively in female insects (Kataoka et al., 1989).

## 4. Discussion

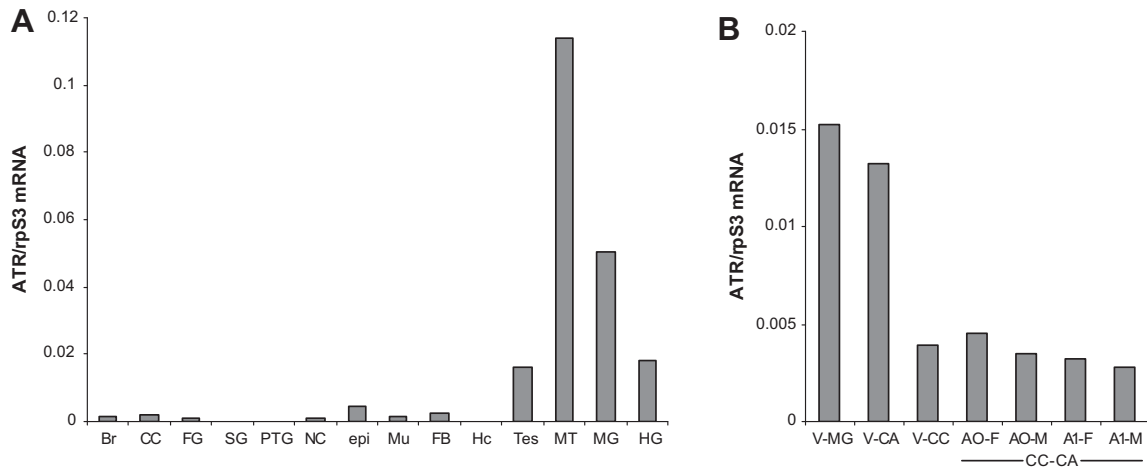
In this study, we used the sequence of the *B. mori* ATR (BNGR-A16) (Yamanaka et al., 2008) to design degenerate oligonucleotide primers to amplify a portion of the homologous cDNA from the larval midgut of *M. sexta*, and determined the full-length sequence following amplification of the 5'- and 3'-ends using RACE. We predicted that the midgut from feeding *M. sexta* larvae would be a good source of Manse-ATR mRNA since Manse-AT robustly inhibits active ion transport across the larval midgut epithelium. This is in contrast to *B. mori*, in which both the response of the midgut to Manse-AT (Lee et al., 1998) and the mRNA level of BNGR-A16 (Yamanaka et al., 2008) are quite low. We considered it highly likely that the cDNA isolated was a receptor for Manse-AT, since a TBLASTN search revealed that the sequence that exhibits the highest identity is BNGR-A16, the *B. mori* AT receptor. Several other insect receptors were identified using this TBLASTN search, notably a closely related receptor from *B. mori* designated BNGR-A5. BNGR-A16 exhibits 73% sequence identity with BNGR-A5 in the region between TM-I and TM-VII, and further differs from BNGR-A5 in the size and sequence of the N-terminal and C-terminal domains and by the insertion of 23 amino acids in BNGR-A5 in the third



**Fig. 3.** Dose-response curves for responses of the effects of peptides in cells expressing Manse-ATR. (A) The sequences of the peptides assayed are aligned and amino acids that are identical to Manse-AT are highlighted in gray. Dose-response curve for bioluminescent responses induced in CHO-WTA11 (B), CHO-PAM28 (C) and HEK293 (D) cells expressing Manse-ATR. Data are the average  $\pm$  SD of three independent measurements done in triplicate and are expressed as percentage of the maximal response. The zero response level corresponds to treatment with buffer only. Peptides tested are Manse-AT (■), Manse-ATL-I (▲), Manse-ATL-II (▼) and Manse-ATL-III (●). All other peptides tested at 10<sup>-5</sup> M did not activate the receptor.

intracellular loop located between transmembrane domains V and VI. The ligand binding specificity of BNGR-A5 has not yet been determined, and the expression profile during two larval stages differs considerably from that of BNGR-A16 (Yamanaka et al., 2008). In larvae, BNGR-A5 is expressed at the highest levels in the testis, while BNGR-A16 is expressed at the highest levels in several larval tissues including the brain, CC–CA, epidermis, and testis.

Other insect sequences identified in the TBLASTN search that exhibit high sequence identity with Manse-ATR include those



**Fig. 4.** Expression of Manse-ATR mRNA. (A) Manse-ATR mRNA levels in tissues of fifth instar larvae were quantified by qPCR and normalized against levels of ribosomal protein S3 (rpS3) mRNA (Jiang et al., 1996). All tissues were dissected from day 1 larvae with the exception of frontal ganglion, prothoracic gland, and testes, which were dissected from day 4 larvae. Values represent the mean of two determinations. Tissues tested are brain (Br), corpora cardiaca (CC), frontal ganglion (FG), salivary gland (SG), prothoracic gland (PTG), nerve cord (NC), epidermis (epi), muscle (Mu), fat body (FB), hemocytes (Hc), testes (Tes), Malpighian tubules (MT), midgut (MG), and hindgut (HG). (B) Manse-AT mRNA levels in day 1, fifth instar larval midgut (V-MG), corpora allata (V-CA), corpora cardiaca (V-CC), and adult CC–CA complexes from day 0 females (AO-F), and males (AO-M); and day 1 females (A1-F) and males (A1-M) were quantified and normalized against levels of rpS3 mRNA.

deduced from mRNA sequences from *T. castaneum* and *N. vitripennis*, and those deduced from genomic sequences from *A. mellifera* and three mosquito species (*A. gambiae*, *Aedes aegypti* and *Culex pipiens*). An AT sequence was deduced from the *T. castaneum* genome sequence (Amare and Sweedler, 2007; Li et al., 2008; Weaver and Audsley, 2008), and the peptide has been shown to stimulate JH biosynthesis in the beetle (Abdel-latif and Hoffmann, 2010), so the presence of a putative ATR was expected. Similarly, in *A. aegypti* and *A. gambiae*, an allatotropin has been identified which is similar in sequence to Manse-AT (Riehle et al., 2002; Veenstra and Costes, 1999), and which stimulates JH biosynthesis by the *A. aegypti* CA (Li et al., 2003). The absence of a similar receptor sequence was already noted in the *D. melanogaster* genome (Yamanaka et al., 2008) which is consistent with the absence of an allatotropin in this insect (Hewes and Taghert, 2001; Vanden Broeck, 2001). A similar sequence to Manse-ATR was unexpectedly found in *A. mellifera* and *N. vitripennis*, insects in which a peptide similar to Manse-AT has not been identified *in silico* using the genome sequence (Hummon et al., 2006) (Hauser et al., 2010). However, Manse-AT-like immunoreactivity was observed in numerous neurons in the CNS of *A. mellifera*, the axons of some terminated near the surface of the CA (Glasscock et al., 2005), and Manse-AT stimulates JH biosynthesis by the CA from feeding-stage larvae, although the peptide concentration required was in the  $\mu\text{M}$  range (Rachinsky and Feldlaufer, 2000) which may result from the use of a peptide from a heterologous insect. It is possible that a hymenopteran AT may have diverged from a Manse-AT-related sequence and acts through the GPCR similar to Manse-ATR. Further research is necessary to determine whether additional AT receptors exist, or whether structurally unrelated peptides act through Manse-ATR and the related GPCRs from other insects.

Examination of the alignments of the ATR (and orexin receptor) sequences (Fig. 2A) reveals the insertion of 29 amino acids in the *A. mellifera* sequence in a region that corresponds to transmembrane domain IV of Manse-ATR. Since the predicted *A. mellifera* sequence was deduced from the genome sequence, we tested for the presence of predicted splice donor and acceptor sequences flanking this insertion using the Splice Site Prediction by Neural network NNSPLICE 0.9 version (Reese et al., 1997), and no such sequence motifs were identified. Nevertheless, seven

transmembrane domains, typical of a GPCR, were identified in the deduced protein using TMHMM, and the C-terminal portion of transmembrane IV was predicted to be localized within the 29 amino acid insertion.

The mammalian receptor that is most closely related to Manse-ATR is the receptor for orexin/hypocretin (Fig. 2A) (Sakurai et al., 1998). Orexins are two peptides, orexin A and orexin B, that exhibit limited sequence identity, but high structural similarity, and are derived from a common precursor protein (Sakurai et al., 1998; Voisin et al., 2003). Mammals contain two orexin receptors (OX<sub>1</sub>R and OX<sub>2</sub>R). OX<sub>1</sub>R is selective for orexin A, but OX<sub>2</sub>R is activated by both orexins with similar potency (Sakurai et al., 1998). The best known functions of orexins are the regulation of wakefulness and feeding; but have additional roles in regulating neuroendocrine homeostasis and gut function (Voisin et al., 2003). Manse-AT does not exhibit sequence identity with orexins, but the sequence similarities between the receptors imply a potential evolutionary link between the two systems.

The Manse-AT gene is expressed as three alternatively spliced mRNAs (Taylor et al., 1996). Proteolytic processing of each translation product is predicted to yield Manse-AT mRNA, and the insertion of either one or two alternative exons in the longer Manse-AT mRNAs is predicted to yield allatotropin-like peptides (Manse-ATL-I, -II and -III, Fig. 3A) that are structurally related to Manse-AT (Horodyski et al., 2001). Alternative splicing of Manse-AT mRNAs is regulated in a stage- and tissue-specific manner and is predicted to yield a diverse set of peptide products that can vary in a dynamic manner during insect development (Horodyski et al., 2001; Lee et al., 2002). Similar alternative splicing of AT mRNAs and the existence of ATL peptides has been shown in several additional lepidopteran insects (Abdel-latif et al., 2003; Sheng et al., 2007; Yin et al., 2005).

It has been demonstrated that Manse-AT and the Manse-ATL peptides possess overlapping biological activities; Manse-AT and Manse-ATL-I stimulate JH biosynthesis by the adult female CA when tested at 20 nM, and each peptide inhibits ion transport across the larval midgut epithelium at 100 nM (Lee et al., 2002). The overlapping set of bioactivities controlled by structurally similar peptides suggested that they act through a common receptor. Our data confirm this, and further show that the ATL peptides exhibit

a greater potency toward Manse-ATR expressed in three different mammalian cell lines (Fig. 3). In each cell line, the most potent peptide was Manse-ATL-I, and the relative potencies for each of the peptides toward Manse-ATR were identical. The greater potency of the ATL peptides compared with Manse-AT suggests that this receptor might be a receptor for ATL peptides. However, it is clear from the data shown in Fig. 3. that Manse-AT is a ligand for this receptor in the nanomolar range. These data do not eliminate the possibility that an additional receptor may exist which has a greater affinity to Manse-AT. A candidate might be the homolog of BNGR-A5, if it is present in *M. sexta*, since it exhibits the greatest identity with BNGR-A16, the *B. mori* ATR. We have so far been unable to amplify cDNA for a second receptor using degenerate oligonucleotide primers. This possibility could be addressed in the future when the genome sequence of *M. sexta* is determined.

Despite the same order of potency, the EC<sub>50</sub> values obtained varied considerably in the cell lines. This could be due to differences in receptor density, posttranslational modifications, or the presence or absence of interacting proteins that could affect the conformational and functional properties of a receptor. Therefore, tests with cloned receptors in heterologous cell lines might not fully reflect the actual situation in the insect. Although most neuropeptides generally function in the nM range, a much higher potency has been observed for Manse-AT using a foregut contraction bioassay. Manse-AT stimulates foregut contractions in *L. oleracea* at concentrations as low as 10<sup>-14</sup> M (10 fM) (Duve et al., 2000), and in *H. armigera* at concentrations as low as 10<sup>-16</sup> M (0.1 fM) (Duve et al., 1999). Aedae-AT stimulates hindgut contractions in *Triatoma infestans* at a concentration reported to be 10<sup>-18</sup> M (Santini and Ronderos, 2007).

The response of Manse-ATR is specific to Manse-AT and Manse-ATL peptides, since this receptor was not activated by any other peptide tested. This includes the FLRFamides F7G and F7D (Kingan et al., 1996). These peptides were tested because, like Manse-AT and Manse-ATL peptides, they also inhibit active ion transport across the larval midgut epithelium (Lee et al., 1998). The effects of Manse-AT and the FLRFamides on the midgut are additive; the addition of FLRFamides to a Manse-AT-inhibited midgut resulted in additional inhibition of ion transport, whereas the addition of Manse-AT did not. This led to our hypothesis that Manse-AT and the FLRFamide act through distinct receptors on the basolateral membranes of the same midgut cells. The finding that F7G and F7D weakly activate the Bommo-myosuppressin receptor (Yamanaka et al., 2005) is consistent with this hypothesis.

In insects, the rate of JH biosynthesis is often correlated with an increase in the intracellular Ca<sup>2+</sup> concentration of the CA cells (Allen et al., 1992; Rachinsky and Tobe, 1996; Rachinsky et al., 2003). In adult *M. sexta*, stimulation of JH biosynthesis is accompanied by increased levels of inositol phosphates (Reagan et al., 1992). In another lepidopteran insect, *Heliothis virescens*, a calcium ionophore mimics the effect of Manse-AT and a Ca<sup>2+</sup> chelator antagonized the stimulatory effects of Manse-AT, suggesting that the peptide affects JH biosynthesis by increasing intracellular Ca<sup>2+</sup> concentrations (Rachinsky et al., 2003). Our observation that the activation of the Manse-ATR in CHO-PAM28 cells is coupled to an increase in Ca<sup>2+</sup> levels (Fig. 3B) is consistent with these previous studies, and suggests that the Manse-ATR acts through G<sub>αq/11</sub>. The increase in cAMP levels in HEK293 cells by activation of Manse-ATR (Fig. 3C) indicates that it may also be coupled through a distinct G<sub>αs</sub> second messenger pathway. Further studies will be needed to determine whether this multiplicity of G protein coupling is genuine or due to the high receptor densities generated in transfected cells.

Our initial investigation into the tissue-specific profile of Manse-ATR expression in feeding larvae clearly demonstrated that the

highest level of mRNA was seen in the Malpighian tubules (Fig. 4A). This tissue is generally not thought of as a target tissue for AT, but Manse-AT has been shown to inhibit fluid secretion by larval *M. sexta* Malpighian tubules (G.M. Coast, personal communication). Furthermore, Manse-AT caused an increase in acid phosphatase and alkaline phosphatase levels in Malpighian tubules of the Colorado potato beetle, *Leptinotarsa decemlineata* (Yi and Adams, 2001). This effect of AT mimics the effects of a JH analog, so it was thought that it was due to its effects on JH synthesis. However, it is possible that Manse-AT may act directly on the Malpighian tubules in *L. decemlineata* (Elekovich and Horodyski, 2003). The high level of Manse-ATR mRNA in the larval midgut (Fig. 4A) is consistent with the activity of Manse-AT and Manse-ATL peptides in the inhibition of active ion transport across the midgut epithelium (Lee et al., 1998). It will be interesting to determine whether the stage-specific effect of these peptides on midgut ion transport is due to fluctuating levels of Manse-ATR which may be reflected in changes in mRNA levels. Significant levels of Manse-ATR mRNA were also detected in the testes (Fig. 4A), suggesting that Manse-AT has a role in the male reproductive system which has not yet been characterized.

The first described target of Manse-AT is the adult female CA in which the peptide stimulates JH biosynthesis (Kataoka et al., 1989). Consistent with this activity, Manse-AT mRNA was detected in the adult female CC-CA complex, although mRNA levels are lower than that observed in the larval midgut (Fig. 4B). Surprisingly, Manse-AT mRNA was also detected in adult male CC-CA (Fig. 4B) although the peptide is inactive in this tissue (Kataoka et al., 1989). This indicates that the sex-specific difference in the activity of Manse-AT is not due to mRNA levels of the receptor. Alternatively, a second Manse-AT receptor might exist whose mRNA levels reflect the peptide's activity on the CA. In *M. sexta* larvae, Manse-AT mRNA levels were higher in the CA compared with the CC, which differs from *B. mori*, in which mRNA for the AT receptor was only detected in the CC (Yamanaka et al., 2008).

Further studies are also necessary to determine whether Manse-ATR mRNA levels undergo tissue-specific alterations during development, as has been shown for *B. mori* (Yamanaka et al., 2008). This would be consistent with the dynamic changes of the primary roles of this neuropeptide family that have been shown for insects (Elekovich and Horodyski, 2003; Weaver and Audsley, 2009).

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