Journal of Insect Physiology 61 (2014) 8–15

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

The role of serotonin in feeding and gut contractions in the honeybee

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ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form 20 November 2013
Accepted 17 December 2013
Available online 27 December 2013

Keywords:
Honeybee
Apis mellifera
Serotonin
5-HT
5-HT receptor
Gut contractions

ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT) is involved in the regulation of feeding and digestion in many animals from worms to mammals. In insects, 5-HT functions both as a neurotransmitter and as a systemic hormone. Here we tested its role as a neurotransmitter in feeding and crop contractions and its role as a systemic hormone that affected feeding in adult foraging honeybees. We found 5-HT immunoreactive processes throughout the gut, including on the surface of the oesophagus, crop, proventriculus, and the midgut, as well as in the ventral nerve cord. mRNA transcripts for all four of the known bee 5-HT receptors (Am5-ht$_{1A,2\alpha,2\beta,7}$) were expressed in the crop and the midgut suggesting a functional role for 5-HT in these locations. Application of a cocktail of antagonists with activity against these known receptors to the entire gut *in vivo* reduced the rate of spontaneous contraction in the crop and proventriculus. Although feeding with sucrose caused a small elevation of endogenous 5-HT levels in the haemolymph, injection of exogenous 5-HT directly into the abdomen of the bee to elevate 5-HT in the haemolymph did not alter food intake. However, when 5-HT was injected into directly into the brain there was a reduction in intake of carbohydrate, amino acid, or toxin-laced food solutions. Our data demonstrate that 5-HT inhibits feeding in the brain and excites muscle contractions in the gut, but general elevation of 5-HT in the bee's haemolymph does not affect food intake.

1. Introduction

The commencement and cessation of feeding is orchestrated by a diverse set of internal cues that provide the brain with information about nutritional state and satiety. In animals as diverse as nematodes and humans, the biogenic amine, serotonin (5-HT), is one of the key signalling molecules regulating feeding, nutrient intake and digestion (Gietzen et al., 1991; Howarth et al., 2002; Liscia et al., 2012; Marston et al., 2011; Song and Avery, 2012).

In many insects, 5-HT neurons innervate the crop and midgut (Budnik et al., 1989; Haselton et al., 2006; Molaei and Lange, 2003; Pietrantonio et al., 2001) indicating that they are likely to play an important role in the movement of food through the digestive tract. This idea has been supported by a recent study in the blowfly (*Phormia regina*) demonstrating that 5-HT applied to the crop increases muscle contractions and crop emptying rate (Liscia et al., 2012). Previous studies have also identified serotonergic varicosities in the foregut and midgut of other insect species including the kissing bug, *Rhodnius prolixus* (Lange et al., 1989), locusts, *Locusta migratoria* (Molaei and Lange, 2003) and *Schistocerca gregaria* (Johard et al., 2003), the mosquito, *Aedes aegypti* (Moffett and Moffett, 2005; Pietrantonio et al., 2001), the stable fly, *Stomoxys calcitrans* (Liu et al., 2011) and the ant species, *Campanotus mus* (Falibene et al., 2012). In these species, innervation of the hindgut is often less evident. The possible presence of 5-HT neurons in the digestive tract and the functional role of 5-HT in the gut has not yet been investigated in the honeybee.

In *R. prolixus*, processes in the mesothoracic ganglion project throughout the body, and in particular, innervate the digestive tract (Lange et al., 1989; Orchard, 2006). These neurons release 5-HT directly into the haemolymph during a blood meal, but also orchestrate contractions of the crop and prime the animal's physiology for rapid diuresis and the digestion of blood (Lange et al., 1989; Orchard, 2006). Experimental elevation of haemolymph 5-HT via direct injection into the thoracic or abdominal haemolymph in cockroaches or flies (Cohen, 2001; Dacks et al., 2003; Haselton et al., 2009) or by feeding 5-HT to ants (Falibene et al., 2012) reduces meal size, but whether this is a general mechanism for the regulation of feeding in insects remains unclear.

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0022-1910
http://dx.doi.org/10.1016/j.jinsphys.2013.12.005

5-HT injected directly in the brain directly reduces the motor function of the honeybee's mouthparts (proboscis). Studies of associative learning in honeybees indicate that 5-HT injected directly into the brain via the median ocellar tract prior to olfactory associative conditioning of the proboscis extension reflex (PER) reduces performance during conditioning (Menzel, 1999). Honeybees can also be trained to learn to withhold their proboscis to odours signalling rewards containing toxins (Wright et al., 2010). When the known 5-HT receptors in the brain are blocked using a cocktail of 5-HT receptor antagonists, bees do not learn to avoid toxins in food. They continue to extend the proboscis and feed even though the reward contains toxins, indicating that 5-HT mediates conditioned withholding of the PER (Wright et al., 2010). These two studies indicate that 5-HT is involved in the control of motor function of PER in bees, but neither has identified whether 5-HT inhibits food consumption once the proboscis is extended. In addition, no one as yet has reported whether haemolymph levels of 5-HT in the honeybee are elevated by feeding, and whether elevation of 5-HT in the haemolymph reduces food consumption by bees.

Here, we tested several hypotheses regarding the role of 5-HT in feeding the brain, gut, and ventral nerve chord of the honeybee. First, we tested whether 5-HT played a role in digestion by using immunohistochemical methods to identify 5-HT processes in the gut and ventral nerve chord. The four known 5-HT receptor homologues in bees have been measured and described from the brain (Blenau and Thamm, 2011; Schlenstedt et al., 2006; Thamm et al., 2010, 2013), but not measured elsewhere. For this reason, we also measured whether 5-HT receptors were expressed in the digestive tract and examined their role in digestion by measuring whether 5-HT affected gut contractions. Because we identified 5-HT immunoreactive processes in the ventral nerve chord, our second hypothesis tested whether systemic levels of 5-HT and/or brain 5-HT affected food intake. We first measured whether feeding elevated haemolymph 5-HT as shown in *R. prolixus* using HPLC methods. To test whether elevation of haemolymph 5-HT reduced food intake, we injected 5-HT into the abdomen and measured the consumption of sucrose solution. To verify that elevation of 5-HT in the brain but not haemolymph affected feeding, we injected 5-HT into the brain prior to assaying the total food consumption of three different types of liquid food encountered by honeybees.

2. Materials and methods

2.1. Insects

Honeybee colonies (*Apis mellifera mellifera*) were obtained from stock of the National Bee Unit (FERA, York, UK). During the months of January–March 2011 bees were maintained in an indoor flight room at a temperature of 28 °C with a 12-h light/dark cycle. During the months of May–September 2011 and 2012, bees were kept outdoors and allowed to forage freely. Adult foraging worker bees were collected in small plastic vials from outside the colony entrance. Foragers were identified as they were flying back into the colony and collected at the entrance.

2.1.1. Immunohistochemistry

Using bees collected as described above, ventral nerve cords (VNC; $N = 4$), and digestive tracts ($N = 8$) were dissected in air and fixed for 1–3 h in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS). Tissue was washed in PBS with agitation (3 changes: 10 min each) and then probed with rabbit anti-5-HT antiserum (Sigma–Aldrich, product code S5545) diluted (1:400) in 10% normal goat serum (Sigma–Aldrich, G9023) and 0.1% Triton X in PBS (NGS/PBST) for 18 h at 4 °C. Control tissues ($N = 4$ for VNC and $N = 8$ for guts) were incubated in diluent only. After incubation, probed and control tissues were first washed in PBS with agitation (3 changes: 10 min each) and incubated in biotinylated goat anti-rabbit antiserum (Vectalabs, BA-1000) in NGS/PBST (1:200) for 2 h at room temperature (RT), then washed in PBS with agitation (3 changes: 10 min each) and incubated in Fluorescein Avidin D (Vectalabs, A-2001) in NGS/PBST (1:200) for 1 h at RT in darkness. The tissue was washed a final time in PBS as before and then mounted on microscope slides under a coverslip in Vectashield mounting medium (Vectalabs, H-1500). Coverslips were sealed with clear nail polish and stored in darkness. Control tissues, which were incubated in diluent instead of primary antibody showed no positive staining, indicating that the secondary antibodies did not bind anything expressed in the tissue. Rabbit anti-5-HT antiserum (Sigma–Aldrich, product code S5545) is a commercially tested antibody previously used in insect preparations (Falibene et al., 2012), and pre-incubation of diluted antiserum with 500 μM 5-HT inhibits specific staining. Guts incubated in 1:400 concentration ($N = 8$) of primary antibody were photographed for the figures in this study, however other concentrations of primary antibody were also tested; 1:200 ($N = 4$), 1:800 ($N = 2$) and 1:1600 ($N = 2$), positive staining was observed although best images were obtained with 1:400.

2.1.2. Microscopy

To obtain stacked images, specimens were examined and photographed under a Confocal Zeiss Axio Imager microscope (with apotome) using an excitatory wavelength of 488 nm. Number of *Z* slices and depth of *Z* slice interval depended on the topology and thickness of tissue. Snap shot images were obtained using a Leica DMRA fluorescent microscope with Hamamatsu GRCA-ER digital camera or Confocal Zeiss Axio Imager microscope. Images were processed using Axiovision 4.8.1 software. Light microscope images were obtained using Leica M205 C.

2.2. Quantitative real-time PCR

Tissue samples were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and served as template for cDNA synthesis. From each sample, two independent cDNA syntheses from 250 ng total RNA were performed using SuperScript III (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was carried out on a Rotor Gene Q (Qiagen Hilden, Germany) by using TaqMan technology with various fluorescent dyes to allow duplex measurements of receptor and reference gene expression. Fluorescent dyes used as 5′-modifications were 6-FAM-phosphoramidite (6FAM), Cy5, Cy5.5 and Yakima Yellow (YAK). BlackBerry quencher (BBQ) was attached to the 3′-end of TaqMan probes. The sequences of the primers and TaqMan probes are presented in Table 1. The PCR was performed with an initial step at 60 °C for 1 min and a denaturation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 60 s. Tissue samples of individual bees were examined in triplicate. Mean copy numbers were calculated using Rotor Gene Q software (Qiagen). Receptor transcript levels were normalized to elongation factor 1α (*Amef-1α*) transcript levels (=100%) using the standard curve method. The standards covered copy numbers from 10^4 to 10^7.

2.3. Assay of crop and proventriculus contractions

Bees were collected from the colony, immediately chill anesthetized, and then pinned to a dissecting plate dorsal side down under 'protophormia saline' (PPS) (Liscia et al., 2012). With the aid of a dissecting microscope, each bee was cut from the final abdominal tergite upwards towards the thorax using dissection scissors; the