Post mating change in physiology of male *Drosophila* mediated by 5-HT

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Abstract

Sex peptides transferred during mating from male to female fly profoundly influence her behaviour and physiology, including an increase in the movement of eggs along the oviduct. In the male ejaculatory duct we have identified peristaltic waves that travel distally with an average frequency of 0.6 Hz. The frequency of peristalsis is increased by 0.1 μM serotonin and completely blocked by serotonin (5-HT) antagonists (IC50 < 1 μM). We also report that mating affects the male reproductive tract; peristaltic waves along the ejaculatory duct are significantly reduced post-copulation by 30%. Serotonergic neurons innervate the ejaculatory duct, but their genetic ablation does not prevent peristalsis. We propose that peristalsis may be modulated by serotonin circulating in the haemolymph. As serotonin is linked with attentiveness in both flies and mammals, our bioassay suggests reduced behavioural sensitivity of the male fly after mating.

Introduction

A key feature of fly copulation is the profound post-mating change in female produced by sex peptides transferred from male to female along with the sperm (Ram & Wolfner, 2007b). This is a well-understood example of integrative physiology, where the peptides stimulate behavioural changes (increased feeding (Carvalho *et al.*, 2006) and reduced receptivity to males (Bastock & Manning, 1955)) together with physiological responses (e.g. acceleration of oogenesis (Soller *et al.*, 1999), ovulation (Heifetz *et al.*, 2005), transport of eggs along the oviduct and oviposition (Herndon & Wolfner, 1995) to facilitate egg-production. The downstream targets of sex peptides may include octopamine, as this neuromodulator increases the contractions of the reproductive tract and relaxes the oviduct (Middleton *et al.*, 2006), so facilitating release of eggs from the ovary and their progress towards the uterus.

Despite a deep understanding of the changes in females, no post-copulatory changes in physiology have been documented in male flies. We have therefore monitored the activity of the male reproductive tract (Fig. 1A). This consists of the paired, coiled testes, where sperm are created from stem cells, two short vas deferens, which store the sperm, a large pair of accessory glands, which produce the sex peptides, and a single ejaculatory duct (ED) which leads to the ejaculatory bulb, a muscular sphincter. We report for the first time a peristaltic rhythm of the ejaculatory duct (ED, Fig. 1A), which persists in isolation, is serotonin-dependent, and this rate of peristalsis is reduced by mating. We conclude that peristalsis is due to serotonin circulating in haemolymph and (as serotonin modulates arousal and mood) we suggest the males will show a post-coital decrease in attentiveness.

Materials & Methods

Ethical considerations

All experiments were done in the fruit fly, *Drosophila melanogaster* anaesthetised with ether. Data were collected from 150 males.

Dissection

We described (Middleton *et al.*, 2006) a method to record the peristaltic contractions of the oviduct of female *Drosophila* (providing full details of the technology and its validation). Here we use the same approach in the male, to record contractions of the ejaculatory duct (ED). Male flies were anaesthetised, covered in HL-3 saline (Stewart *et al.*, 1994), and the abdomen cut open. The ED was cut close to the ejaculatory bulb and the complete reproductive tract placed on a microscope slide in fresh HL-3 saline. All the data in this report comes from flies anaesthetised with ether. Continuous peristalsis along the ED is also seen following carbon dioxide anaesthesia.

Optical recording and analysis

Video micrographs were recorded to PC-compatible computer disk at 5 frames/s (resolution 768x576 pixels, uncompressed RGB format). The video was analysed off-line, using custom software (avi_line, http://biolpc22.york.ac.uk/avianal/avi_line/). A vector crossing the ED is selected. The change in intensity along this line between successive frames is determined by taking the difference in brightness between successive frames at each pixel, squaring this, taking the average along the vector (Fig. 1C) and exporting the mean square to an Excel format file.

Fly stocks

Drosophila melanogaster were raised on standard yeast-sugar-agar medium at 25 °C (Middleton *et al.*, 2006). All males were tested 7 - 10 days after emergence, when wild type and *fru* mutants are at their peak of sexual activity (O'Dell, 2003). We used our

male laboratory Canton-S flies in most experiments. For the data in Fig. 4, we used the following transgenics: (i) *dTRHn-*, a null for the neuronal enzyme (tryptophan hydroxylase) that synthesises serotonin (Neckameyer et al., 2007d) (ii) *pBac(Ambegaokar et al., 2010)DTRH^{c01440}* (referred to as *pBacTRH*) which is hypomorphic for serotonin throughout the adult CNS, but appears to be a null for serotonin expression in the male specific SAbg neuronal cluster (Neckameyer et al., 2007c) (iii) *fru^{3}/fru^{3}* a null for the Fru^{M} isoform (Billeter et al., 2006b) and (iv) *fru$^{\Delta C}$/fru^{3}* a null for the Fru^{MC} isoform (Billeter et al., 2006a). In both these *fru* mutants, the serotonergic innervation of the ED is not present.

Chemicals

All chemicals were obtained from Sigma, UK. Serotonin and its antagonists were dissolved in HL-3 saline stabilised with 0.1% ascorbic acid to prevent oxidation. Solutions were gently added to the microscope slide and the final dilution was reported.

Results

Upon dissection, the ED is spontaneously active, with peristaltic waves running posteriorly, efficiently moving sperm and seminal proteins along the duct (Fig. 1, Movie S1). The waves appear to start just where the paired vas deferens fuse, and to progress down the ED. The anterior part of this duct is thick-walled and shows a strong, large amplitude rhythm; more posteriorly, the ejaculatory duct is thinner and the rhythm less pronounced. The frequency of the rhythm is very regular, remaining stable for at least 20 minutes (Fig. 1D).

For males kept in mixed-sex vials, the mean frequency of contractions is 0.60 ± 0.22 Hz (± standard error, N=35). To test if this is affected by copulation, we kept males singly in vials (preventing all social interaction), or crowded in male-only vials (preventing mating). These both have a significantly higher rate of ED peristalsis (0.78 ± 0.15, Student's t_{40df}=3.37 P<0.01; 0.71 ± 0.04, t_{42df}=2.56 P<0.02; Fig. 2) than the mixed sex vials,

where mating occurs regularly. To confirm the effect of mating, we allowed isolated males to copulate, examining the ED 5 or 30 minutes afterwards. 5 minutes post-mating there is no change in the frequency of peristalsis, but 30 minutes post-mating it is one-third less (0.52 ± 0.27 Hz, t_{12df}=2.8, P<0.05). This is not significantly different from males kept in mixed-sex vials. Mating has a clear effect on the rate of peristalsis.

The ED is innervated by male-specific serotonergic neurons from the abdominal CNS (Billeter & Goodwin, 2004a;Lee et al., 2001b). We therefore hypothesised that these neurons release serotonin (5-hydroxytryptamine, 5-HT) onto the ED to activate peristalsis. Applying 0.1 μM serotonin increased the frequency of peristaltic waves (mean increase 0.25 ± 0.08 Hz, paired t-test, t_{7df}=3.05, P = 0.018). As little is known of the selectivity of serotonin receptors in adult *Drosophila*, we tested our hypothesis using three serotonergic antagonists, (methiothepin, cyproheptadine, and mianserin) effective in vertebrates and cockroaches (Troppmann *et al.*, 2007). Each of the antagonists reduce the frequency of peristalsis, and the block increases with concentration. Sample recordings from preparation treated with methiothepin are shown in Movies 2 (before) and 3 (with 1 μM methiothepin). At 10 μM (the concentration tested in cockroaches), all three antagonists completely block ED peristalsis (Fig. 3), with block being rapid (<1 minute). Methiothepin has a lower IC50 (0.1 μM) than cyproheptadine (0.7 μM) or mianserin (0.8 μM). The effectiveness of all three antagonists indicates that ED peristalsis is mostly serotonin–dependent *in vitro*.

We also tested whether male-specific abdominal serotonergic neurons are necessary for *in vitro* activity of the ED, using four approaches to reduce or ablate this serotonergic innervation (Fig. 4). We used two types of males which the synthesis of serotonin is restricted: in the *dTRHn-* males serotonin is only synthesised in non-neuronal cells, while in the *pBacTRH* no serotonin is synthesised by the male specific, normally serotonergic abdominal cluster which innervates the ED. We also used two *fru* mutants in which this cluster of cells does not develop. All lines showed wild type levels of ED peristalsis (ANOVA, $F_{4,77df}$=0.58, P=0.68). We infer that, at least *in vitro*, abdominal

neurons are not the source of serotonin and that our bioassay likely reports serotonin in the haemolymph.

Discussion

Our data show that the ejaculatory duct (ED) peristaltic waves occur in the isolated preparation, are serotonin–dependent, and are reduced in frequency after copulation.

The occurrence of the ED peristaltic waves in the isolated preparation suggests that they are myogenic, with a pacemaker site near the anterior end of the duct, at its junction with the vas deferens. Myogenic rhythms have been reported from the female reproductive tract of diverse insects (Middleton *et al.*, 2006;Cook & Peterson, 1989;Orchard & Lange, 1985) as well as from insect viscera, but activity in the male reproductive tract is not well known. Unlike most of the other myogenic rhythms, e.g. the fly oviduct pattern, which occurs in bursts (Middleton *et al.*, 2006), or the locust gut, which shows sporadic contractions (Holman *et al.*, 1991;Oldfield & Huddart, 1982), the fly ED rhythm is very regular.

Myogenic rhythms in insect female reproductive tracts are modulated by a range of neurohormones, including serotonin (Lange, 2004;Messer & Brown, 1995), peptides (e.g. CCAP, (Donini *et al.*, 2001), SchistoFLRF-amide (Lange *et al.*, 1991)) and also by locally released modulators (octopamine, (Middleton *et al.*, 2006), glutamate (Rodriguez-Valentin *et al.*, 2006)). The complete block by serotonin antagonists suggests that this ED rhythm is serotonin-dependent. The innervation of the ED by serotonergic neurons (Billeter & Goodwin, 2004b;Lee et al., 2001a), and the high density of expression of serotonin receptor genes in the male reproductive tract (Chintapalli *et al.*, 2007) suggested the possibility of local serotonin release rather than hormonal action. Although blocking synthesis of serotonin in all neurons (*dTRHn-, pBacTRH* mutants (Neckameyer et al., 2007b)) reduces locomotion, we saw no effect on the ED peristalsis. Similarly, genetic ablation of the ED innervation (fru^3/fru^3, $fru^{\Delta C}/fru^3$ mutants (Billeter et al., 2006c)) does not affect the rhythm, so that we conclude that hormonally-

circulating serotonin (rather than locally released serotonin) is likely to bind to the receptors and remain to activate the ED rhythm *in vitro*. While serotonin is excitatory to the fly ED, in mammals serotonin is generally held to inhibit sexual processes. However, these effects are mainly within the CNS, in the lateral hypothalamic area or the spinal genitourinary tracts (Hull & Dominguez, 2007;Olivier *et al.*, 2006). Nonetheless, there are reports that serotonin also has peripheral effects, increasing the ejaculation of rats (Yonezawa *et al.*, 2005) and the peristaltic rhythm of their vas deferens (Hay & Wadsworth, 1982), which has multiple serotonergic receptors (Kim & Paick, 2004). As SSRIs (selective serotonin reuptake inhibitors) are under trial as a treatment for premature ejaculation (Giuliano & Hellstrom, 2008), an evolutionarily conserved role for serotonin in the male reproductive tract is of substantial interest.

Although post-coital changes in physiology are well documented in the female fly (Ram & Wolfner, 2007a), Galen's observation "Triste est omne animal post coitum, praeter mulierem …" [After copulation every animal feels sad except the woman …] of post-coital tristesse in the male has been less well followed-up. Only from a few insects have post mating changes in behavioural physiology been reported. These include a parasitic wasp, where the change lasts less than 5 min (Fischer & King, 2008), - perhaps due to exhaustion - and noctuid moths (duration ~1day) (Gadenne *et al.*, 2001) or earwigs (Rankin *et al.*, 2009), where the reduction is proposed to allow time for replenishment of sperm and/or sex peptides. In the male fly, the accessory glands do show a post-mating increase in gene expression (Herndon et al., 1997), while a whole body screen indicates changes in gene expression already begin during courtship (Carney, 2007).

Our data, with a decline in peristalsis at 30 rather than 5 minutes suggests that exhaustion is not an explanation (especially given copulation normally lasts ~20 min, (Fowler, 1973)); rather we postulate that the change in serotonin is the result of a physiological cascade, possibly initiated by a female secretion or from a reduction in the pressure/volume of the males' own accessory gland. The roles documented for

amines, in both flies (Dierick & Greenspan, 2007) and vertebrates (Di Giovanni *et al*., 2008), would imply post-mating reduction of male attentiveness and social interactions. Taken together, we suggest that such a reduction in serotonin may be indicative of a wider post-coital coordinated change in male behavioural physiology.

Supplementary Movie Legends

Movie 1: Video micrograph of peristalsis along the ED, showing emission of seminal fluid from the distal end of the duct. Video frame rate increased 3x, and compressed using default Quicktime settings. Same recording, from an isolated male, as Fig. 1A, where the Scalebar is shown.

Movie 2: Recording from a second preparation showing normal peristalsis along the ejaculatory duct. Video speeded up x3; all settings as in Movie 1.

Movie 3: Recording from the same preparation as Movie 2, but in 1 μM methiothepin, showing reduced ejaculatory duct peristalsis. Video speeded up x3; all settings as in Movies 1 & 2.

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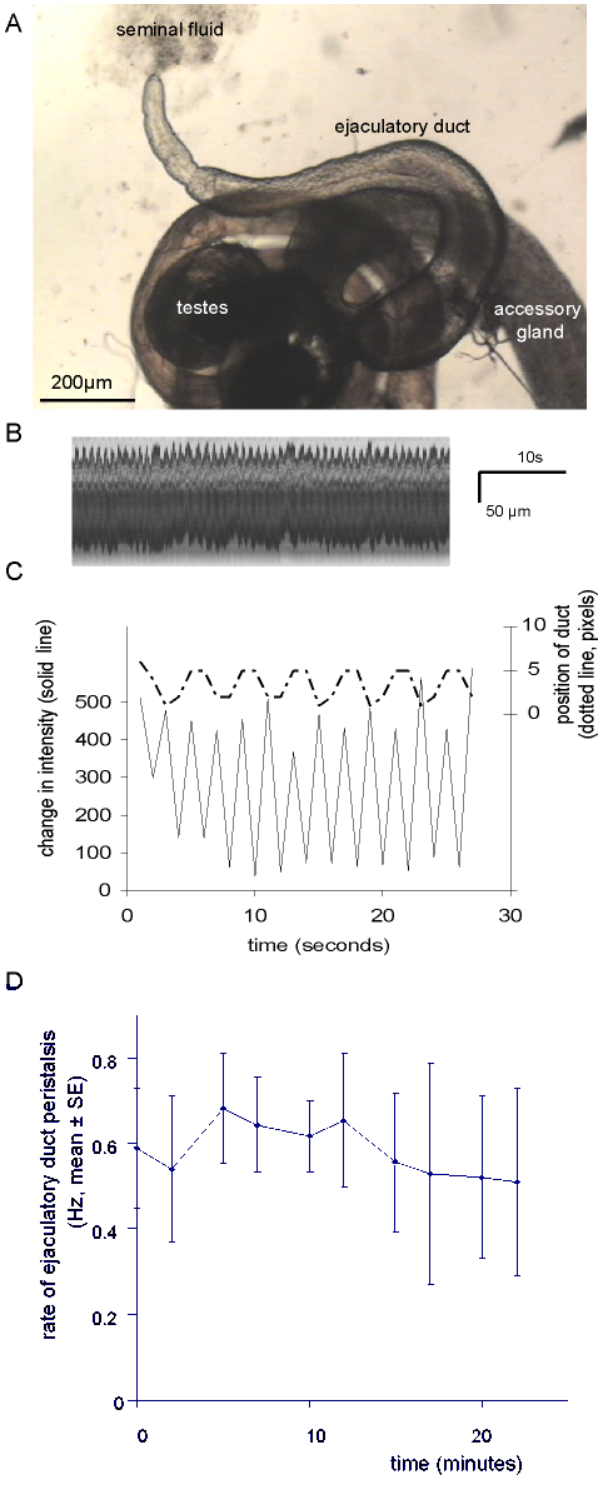

Fig. 1. A. Overview of male *Drosophila* reproductive tract, showing the ejaculatory duct (ED), the coiled testes and accessory gland. Peristaltic waves run down the ED, transporting seminal fluid, expelled in the top left of the frame (see Movie S1). B. A plot of the width of the ED (vertical) against time (horizontal) showing the rhythmic changes in duct diameter. C. Quantitative assay of a vector across the duct, showing both the movement of the edge of the duct (dotted line), and the change in intensity (solid line). Note that the change in intensity signal has a much better signal/noise ratio than the position of the edge of the duct. Both dilation and contraction of the duct results in an increase in the change of intensity (Middleton et al., 2006). D. The ejaculatory duct rhythm persists consistently for 20 minutes, mean ± standard error of 8 preparations.

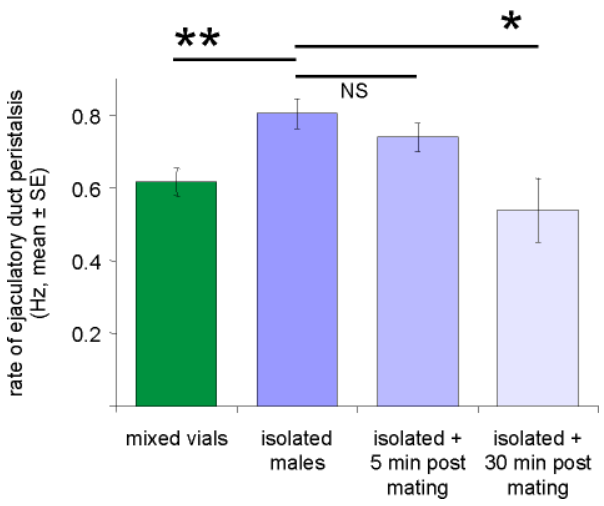

Fig. 2. Mating reduces peristalsis in the ED. Males kept in mixed-sex vials show significantly less contractions than isolated males (**P=0.002). Males kept isolated, mated and then dissected 5 minutes later, show no significant difference from the isolated males, but peristalsis is 33% reduced in isolated males dissected 30 min after mating (*P=0.017).

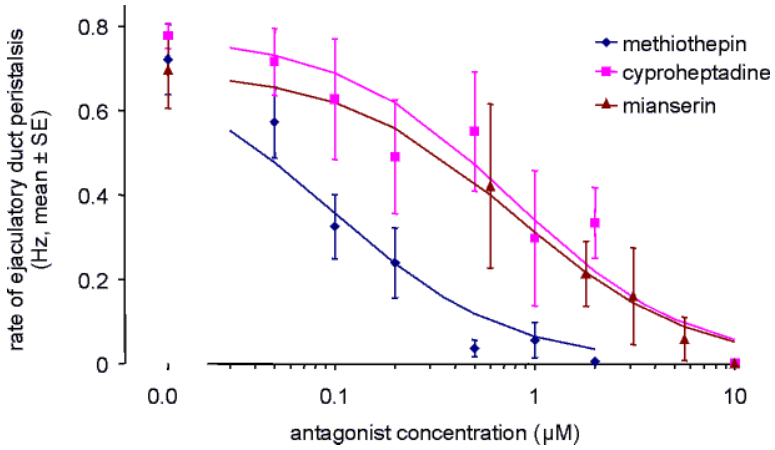

Fig. 3. Rhythmic peristalsis of the ejaculatory duct requires serotonin. Dose response curves for three serotonergic antagonists (methiothepin, cyproheptadine and mianserin), each with an IC50 below 1 µM and showing complete block of the ED contractions by 10 µM.

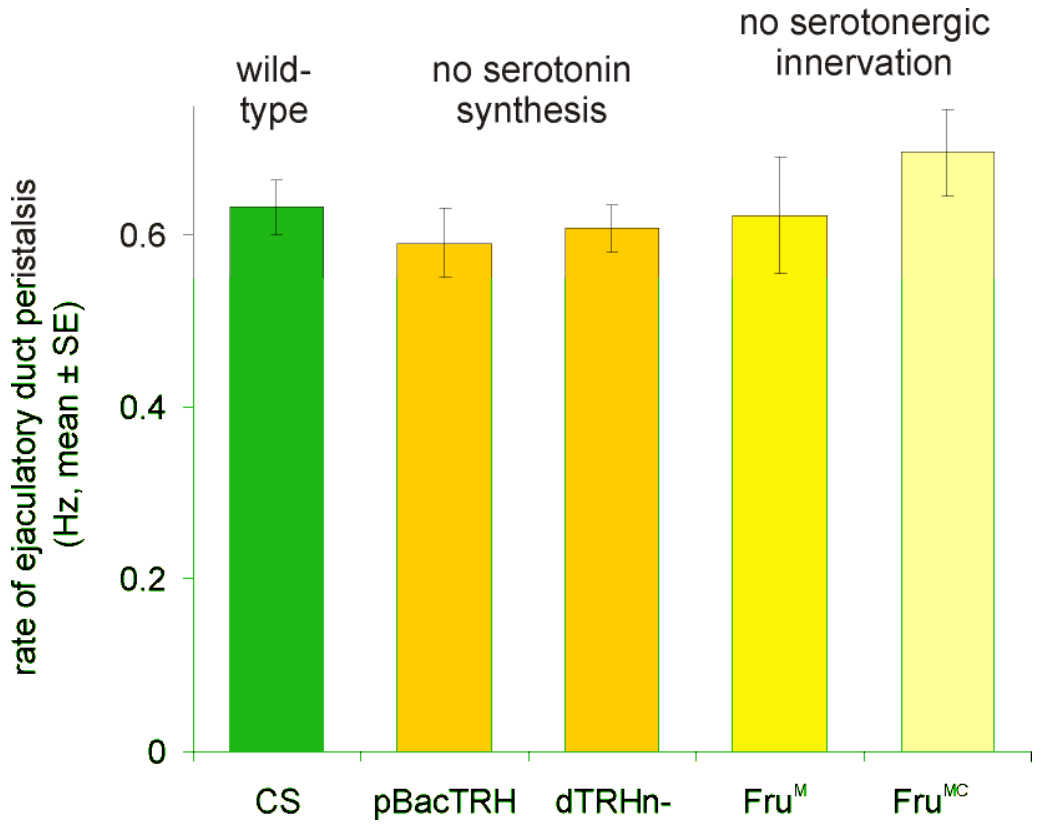

Fig. 4. Reduction of the serotonergic innervation of the ED does not affect peristalsis (ANOVA: $F_{4,80df}$=0.57, P=0.68). The *dTRHn-* line is a null for serotonin synthesis throughout the CNS, though serotonin is synthesised elsewhere, e.g. in the fat bodies (Neckameyer et al., 2007a). The *pBacTRH* line appears to be a null for serotonin in the male specific, normally serotonergic abdominal cluster which innervates the ED. Both *fru* mutants fail to develop the male specific serotonergic neurons, and have no serotonergic neuronal fibres running over the ejaculatory duct. All data from flies 7-10 days old from mixed-sex vials.