Article

THE OCTOPAMINE RECEPTOR octß2R IS ESSENTIAL FOR OVULATION AND FERTILIZATION IN THE FRUIT FLY *Drosophila melanogaster*

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The biogenic monoamine octopamine is essential for ovulation and fertilization in insects. Release of this hormone from neurons in the thoracoabdominal ganglion triggers ovulation and sperm release from the spermathecae. Here we show that the effects of octopamine on ovulation are mediated by at least two different octopamine receptors. In addition to the Oamb receptor that is present in the epithelium of the oviduct, the octβ2R receptor is essential for ovulation and fertilization. Octβ2R is widely expressed in the female reproductive tract. Most prominent is expression in the oviduct muscle and the spermathecae. Animals deficient in expression of the receptor show a severe egg-laying defect. The corresponding females have a much larger ovary that is caused by egg retention in the ovary. Moreover, the very few laid eggs are not fertilized, indicating problems in the process of sperm delivery. We assume that octβ2R acts in a similar way as β2-adrenoreceptors in smooth muscles, were activation of this receptor

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induces an increase in cAMP levels that lead to relaxation of the muscle. Taken together, our findings show that octopaminergic control of ovulation and fertilization is more complex than anticipated and that various receptors located in different cells act together to enable a well-orchestrated activity of the female reproductive system in response to copulation. © *2014 Wiley Periodicals, Inc.*

Keywords: octopamine; ovulation; Drosophila; fertilization

INTRODUCTION

The biogenic monoamines octopamine (OA) and tyramine (TA) act as functional counterparts of epinephrine and norepinephrine in invertebrates. They have widespread neuromodulatory roles in almost all invertebrates studied so far (Roeder, 1999, 2002, 2005). Similar as known for epinephrine and norepinephrine, both monoamines control a great variety of different behaviors and sense organs as well as the performance of various peripheral organs (Roeder, 1999). Among the modulatory effects on peripheral organs, their role in controlling muscle contractions is well documented (Evans, 1981). This modulation is operative in skeletal and visceral muscles. To unravel the different physiological layers of OA and TA activities, fruit flies unable to produce either OA or both monoamines have been highly instructive. *Drosophila* defective in the tyramine-ß-hydroxylase (TßH), the enzyme that converts TA to OA are devoid of OA (Monastirioti et al., 1996). TßH-deficient flies show a complex set of phenotypes, but the most conspicuous one is female sterility. This inability to lay fertilized eggs could be recovered by addition of exogenous OA, indicative for a functional role of OA in this process rather than a developmental impairment that is induced by the OA deficiency (Monastirioti et al., 1996). The female sterility of TßH-deficient animals could be rescued by targeted expression of this enzyme in a subset of octopaminergic neurons of the thoracoabdominal ganglion that innervate the oviduct and the spermatheca further supporting the hypothesis that OA plays a crucial role in the control of ovulation (Monastirioti et al., 1995, Monastirioti, 2003).

Egg production starts in pairwise organized ovarioles and egg maturation occurs during the transport within the ovary. Mature eggs are released from the ovary to the oviduct (ovulation) and finally transported to the uterus, were fertilization occurs, which is followed by egg-laying (Middleton et al., 2006). The entire female reproductive system is under strict hormonal control. A variety of hormones participate in the various control systems acting in different temporal scales. Whereas classical hormones involved in the general control of reproductive processes such as the juvenile hormone and the ecdysteroids act on the longer time frame (Simonet et al., 2004), others directly control, for example, the transport of eggs to and within the oviduct. For the latter control system, two neuroactive compounds, namely, glutamate and OA execute this complex control system. Whereas glutamate induces oviduct contractions, OA inhibits these contractions and leads to relaxation (Rodriguez-Valentin et al., 2006).

The role of OA in controlling various aspects of female reproduction appears to be slightly more complex as it controls various successively induced steps to enable laying of fertilized eggs. OA release increases the strength of peritoneal sheath contractions while the oviduct muscles are relaxed. This combined activity ensures that mature eggs are expelled from the ovaries and that the relaxed oviduct is able to accept the eggs and guide them to the uterus (Middleton et al., 2006). This effect is OA-specific. This complex

modulatory system ensures that ovulation is induced only if sperm is available. Otherwise, nonfertilized eggs would be laid, thereby reducing reproductive fitness dramatically. Thus, only in mated female that have sperm deposited in their spermatheca should ovulation be triggered. During mating, a number of seminal compounds are transferred from males to females including the seminal protein ovulin (Rubinstein and Wolfner, 2013). This protein induces ovulation in females, thus representing the major signal that links mating and ovulation. Ovulin induces the increased peritoneal sheath contractions as well as the decreased tonus of the oviduct muscles in an indirect manner, obviously utilizing the endogenous octopaminergic (OAergic) system of the female fly (Rubinstein and Wolfner, 2013).

In *Drosophila*, the effects of OA are mediated via a set of four different OA receptors that all belong to the G-protein coupled receptor superfamily (Maqueira et al., 2005). Whereas the Oamb receptor shows similarities to mammalian α-adrenergic receptors, the other three (OA2/octß1R, octß2R, octß3R) share substantial similarities with mammalian ß-adrenergic receptors. Oamb is essential for mediating the effects of OA on egg laying, meaning that Oamb-deficient flies phenocopy the OA-deficient ones regarding their female sterility (Lee et al., 2003) Restoring expression of the Oamb receptor in the oviduct of Oamb deficient animals only, could save the egg-laying deficiency (Lee et al., 2009). Thus, Oamb expressed in the oviduct muscle seems to transmit the effects of OA on inducing ovulation in the fly, via activation of Ca^{2+}-signaling in epithelial cells.

Here, we show that another OA receptor, the octß2R, is essential for ovulation but also for fertilization. The receptor is expressed in different parts of the female reproductive organ including the oviduct and the spermatheca. Thus, our work adds a second receptor that allows a better understanding about how OA controls ovulation and fertilization in female *Drosophila* flies. During final preparation of the manuscript, we became aware that a manuscript with similar major findings has been published very recently (Lim et al., 2014).

MATERIAL AND METHODS

Fly Strains

The Octβ2R-deficient strain carries a *piggyBac* transposon (f05679; Bloomington strain 18896) in the fifth exon of the *octβ2R* gene. The control strain was the parental strain w^{1118}. The RNAi line for knockdown of *octβ2R* expression was obtained from the Vienna *Drosophila* Resource Center (104524/KK; VDRC). Octβ2R-GAL4 was created in our lab utilizing the pPTGAL vector containing the presumptive promoter region of the *octβ2R* gene (2.5 kb upstream of the transcriptional start). The *tβh*-deficient line has already been described (Monastirioti et al., 1996) and was generously provided by Henrike Scholz (Cologne, Germany). Other transgenic stocks including tubP-GAL4, nsyb-GAL4, Mef2-GAL4, and UAS-mCD8-GFP were obtained from the Bloomington Stock Center. All stocks were raised at 25°C on standard yeast-cornmeal medium with 50–60% relative humidity under a 12 h light/dark cycle (Hoffmann et al., 2013).

Copulation Test

The test was performed in the morning at a temperature of 25°C. Single virgin females, 1 to 5 days old, were placed together with two wild type w^{1118} males in a mating chamber

(2 cm in height, 1.5 cm in diameter) supplied with a wetted piece of filter paper. The flies were allowed to recover for 10 min. Copulation was scored every 5 min and the number of copulations within 1 h was recorded. At least 20 virgin females were assayed for each genotype. Statistical analysis was performed using unpaired *t*-test with the Prism 6.0 program package.

Egg Laying Assay

Mated females collected from larger copulation setups were shifted to fresh vials. Five females in one vial were fed with yeast paste at 25°C and the number of laid eggs was counted every 24 h. At least four vials per genotype were analyzed. Statistical analyses were performed using GraphPad Prism Software 6.0 using ANOVA and the unpaired two-tailed Student's *t*-test. All data are presented as mean values ± SEM. For the RNAi-experiments, the number of laid eggs per day was counted.

Quantification of Ovary Sizes

Ovaries of mated females were prepared under the stereomicroscope. Ovaries of 1 day old females and those of 15 day old females were analyzed. Pictures of ovaries were taken and the areas of individual ovaries were measured using the Cell sense program package (Olympus, Hamburg, Germany). Statistical analyses were performed using GraphPad Prism Software 6.0 using ANOVA and the unpaired two-tailed Student's *t*-test. All data are presented as mean values ± SEM.

DAPI Staining

Eggs were collected from the surface of the medium within 2 h after oviposition, washed with PBS and fixed in 4% paraformaldehyde for 5 min. After washing, the eggs were mounted on slides and stained with DAPI (Carl Roth GmbH, Karlsruhe, Germany) for 10 min before observation with epifluorescence microscopy (Olympus, Hamburg, Germany).

Immunohistochemistry

To identify the sites of expression the *octβ2R*-Gal4 line was crossed with a UAS-*gfp* line and the F1-generation of this cross was analyzed (Brand and Perrimon, 1993). Female reproductive system were dissected in PBS under microscope, fixed in 4% paraformaldehyde for 30 min at room temperature, and washed three times in PBT (0.3% Triton X-100). After blocking for 30 min in buffer (1 × PBS + 1% Triton X-100 + 10% goat serum), the specimens were incubated in primary antibody (rat anti-GFP; 1:500 in blocking buffer) overnight at 4°C, after washing three times, the secondary antibody was applied (donkey anti rat IgG; 1:500 DyLight 488). The tissue was directly mounted on a slide with one drop of FocusClear (CelExplorer) medium. Each image was photographed and analyzed by using a Carl Zeiss HPX120 microscope (Zeiss, Göttingen, Germany) and Zeiss AxioVision 4.8 software (Rahn et al., 2013).

RNA Extraction, Reverse Transcription, and qPCR

Adult female reproductive systems were dissected in Hemolymph-like saline buffer (HL3; 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM

Trehalose, 115 mm Sucrose, 5 mM HEPES (pH 7.1)). They were divided into the following parts: ovaries, oviduct, spermatheca, uterus/seminal receptacle. Total RNA was extracted using RNAmagic (Bio-Budget technologies, Krefeld, Germany) using the Bead Ruptor system (BioLab Products, Bebensee, Germany). The isolated RNA was free from contaminating DNA. cDNA-synthesis, and PCR was essentially performed as already described (Faisal et al., 2014). In brief, cDNA-synthesis was performed at 50°C for 60 min using a conventional oligo(dT) primer and SuperScript III reverse transcriptase (Life technologies, Darmstadt, Germany). PCR amplification was carried out using TaqDNA polymerase (Life technologies, Darmstadt, Germany) in a total volume of 25 µl and the gene specific primers were used for PCR: OAMB (forward 5′-CGG TTA ACG CCA GCA AGT G-3′; reverse 5′-AAG CTG CAC GAA ATA GCT GC-3′), OA2 (forward 5′-GGC AAC GAG TAA CGG TTT GG-3′; reverse 5′-TCA TGG TAA TGG TCA CGG GC-3′), Octβ2R (forward 5′-TCC TGT GGT ACA CAC TCT CCA-3′; reverse 5′-CCA CCA ATT GCA GAA CAG GC-3′), Octb3R (forward 5′-TGT GGT CAA CAA GGC CTA CG-3′; reverse 5′-GTG TTC GGC GCT GTT AAG GA-3′), and rpl32 (forward 5′-CCG CTT CAA GGG ACA GTA TC-3′; reverse 5′-GAC AAT CTC CTT GCG CTT CT-3′).

RESULTS

To characterize the contribution of different OA receptors to the phenotypes observed in OA-deficient flies, we used a set of flies impaired in the expression in one of the four different OA receptors (Oamb, Oa2/octß1R, octß1R, octß1R). Analogous to OAMB-deficient flies that were almost unable to lay eggs, *octβ2R*-deficient animals showed a very similar phenotype, being unable to lay fertilized eggs. To quantify this effect, we set up groups of five females each together with the corresponding males and counted the number of eggs laid per day. The w^{1118} strain served as the control (Fig. 1A). Whereas w^{1118} flies showed a high level of egg laying with a maximum at day 3, the *octβ2R*-deficient flies showed only negligible egg-laying (Fig. 1A). This effect was female specific as we observed the same result if females of the *octβ2R*-deficient flies were crossed to w^{1118} males, while crossing of *octβ2R*-deficient males with w^{1118} deficient males gave normal egg-laying rates. We determined whether mating was impaired and used cohorts of males and females to quantify the number of copulations. Whereas w^{1118} and *tβh*-deficient flies show almost identical copulation rates, the *octβ2R*-deficient flies showed a strong delay in the copulation rates (Fig. 1B). The very few eggs that were laid in the *octβ2R*-deficient animals always failed to develop, because they were not fertilized (Fig. 1C, D). Eggs of *octβ2R*-deficient animals show after DAPI-staining only a single large nucleus (Fig. 1C, arrow), which is typically seen in nonfertilized eggs (Ejima et al., 2004), whereas eggs from control animals show a complex pattern of DAPI-positive signals as seen in a developing embryo (Fig. 1D).

To identify the underlying reason for this phenotype, we analyzed the ovaries of control and *octβ2R*-deficient flies. Ovaries of the latter ones are much larger than those of control animals (Fig. 2). This holds true for very young (1D; Fig. 2A–D, G) as well as for older females (15d; 2E, F, H). Usually, eggs are organized in the ovary in rows of maximal two eggs (Fig. 2D). In the *octβ2R*-deficient animals we observed up to five eggs in a row (Fig. 2C), indicative of severe problems with the process of ovulation. A quantitative evaluation of the different ovary sizes was performed using photographs taken from isolated ovaries followed by quantification of the corresponding areas. Both, for 1 day old

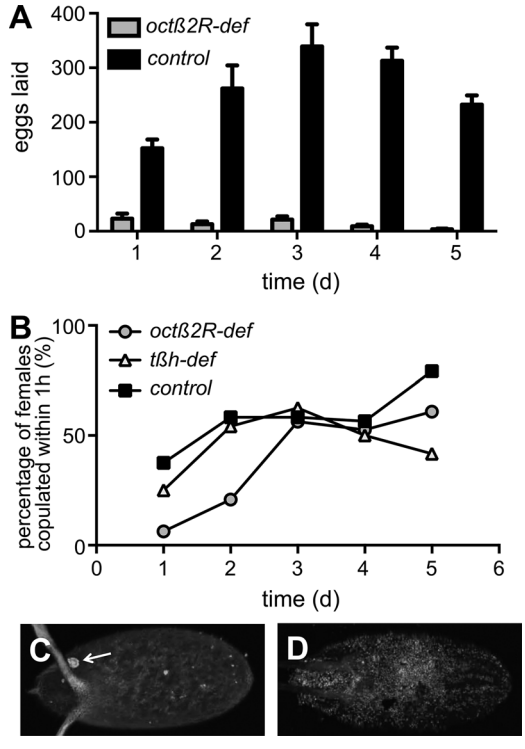

Figure 1. *Octβ2R*-deficient animals have a reduced ovulation rate and show a delayed copulation. Flies deficient in the *octβ2R* (gray bars) show a reduced egg-laying rate (A) if compared with that of the wild-type control (black bars). All values are statistically different between the *octβ2R*-deficent animals and the matching controls ($N = 10$; $P < 0.0001$). The number of copulations (B) was scored in *octβ2R*-deficient animals (gray circles), tyrosine-ß-hydroxylase-deficient flies (gray triangles) and control animals (black squares). Females of the corresponding genotype were confronted with wild-type males. DAPI-staining of *octβ2R*-deficient animals revealed only a single positive signal (C, arrow), while that of control animals showed a complex pattern (D).

females (Fig. 2G) as well as for 15 day old females (Fig. 2H), these differences are highly significant ($P < 0.0001$).

In the next step, we aimed to elucidate the expression pattern of the *octβ2R* within the female reproductive organ. For this, we produced a Gal4-line containing the presumptive *octβ2R*-promoter. Crossing this line with a UAS-*gfp* line utilizing the binary Gal4/UAS-system allowed us to visualize the expression sites of the receptor gene within the female reproductive organ (Fig. 3A). Expression is seen in the oviduct, but also in other structures including the spermatheca (Fig. 3B). To obtain further information about the expression in these different parts of the female reproductive organ, we performed RT-PCR experiments, with all four different OA receptor genes (Oamb, Oa2, octß2R, octß3R). Thus, we manually isolated the female reproductive system of young virgin females and of older mated females and dissected different parts including the ovary, the oviduct, the spermatheca, and the uterus/seminal receptacle to isolate RNA usable for cDNA synthesis. As a loading control the rpl32 gene was employed (Fig. 3C top). All four different OA receptors were tested. Except the *Oa2* receptor that is almost not present in the female reproductive organ, all others show a relatively broad expression in the different parts of this structure (Fig. 3C). While the Oamb and the *octβ2R* show very strong signals in

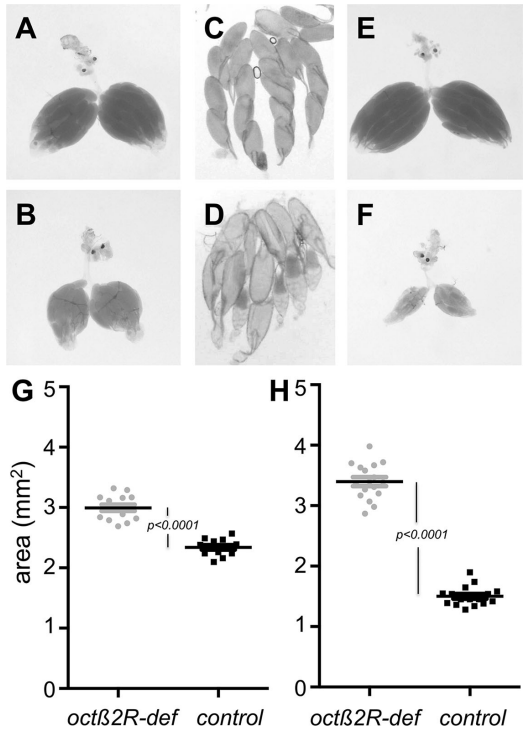

Figure 2. The ovaries of *octβ2R*-deficient flies are significantly larger than those of control females. Ovaries of *octβ2R*-deficient 1 day old females (A) are larger than those of control females (B) of the same age. Ovaries of 15 day old *octβ2R*-deficient females (E) and ovaries from females of the same age are also shown (F). Rows of ovaries containing eggs of *octβ2R* (C) and control animals are displayed (D). To quantify this effect, the area taken by the ovaries was measured (C, $N > 8$, $P < 0.0001$) from 1 day old females (G) and 15 day old females (H).

the oviduct, expression levels of the *octβ3R* are slightly lower. Regarding the *octβ2R*, the broad presence in the different parts of the female reproductive system is obvious, with maximal expression in the oviduct and in the spermatheca (Fig. 3C).

To further dissect the effects the role of the *octβ2R* in the process of ovulation, we used RNAi targeted against the corresponding gene. Using three different Gal4-lines driving expression into different organs, we could identify the site of *octβ2R* action for OA control of ovulation. Using the tubulin-promoter Gal4 line, that drives expression in most organs of the fly, we observed a downregulation of the ovulation rate by approximately 80% (Fig. 4). On the other hand, targeting the RNAi-effect to the nervous system with the nsyb-Gal4 driver had no effect on the ovulation rate. On the other hand, using the muscle-specific mef2-Gal4 driver to decrease the expression of the *octβ2R* resulted in a significant decrease of the ovulation rate indicating that expression of this receptor in the oviduct muscle is responsible for transmitting the effects on ovulation (Fig. 4).

DISCUSSION

Our experiments extend previous knowledge about the neurohormonal control of ovulation and fertilization. To maximize successful reproduction, the activities of the ovary,

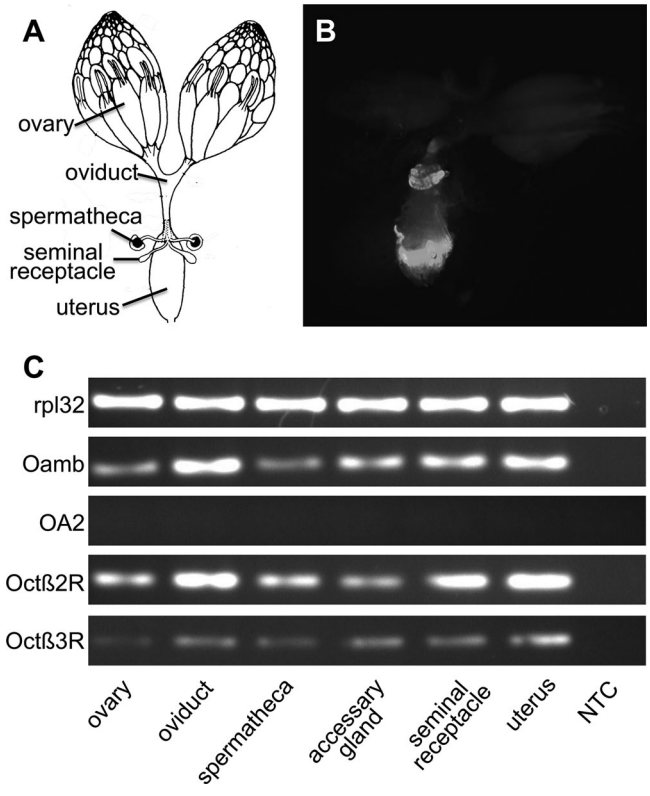

Figure 3. Expression analysis of octopamine receptors in the female reproductive system. The female reproductive organ consists of different regions including the ovary, the oviduct, the spermatheca, the seminal receptacle, and the uterus (A, modified after (Middleton et al., 2006). The *octß2R*-promoter-Gal4 line (crossed to UAS-gfp) revealed expression in different parts of the female reproductive organ (B). Reverse transcriptase-PCR analysis of expression of the different OA receptors in the different substructures of the female reproductive organ (C). Rpl32 is used as a loading control. NTC means no template control.

the oviduct, and sperm storage organs have to be highly coordinated with the availability of sperm (Sun and Spradling, 2013). In insects, OA, the invertebrate equivalent of epinephrine and norepinephrine is central to this complex neurohormonal control system (Monastirioti et al., 1996). OA containing neurons in the thoracoabdominal ganglion innervates all major parts of the female reproductive organ, including the ovary, the oviduct and the spermatheca (Lange and da Silva, 2007; Monastirioti, 2003). Its essential role for successful ovulation has already been shown utilizing OA-deficient animals. Moreover, restoring the ability to produce OA in neurons only was sufficient to rescue the egg-laying phenotype. Locally, within the female reproductive organ, the OA receptor Oamb is essential for this process. It is present in the epithelial cells to produce secretions that are required for ovulation upon stimulation with OA (Lee et al., 2003, 2009). In the current project, we show that the octopaminergic control of egg-laying is more complex than previously anticipated. A second OA receptor, the *octß2R* is essential for transmitting another component in the well-orchestrated process of ovulation. In contrast to the Oamb receptor, whose activation induces a rise in cytosolic Ca^{2+}-levels, octß2R activation leads to an increase of cAMP in all cells expressing this receptor. Increasing the levels of cAMP is known to induce relaxation in vertebrate smooth muscles (Kotlikoff and Kamm, 1996;

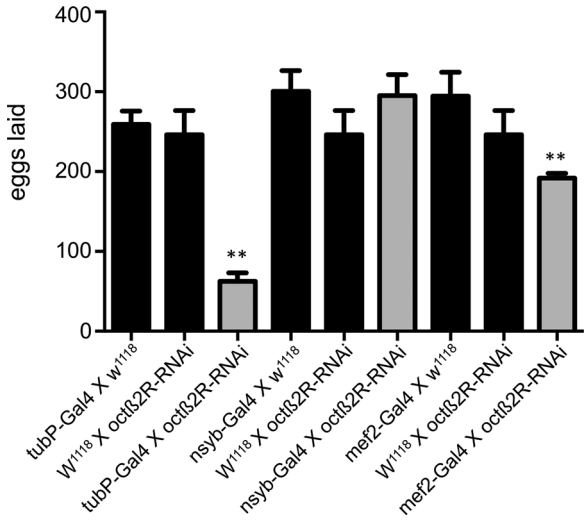

Figure 4. RNAi-mediated gene silencing of the *octß2R* revealed that visceral muscles as their site of action. Egg-laying was quantified in different RNAi experiments. Controls (black bars) are parental lines crossed to the genetic background of all RNAi liners (w^{1118}). Egg-laying was quantified in groups of five females and listed as eggs laid per day ($P < 0.005$).

Tanaka et al., 2005). Activation of ß2-adrenergic receptors in smooth muscles controlling the lumen of arteries or bronchi induces exactly this relaxation. Mechanistically, the link between cAMP level increase and muscle relaxation should involve a Ca^{2+}-downregulation leading to an inhibition of Ca^{2+}/calmodulin mediated signaling and activity of the myosin light chain kinase or it involves regulation of K^{+}-channels such as the MaxiK channel (Kotlikoff and Kamm, 1996; Tanaka et al., 2005). Following the model proposed by Lee et al. (Lee et al., 2009), these seemingly contra-productive activities, increasing Ca^{2+}-mediated signaling via the Oamb, while the octß2R does the opposite, may nevertheless come together, in the case that both receptors are expressed in different cells within the oviduct system. *Oamb* is supposed to be expressed in the epithelial cells were it induces fluid secretion, which is essential for ovulation. The *octß2R* on the other hand is expressed in the visceral oviduct muscle, where it induces relaxation via increase in cAMP levels. OA-induced relaxation of the oviduct muscle appears to be mediated via the octß2R. Moreover, it might be operative in all insects, as locusts show a very similar type of OA action on contraction output of the oviduct muscles (Lange and Orchard, 1986; Orchard and Lange, 1986; Nykamp and Lange, 2000). The role of octß2R in the control of the female reproductive system is not confined to the oviduct muscles. We observed very high expression levels also in other parts of the female reproductive tract, most notably in the ovaries and, at even higher levels, in the spermatheca. Especially the expression in the spermatheca is of interest, as this might explain the lack of fertilization observed in the few eggs that are laid in the *octß2R*-deficient animals. In locusts, the OA/TAergic control of the spermatheca is essential for successful fertilization, which may thus also be the case in the fly (Lange and da Silva, 2007).

Thus, our results imply that OAergic neurohormonal signaling orchestrates a very complex processor in the reproductive system that ensures that ovulation and fertilization are well-coordinated. For this, copulation is the primary signal that triggers release of OA from neurons located in the thoracoabdominal ganglion. As mentioned above, this

induces contraction/relaxation of the ovary/oviduct muscles mediated via octß2R as well as the release of secretions mediated by the Oamb. Moreover, an OA signal initiated in an identical temporal situation targets the spermatheca. This signal induces contraction of the spermatheca, which expels sperms into the uterus to ensure proper fertilization. The role of the third OA receptor in this organ, the octß3R is completely unknown, although it is expressed in all major parts of the female reproductive system.

Although, OA is obviously of central importance for controlling ovulation and fertilization, other hormonal signals also influence this complex process. These other hormones act at different temporal scales, where they are involved in e.g. maturation of the female reproductive system (Simonet et al., 2004). The role of other hormones directly acting on the oviduct, especially that of the diverse set of peptide hormones is much less understood.

Early studies utilizing mammals revealed a surprisingly high degree of similarities regarding the architecture of adrenergic control of ovulation. In rats, application of adrenergic agonists was able to increase ovulation in rats, while that of adrenergic antagonists had the opposite effect (Kannisto et al., 1985).

Taken together, our results imply that the octß2R is an essential part of the OAergic control system required to temporarily connect ovulation and fertilization in female *Drosophila*. Moreover, taken the similar signaling properties as ß-adrenergic receptors into account, this system might be very useful as a model for smooth muscle control by aminergic compounds in the future.

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