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Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in *Drosophila melanogaster*

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Abstract

Octopamine is an important neuroactive substance that modulates several physiological functions and behaviors of invertebrate species. Its biosynthesis involves two steps, one of which is catalyzed by Tyramine β-hydroxylase enzyme (TBH). The *Tβh* gene has been previously cloned from *Drosophila melanogaster*, and null mutations have been generated resulting in octopamine-less flies that show profound female sterility. Here, I show that ovulation process is defective in the mutant females resulting in blockage of mature oocytes within the ovaries. The phenotype is conditionally rescued by expressing a *Tβh* cDNA under the control of a *hsp70* promoter in adult females. Fertility of the mutant females is also restored when TBH is expressed, via the GAL4-UAS system, in cells of the CNS abdominal ganglion that express TBH and produce octopamine. This neuronal population differs from the dopamine- and serotonin-expressing cells indicating distinct patterns of expression and function of the three substances in the region. Finally, I demonstrate that these TBH-expressing cells project to the periphery where they innervate the ovaries and the oviducts of the reproductive system. The above results point to a neuronal focus that can synthesize and release octopamine in specific sites of the female reproductive system where the amine is required to trigger ovulation.

Keywords: Octopamine; TBH-immunohistochemistry; GAL4-UAS rescue; Oviduct innervation; *Drosophila*; Female sterility; Ovulation; Abdominal ganglion; Nervous system

Introduction

The biogenic amine Octopamine acts as a neurotransmitter, a neuromodulator, and a neurohormone in invertebrates (reviewed in Roeder, 1999). It is considered to be the functional homologue of noradrenaline in these species, and it affects several aspects of their physiology and behavior by altering neuronal activity at central and peripheral targets. In insects, the function of octopamine has been associated with processes such as neuromuscular transmission in locusts (Malamud et al., 1988; Whim and Evans, 1988; Walther and Zittlau, 1998), feeding and sting behavior in bees (Braun and Bicker, 1992; reviewed in Bicker and Menzel, 1989; Burrell and Smith, 1995), phototaxis and learning in bees and fruit flies (Dudai et al., 1987; Hammer and Menzel, 1998 and references therein), and modulation of reproductive organs (Orchard and Lange, 1985; Clark and Lange, 2003). The physiological role of octopamine is considered to be restricted to invertebrates, thus studies on octopamine regulated processes, besides their inherent scientific importance, may also lead to the development of advanced pest control strategies.

Earlier studies reported on the development of a model system in *Drosophila* to study octopamine's requirement in physiological functions and behaviors in insects. Specific neuronal cell populations containing octopamine were mapped in larval and adult CNS, and sites of octopaminergic innervation in the periphery were also identified (e.g., specific neuromuscular junctions) (Monastirioti et al., 1995). Moreover, the molecular genetic approach was undertaken, the gene coding for Tyramine β-hydroxylase (TBH) enzyme in the octopamine biosynthetic pathway was isolated, and mutations in the respective locus that resulted in octopamine-less flies were generated ($T\beta h^{nM18}$) (Monastirioti et al., 1996). The octopamine-less flies survive to

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adulthood, have normal external morphology, but they exhibit defects when tested in different behavioral or physiological assays. The development of ethanol tolerance is impaired in the mutants (Scholz et al., 2000), while they also show reduced aggressive behavior (Baier et al., 2002) and alterations in stress reactivity (Gruntenko et al., 2000). Moreover, the mutant flies were recently shown to have sugar learning defects implicating octopamine in acquisition of sugar memory in *Drosophila* (Schwaerzel et al., 2003). A very profound phenotype that has been discussed in an earlier report (Monastirioti et al., 1996) involves female fecundity. Mutant females, although they mate, retain their eggs exhibiting characteristic swollen abdomens. This defect is functionally connected to the octopamine deficit as it can be rescued by feeding the flies on octopamine supplemented food.

There is not much known about the developmental and molecular mechanisms that underlie the hormonal and neuronal control of the egg laying process in *Drosophila*. Juvenile Hormone and Ecdysone are implicated as the major hormones in the control of vitellogenesis and oocyte maturation by regulating yolk protein synthesis and uptake into the ovary (Soller et al., 1999; Buszczak et al., 1999). Egg laying can also be stimulated by male-specific peptides (Acps) that are transferred to the females by copulation (Chen et al., 1988; Aigaki et al., 1991; Herndon and Wolfner, 1995; reviewed in Wolfner, 1997). On the other hand, either the innervation of the female reproductive system or its function in response to neural signals is poorly understood. An egg laying defect is one of the phenotypes observed in mutants of the *dissatisfaction* gene, and this defect has been correlated to the absence of motor neuronal innervation on uterine muscles (Finley et al., 1997). However, specific neurons or neuronal populations with particular neurochemical properties responsible for this innervation have not been identified.

The egg retention phenotype of the $T\beta h^{nM18}$ mutant flies that is correlated with the absence of octopamine implicated for the first time an important neuroactive molecule in the egg laying process (Monastirioti et al., 1996). The mutants and the molecular genetics of the *Tβh* gene offer useful tools in the investigation of the neuronal networks that affect female reproduction as well as of the octopamine requirement in this process. In this report, I show that the $T\beta h^{nM18}$ mutant females are defective in ovulation, and I provide rescue of this phenotype by conditionally expressing the *Tβh* gene in the adult females under the control of the heatshock 70 promoter. By immunohistochemical analysis, I also show that TBH/octopamine-expressing cells that reside in the abdominal ganglion of the thoracic CNS innervate the ovaries and oviducts, the sites that are presumably not functioning properly in the $T\beta h^{nM18}$ nonovulating females. Moreover, I was able to correlate these specific octopaminergic neurons with the ovulation defect by monitoring rescue of the mutant phenotype, when *Tβh* expression is targeted to this neuronal subpopulation. These data identify a cell focus of defined chemical specificity that innervates the female reproductive system and point to octopamine as a key neurochemical factor for ovulation in *Drosophila*.

Materials and methods

Preparation of ovaries for oocyte staging

Ovary dissections were performed from $T\beta h^{nM18}$ and Canton-S females of same age. Virgin females were collected in the evening of the day of hatching, from vials that were emptied in the morning of that day, and were kept in vials supplemented with freshly prepared living yeast for 5 days before mating. Ovaries were dissected 24 h later in standard PBS solution, fixed in 4% paraformaldehyde, washed in PBS, and mounted in 80% glycerol in PBS. The ovarioles of each ovary pair were spread out, and the stages of oocytes were determined according to King (1970).

Heat shock sterility rescue

Generation of strains bearing a transgene of the heatshock inducible form of the *Tβh* gene (*hsp-Tβh*) are described elsewhere (Schwarzel et al., 2003). For the heat shock rescue, 1- to 2-day-old homozygous mutant virgin females bearing 2 copies of the *hsp-Tbh* transgene in the 3rd chromosome ($T\beta h^{nM18}/T\beta h^{nM18}$; *hsp-Tβh/hsp-Tβh*) were mated, in mass (5–10/vial), to Canton-S males for 3 days. They were then transfered to a new vial and were given 1-h heat shock at 37°C for 1 or 2 times spaced by 3 h. Egg laying was monitored 24 h after the last heat shock. In the case of one heat shock, egg laying of transgene bearing mutant females was also monitored 48 and 72 h after heatshock. Same procedure was followed for the control Canton-S females, egg laying of which was monitored for a 24-h period with or without heatshock. Approximately 20–30 females were tested in each experiment. A total of 66 Canton-S females (36 and 30 with or without heatshock, respectively) and 148 of mutant females bearing the *hsp-Tbh* transgene (35 for the two heatshocks) were tested. The number of eggs per female was calculated as the total number of eggs produced by the total number of flies assayed.

Immunohistochemistry

Adult thoracic CNS's from experimental females joined to internal genitalia (ovaries, oviducts, uterus, spermathecae) with intact abdominal nerves were dissected in standard PBS and processed for TBH immunohistochemistry (Monastirioti et al., 1996) and DAB staining using an anti-rat HRP-conjugated secondary antibody (Jackson Immu-

noResearch Labs, West Grove, PA) diluted 1:100 in PBT. For the double labeling experiments, dissected adult female thoracic CNS's were fixed in PBS plus 4% paraformaldehyde for 1 h at room temperature. Incubations for each of the primary antibodies were performed at 4°C O/N followed by 6 h incubation at room temperature with the respective secondary antibody. As primary antibodies, I used the anti-TBH rat antibody (1:25 in PBT) (Monastirioti et al., 1996), and anti-β-galactosidase rabbit antiserum (1:4000 in PBT) (Cappel). As secondary antibodies, I used a-rat FITC conjugated (1:100 in PBT) and a-rabbit Cy3-conjugated (1:1000 in PBT) from Jackson ImmunoResearch Labs, West Grove, PA). At least five samples from each genotype were examined. Transmitted light images were obtained on a Leica Diaplan microscope. Fluorescent samples were observed by using a Leica SP confocal microscope and sections, 1.2–1.5 μm each, of the CNS abdominal ganglion region that includes all TBH and β-galactosidase-expressing cells in each line were taken. Projections were made from sections that include neurons which coexpress both antigens, as in *c164-GAL4* (6 of 20/1.2 μm), *OK348-GAL4* (4 of 9/1.5 μm), and *ptc-GAL4* (5 of 11/1.5 μm) or neurons expressing either TBH or β-gal antigens as in *Ddc-GAL4* (7 of 13/1.2 μm).

Plasmid constructions and fly transformation

The construction of the *hsp-Tβh* transgene that has the *Tβh* cDNA (Monastirioti et al., 1996) under the control of the *hsp70* promoter is described in Schwaerzel et al. (2003). The *UAS-Tβh* transgene was generated by cloning a 3.0-kb *Eco*RI fragment that contains the *Tβh* cDNA, downstream of the UAS sequences at the *Eco*RI polylinker site of the pUAST fly transformation vector (Brand and Perrimon, 1993). DNA of the plasmid was used to transform yw^{67c23} embryos and independent insert-bearing transformant lines were isolated by standard genetics.

Fly stocks and genetics

The $T\beta h^{nM18}$ mutation has been isolated in a P-excision screening of a P-element inserted in the *Tβh* locus (Monastirioti et al., 1996). It represents a small deletion in the ATG region of the *Tβh* gene (unpublished data) and it is kept balanced with the *FM6l* chromosome. $T\beta h^{nM18}$*/FM6l* stocks carrying different insertions of a *UAS-Tβh* transgene or the enhancer detection-GAL4 insertions over balancers were generated by standard genetics. For the GAL4-UAS rescue experiments, two different UAS lines (UAS32, UAS15), bearing the *UAS-Tβh* insert on the 2nd and 3rd chromosome, respectively, were tested. The following GAL4-expressing lines were used: *c164-GAL4* (Torroja et al., 1999), *OK348-GAL4* (Conolly et al., 1996), *ptc-GAL4* (Wilder and Perrimon, 1995), *79B-GAL4* (R. Greenspan, unpublished), and *Ddc-GAL4* (Li et al., 2000).

Virgins $T\beta h^{nM18}$*/FM6l; enhancer-GAL4/Balancer* were mated to males $T\beta h^{nM18}$*/Y; UAS-Tβh/Balancer*. Female progeny of the different genotypes resulting from the cross were tested for fertility in mass or in single fly experiments. Numbers of females that were tested from each genotype are: 52 for *c164-GAL4*, 59 for *OK348-GAL4*, 47 for *ptc-GAL4*, 35 for *79B-GAL4* or *Ddc-GAL4*. Fertility assays were done at 25°C as described in Monastirioti et al. (1996). For the double labeling experiments, males from each of the enhancer-GAL4-expressing lines were mated to virgins from the *UAS-LacZ* strain (Brand and Perimmon, 1993) and thoracic CNS's from the female progeny were processed for TBH and β-galactosidase immunohistochemistry.

Results

Octopamine-less flies are defective in ovulation

Flies in which octopamine biosynthesis is blocked have been previously generated via null mutations at the *Tβh* locus ($T\beta h^{nM18}$). Females homozygous for these mutations, although they normally mate, don't deposit eggs (Monastirioti et al., 1996). They all exhibit characteristic swollen abdomens with ovaries full of late stage oocytes that remain so even when the flies are very old (at least 20 days old) (Fig. 1A and B). As $T\beta h^{nM18}$ virgin females do not deposit any eggs as well, in contrast to what the wild type virgins do, the defect must be attributed to an endogenous physiological problem that has been induced by the absence of octopamine in these mutants.

In order to get insights into the sterility phenotype of the octopamine-less females, I dissected their reproductive system and I monitored the oocyte stages contained into their ovaries. Stage 14 oocytes were found in the ovaries of all the mature mated females dissected, suggesting that egg development proceeds normally. Moreover, ovarioles of these females contained four to five stage 14 oocytes, instead of the normal number of one to two, while oocytes between stage 7 and 13 were essentially missing (Fig. 1C), a characteristic of "backed-up" ovarioles that are generated when egg laying is inhibited.

Two different steps of the egg laying process can be defective in the mutant flies: the passage of eggs from ovaries to oviducts (ovulation) or the extrusion of the eggs from the uterus to the outside through the ovipositor (oviposition). The mutant females extrude their ovipositor and contract their abdomen; however, they never extrude an egg even when the tip of their abdomen is mechanically squeezed, suggesting that no egg ever resides into their uterus. Indeed, in all dissections performed, in more than 40 mutant females, I never detected any egg in the lower genital tract (i.e., uterus or ovipositor). On the contrary, many stage 14 oocytes were detected in the ovaries and in 2 cases in the ovary calyx area. All the above observations lead to the conclusion that the sterility deficit of the octopamine-less flies is rather in the ovulation step of the egg laying process than in the development or oviposition of the oocytes.

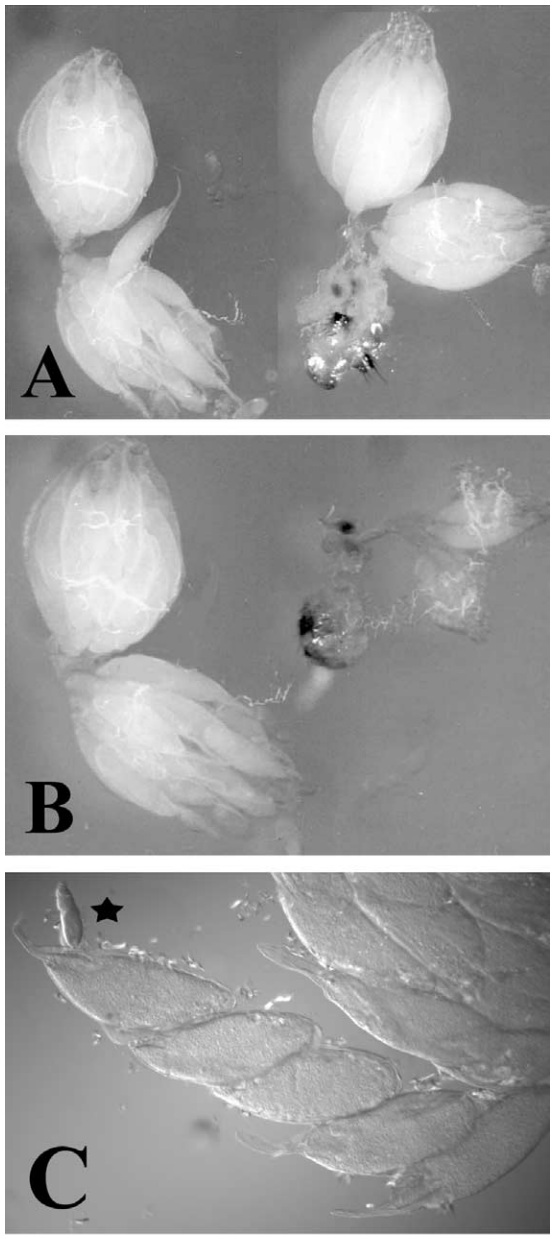

Fig. 1. Photographs of ovaries from $T\beta h^{nM18}$ mutant (left in A and B) and wild-type (Canton-S) (right in A and B.) females. (A) Ovaries from mature (6 days old) females bearing late stage oocytes. (B) Ovaries from aged (>20 days old) females. Note that the mutant ovaries are still full of mature oocytes, while wild type ovaries have emptied their contents. Pictures were taken from a stereomicroscope with a 5× magnification lens. (C) Photomicrograph of a dissected ovariole from a $T\beta h^{nM18}$ mutant ovary. At least four oocytes with chorion appendages characteristic of late stage oocytes are included. Asterisk indicates the presence of early stage oocytes in this ovariole, while the intermediate stages are missing.

Conditional rescue of the sterility defect of octopamine less flies

We have previously shown that the sterility phenotype of the $T\beta h^{nM18}$ females is due to their octopamine deficit and it is attributed to changes in the genomic region of the *Tβh* locus (Monastirioti et al., 1996). In this study, I confirm that it is the absence of *Tβh* gene that causes the mutant defect by restoring *Tβh* function and rescuing sterility of $T\beta h^{nM18}$ mutant through expression of a wild-type *Tβh* cDNA downstream of the *hsp70*-promoter. When the transgene bearing mutant flies are raised in 25°C, I can detect few eggs presumably due to the leakiness of the *hsp70* promoter (Fig. 2), while when raised in 18°C, the females are completely sterile. They start, though, laying their eggs within a few hours after heat shock is given and continue to lay eggs for almost 3 days (Fig. 2) following the same rhythmicity as the wild type females (more egg laying around dusk time) (unpublished observations). Rescue of the egg laying deficit is evident when heat shock is applied to different age groups of females as well as in mature virgins. Fertility of the rescued females 24 h after 1 h of heatshock is reaching 50–60% of the wild type levels (Fig. 2), and it approximates the wild type ones when two spaced heat shocks of 1 h each are given to them.

TBH immunoreactive cells innervate the female reproductive system

The effect of octopamine on ovulation could be due to its direct action on the activity of the oviductal muscles or

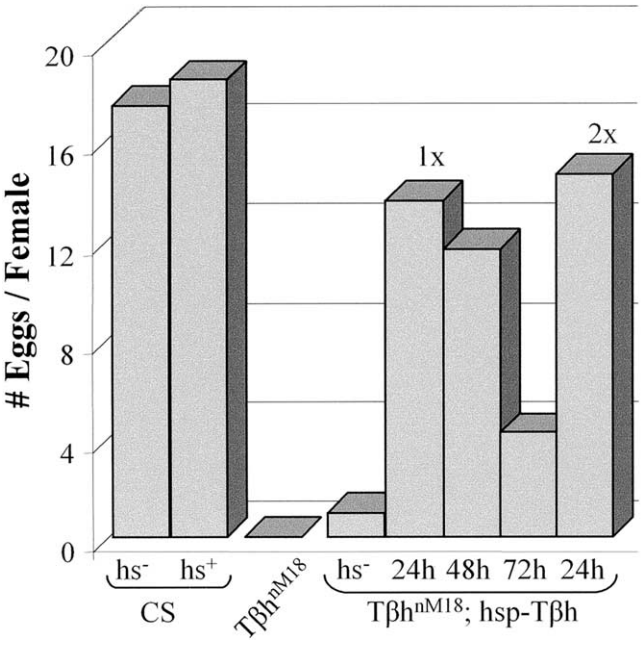

Fig. 2. Conditional rescue of the $T\beta h^{nM18}$ female egg-laying defect. Non heat-shocked $T\beta h^{nM18}$ females bearing a heat shock inducible *Tβh cDNA* ($T\beta h^{nM18}$;*hsp-Tβh//hs-*) lay few eggs presumably due to leakiness of the heat shock promoter. Twenty-four hours after one heat shock (1×, 24 h), egg laying of the rescued females reaches almost 60% of the wild type value (CS, Canton-S) measured for the same period and it continues for at least 2 more days (48 h, 72 h) but with reduced values. More egg laying is monitored 24 h after two spaced heatshocks (2×, 24 h).

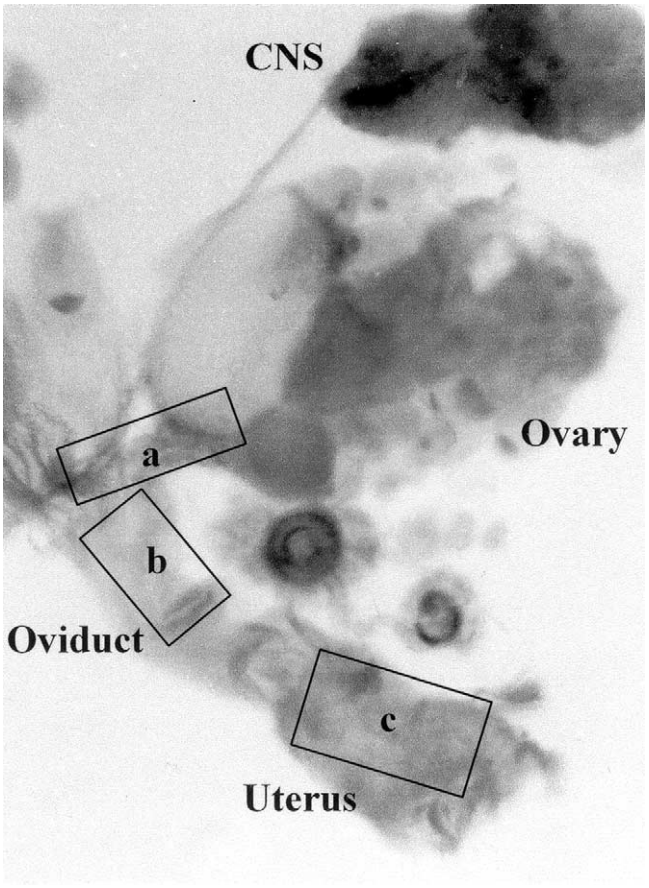

Fig. 3. Photomicrograph showing a representative whole-mount preparation of the female reproductive system and thoracic CNS joined by the abdominal nerves. Boxes indicate the regions that are presented in Fig. 5 in high magnification.

indirectly through its putative effects on the endocrine system. In the first case, octopamine could be locally released in the reproductive system or could target it through haemolymph. The female reproductive system is extensively innervated by neurons located in the abdominal ganglion of the thoracic CNS (Demerec, 1950). On the other hand, several unpaired octopamine-immunoreactive (OA-IR) cells have been detected in the ventral midline of this ganglion (AC, abdominal cluster) (terminology as in Monastirioti et al., 1995), thus I questioned whether any of these neurons innervate the reproductive system. Immunohistochemistry was performed in whole-mount preparations of wild type female thoracic CNS and reproductive system joined with intact abdominal nerves (Fig. 3) using the a-TBH antibody that specifically detects OA cells in all parts of the CNS (Monastirioti et al., 1996) and OA processes in the larval body wall muscles (unpublished observations).

Several TBH immunoreactive (TBH-IR) cell bodies are detected in the ventral midline of the abdominal region of the adult thoracic CNS (Fig. 4A), where the OA cells have been previously detected (Monastirioti et al., 1995). These cells send axons toward the dorsal neuropil (not shown), and then into the abdominal nerve (Fig. 4B). The IR axons reach the reproductive system where they ramify and target different parts of it. Fig. 5 shows that the immunoreactivity is primarily localized in the oviducts and ovaries. Strings of IR vesicles are observed in the ovarian sheath (Fig. 5D), on both lateral oviducts and common oviduct (Fig. 5E), while a dense network of IR fibers is primarily detected at the base of both ovaries (calyx) (Fig. 5A and D). In contrast, no immunoreactivity was detected on the uterus muscles (Fig. 5F). TBH-IRy is evident in the reproductive system of newly emerged females (less than 8 h old) (Fig. 5B), while it is missing from the respective regions in the $T\beta h^{nM18}$ mutant females (Fig. 5C). The immunoreactivity pattern of the TBH-expressing cells and axons suggests that octopamine is being locally released in the vicinity of ovaries/oviducts where it might modulate contractility of the visceral muscles, thus influencing the ovulation process.

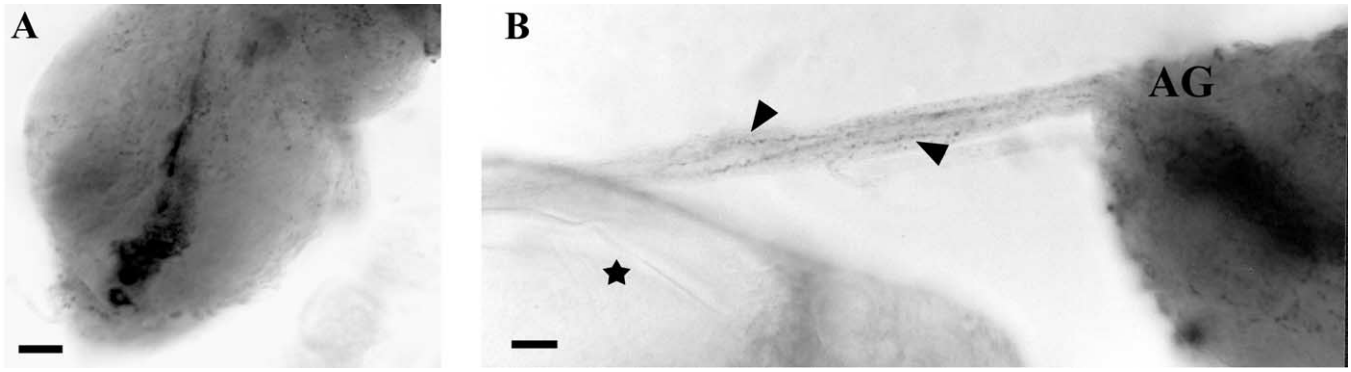

Fig. 4. Photomicrographs of whole-mount preparations of adult thoracic CNS and abdominal nerves from a wild type female. TBH-IRy was visualized with an HRP-conjugated secondary antibody. (A) TBH-expressing cells in the midline of the metathoracic and fused abdominal ganglia. Scale bar, 50μm. (B) Immunoreactive fibers (arrowheads) from the TBH-expressing cells (out of focus) of the abdominal ganglia (AG) traveling toward the reproductive system via the abdominal nerve. Asterisk marks an oocyte (out of focus) and indicates the position of the reproductive system (left) relative to the thoracic CNS (right) (see also Fig. 3). Scale bar, 25 μm.

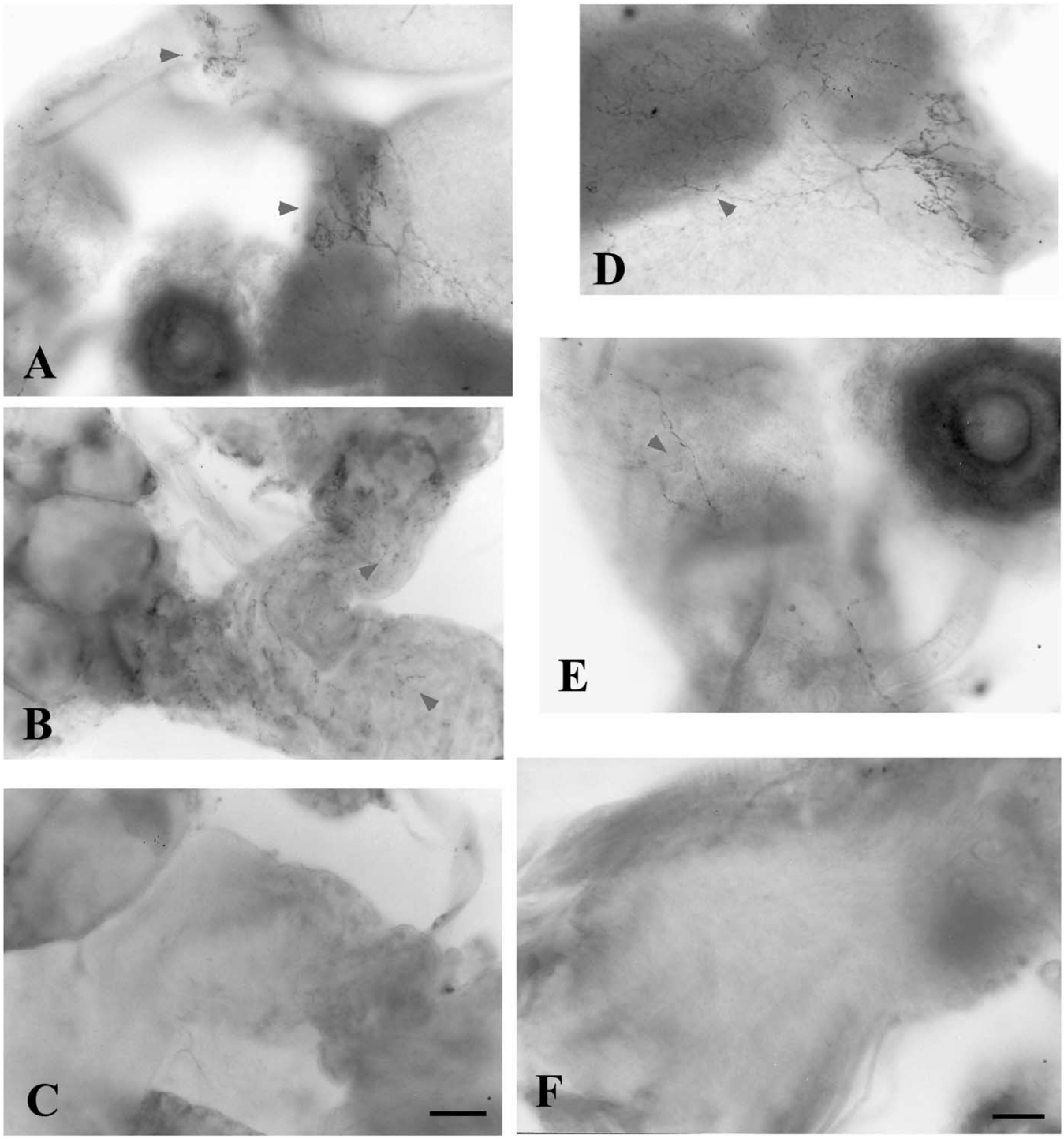

Fig. 5. Photomicrographs of different regions of the wild type (except in C) female reproductive tract showing TBH-IR fibers visualized with an HRP-conjugated secondary antibody. Regions in (A–D) correspond to the a box, while (E) and (F) correspond to the b and c boxes of Fig. 3, respectively. (A) Networks of IR fibers at the calyx region of both ovaries (arrowheads). (B) IR processes in the lateral oviducts of a wild type, newly emerged, female (note the immature oocytes within the left ovary). (C) Similar region as in (A) and (B) but from $T\beta h^{nM18}$ female reproductive system. Note the absence in immunoreactivity. (D) Higher magnification of (A) showing the immunoreactive fiber network in the calyx of one ovary and the processes extending in the ovary epithelium (arrowheads). (E) The arrowhead shows IR fiber in the common oviduct. (F) No immunoreactivity is observed in the uterus. Scale bar in (A–C) 50 μm, while in (D–F) 25 μm.

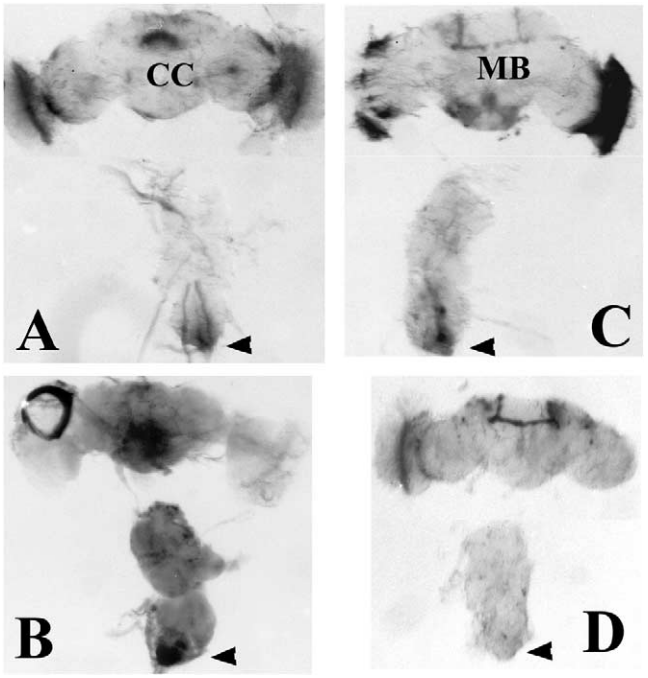

Fig. 6. Whole-mount adult brains and thoracic CNSs from the four different *enhancer-GAL4* expressing lines used in the study, were processed according to β-galactosidase activity staining technique. (A) *OK348-GAL4*. (B) *c164-GAL4*. (C) *ptc-GAL4*. (D) *79B-GAL4*. Arrows indicate the abdominal ganglion region at the tip of the thoracic CNS. CC, central complex; MB, mushroom bodies.

Targeted expression of TBH in abdominal ganglion cells rescues the sterility of octopamine-less females

The sites of TBH expression and presumably of octopamine release in the reproductive system perfectly correlate with the observed ovulation phenotype of the $T\beta h^{nM18}$ mutant females. However, rescue of the sterility phenotype by restoring octopamine biosynthesis in the respective cells offers a more substantiated proof of the above correlation. Thus, I used the binary GAL4-UAS system (Brand and Perrimon, 1993) to restore TBH expression, in otherwise mutant flies, selectively in cells of the abdominal ganglia and then monitor rescue of the sterility phenotype.

Several GAL4 enhancer trap lines, kindly provided by different laboratories, were tested for their potential to drive expression of a *UAS-βgal* transgene in the adult abdominal ganglion, and four of them selected for the study are shown in Fig. 6. The lines show overlapping patterns of expression in different parts of the adult CNS; for example, *OK348-GAL4* (Fig. 6A) and *c164-GAL4* (Fig. 6B) strongly express in the central complex while *ptc-GAL4* (Fig. 6C) and *79B-GAL4* (Fig. 6D) in mushroom bodies. However, a common site of expression between the lines *OK348-GAL4, c164-GAL4,* and *ptc-GAL4* is the tip of the thoracic CNS (arrow), and this site of expression is missing from *79B-GAL4* line which was used as the negative control line in the study.

Fig. 7 shows that the three GAL4 drivers (*c164-GAL4, OK348-GAL4,* and *ptc-GAL4*) that can direct expression of a *UAS-Tβh* transgene in the abdominal ganglion induce rescue of the $T\beta h^{nM18}$ egg-laying deficit ($T\beta h^{nM18}$*;* G/U). Females with genotypes that are internal controls of the crosses, bearing either the *enhancer-GAL4* or the *UAS-Tβh* transgenes ($T\beta h^{nM18}$*;* G/Bal and $T\beta h^{nM18}$*;* U/Bal), remain sterile. Same results were obtained when a different *UAS-Tβh* insertion (UAS15) was used to discount a *UAS-Tβh* insertion specific effect. *c164-GAL4* and *ptc-GAL4* drivers rescue sterility to wild type levels, while *OK348-GAL4* drives rescue up to 60%. Penetrance for this line is less than 100% (data not shown). On the contrary, the *79B-GAL4* driver that does not express in the abdominal ganglion region but it shows expression in other regions of the adult CNS did not promote any rescue (Fig. 7). It is worth noting that expression sites of this line have overlaps with *ptc-GAL4* line that rescues the phenotype. Finally, rescue of the egg laying phenotype is irrespective of mating as mature virgin Tbh^{nM18} females carrying both components of the binary system deposit eggs as the wild type virgin females do (not shown).

Based on these results, I conclude that expression of *Tyramine β-hydroxylase* gene in cells of the abdominal ganglion is required for the egg laying process.

Coexpression of the enhancer GAL4 drivers and TBH in neurons that innervate the reproductive system

In order to see whether TBH expression is driven by the *enhancer-GAL4* lines in the regions where TBH immunoreactivity is observed in wild type females, TBH immunohistochemistry was performed in dissected tissues of the $T\beta h^{nM18}$ mutant females with fertility restored by the GAL4 drivers. In Fig. 8, representative stainings for *c164-GAL4* (Fig. 8A and B) and *ptc-GAL4* (Fig. 8C–E) lines show immunoreactive cells in the abdominal ganglion and processes in the ovary calyx, oviducts, and ovaries. Similar results were obtained with the *OK348-GAL4* line (not shown).

The fact that TBH-IR cells and processes were observed in tissue areas of the above females that are normal sites for TBH expression correlates well with the rescue of the sterility of these females. Here the assumption is that, if TBH is expressed in non-TBH cells, no rescue will be provided as production of octopamine in particular cells requires a complete biosynthetic machinery (substrate and cofactors for TBH) and not just the expression of one biosynthetic enzyme. Indeed, double labeling experiments for wild type TBH expression and β-galactosidase expression under the control of the three GAL4 drivers show that cells in the abdominal ganglion coexpress TBH and the different enhancers (Fig. 9). Inspection of all confocal sections showed that, in the cases of *c164-GAL4* and *ptc-GAL4,* there are also cells expressing either TBH or the enhancer alone (not shown), while in the case of *OK348-GAL4,* the driver is expressed in three to four cells that are all TBH-positive (Fig. 9B). Whether

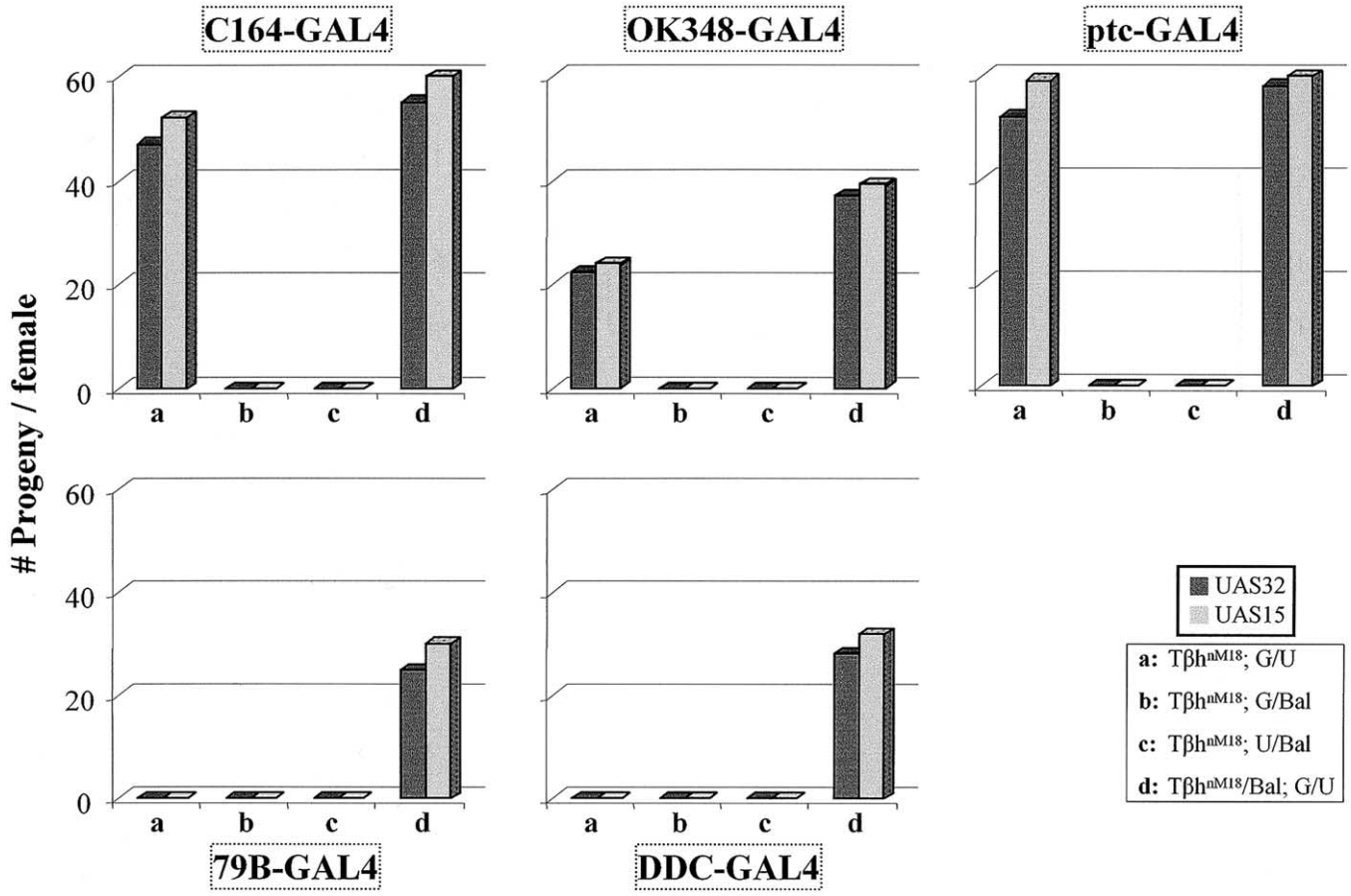

Fig. 7. Rescue of $T\beta h^{nM18}$ female sterility by expressing TBH in cells of the abdominal ganglion. All charts include results from the four female genotypes resulting from the crosses between enhancer-GAL4 and *UAS-Tβh* strains as described in Materials and methods. *a* denotes the female group with mutant background bearing both GAL4 and UAS transgenes. *b* and *c* represent the internal negative control female groups that are mutants and have either the GAL4 or the UAS transgene. *d* is the internal positive control of females that are fertile as they carry one copy of the wild type *Tβh* gene. Rescue of mutant phenotype (*a* group in all charts) is evident for *c164-GAL4*, *OK348-GAL4*, and *ptc-GAL4* drivers but not with *79B-GAL4* or *Ddc-GAL4* drivers. Different gray tones in the bars indicate two different *UAS-Tβh* transgenes used (UAS32, UAS15).

there are cells that coexpress TBH and two or all three enhancers simultaneously is not clear at this point.

TBH expression in dopamine and serotonin cells does not rescue sterility

Earlier studies had shown that a set of neurons in the midline of the adult abdominal ganglia express the catecholamine dopamine (Budnik and White, 1988; reviewed in Monastirioti, 1999) but till now, no particular function has been assigned to them. In order to test whether any of the cells expressing Dopamine also express TBH/ octopamine and are possibly involved in the ovulation control, I performed two types of experiments. I used a *Ddc-GAL4*-expressing line (Li et al., 2000) to express TBH in the $T\beta h^{nM18}$ mutant background and monitor sterility rescue, and I performed double labeling experiments as before to detect any collocalization between TBH and DDC expression. The *Ddc-GAL4* driver directs expression in both dopamine and serotonin cells as Dopa decarboxylase enzyme is utilized in the biosynthetic pathways of both neurotransmitters. No rescue of the sterility defect of the $T\beta h^{nM18}$ females bearing both *Ddc-GAL4* and *UAS-Tβh* transgenes is observed (Fig. 7), while the immunohistochemical experiment revealed that TBH-expressing cells in the abdominal ganglion are different from the DDC ones (Fig. 9D).

The above results show that, although expression of the driver is localized in the abdominal ganglion, it cannot promote rescue of the sterility phenotype as there is no expression of DDC in the TBH cells that are required for the innervation of ovaries and oviducts. They also indicate that, within the abdominal ganglion, the three neuroactive molecules, octopamine, dopamine, and serotonin, share different cell populations.

Discussion

Various physiological events take place during egg laying process in *Drosophila*, including ovulation, egg activation, fertilization, and oviposition. Among them, the ovula-

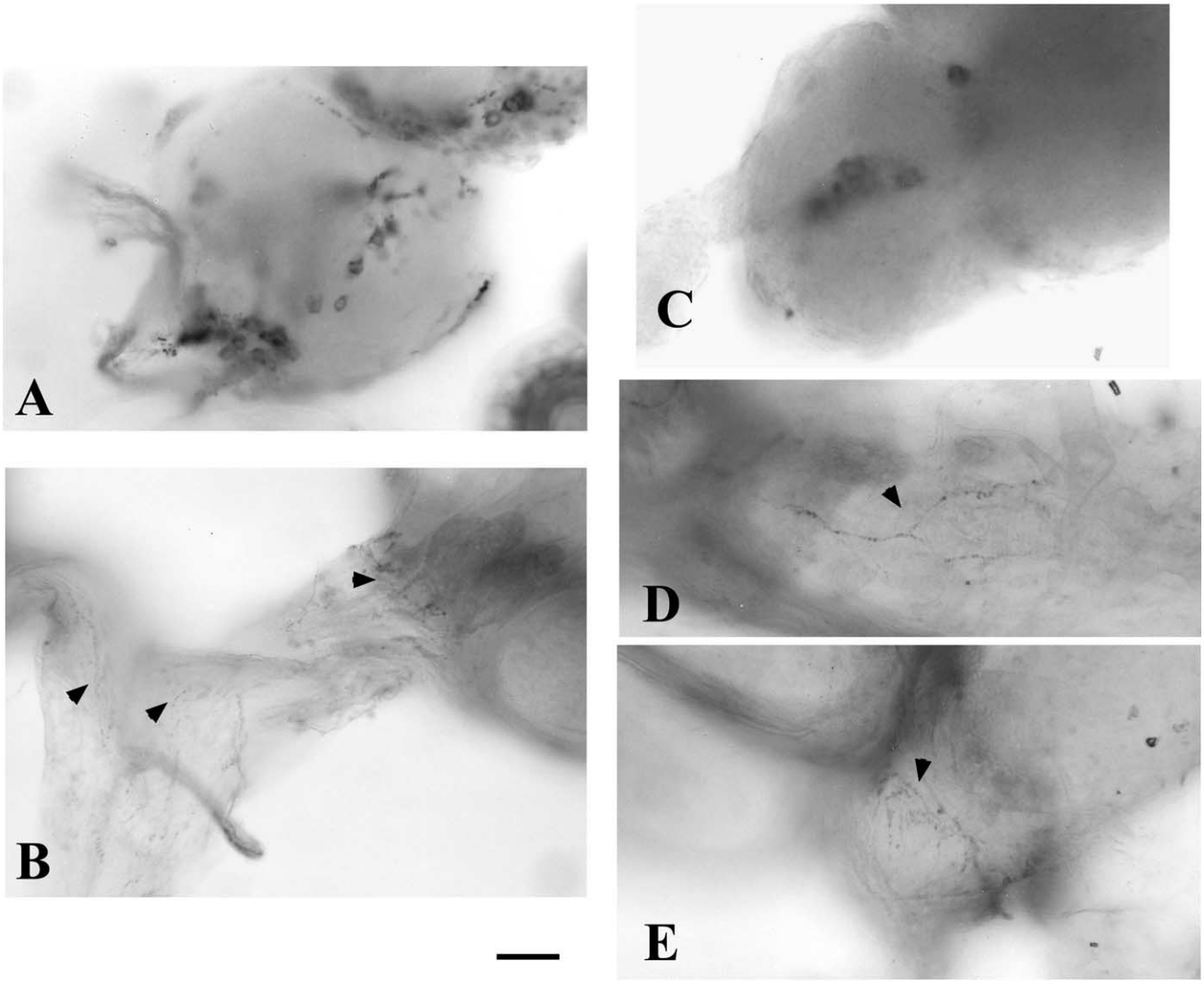

Fig. 8. TBH-IR cells and processes in the thoracicoabdominal ganglia (A, C) and reproductive tract (B, D, E) of $T\beta h^{nM18}$ females bearing *enhancer-GAL4* and *UAS-Tβh* transgenes. (A, B) $T\beta h^{nM18}$*; c164-GAL4/UAS-Tβh*. Arrowheads show IR fibers in the ovary calyx and oviducts. (C–E) $T\beta h^{nM18}$*; ptc-GAL4/ UAS-Tβh*. Arrowheads in (D) and (E) show IR fibers in the ovary and ovary calyx respectively. Scale bar, 50 μm.

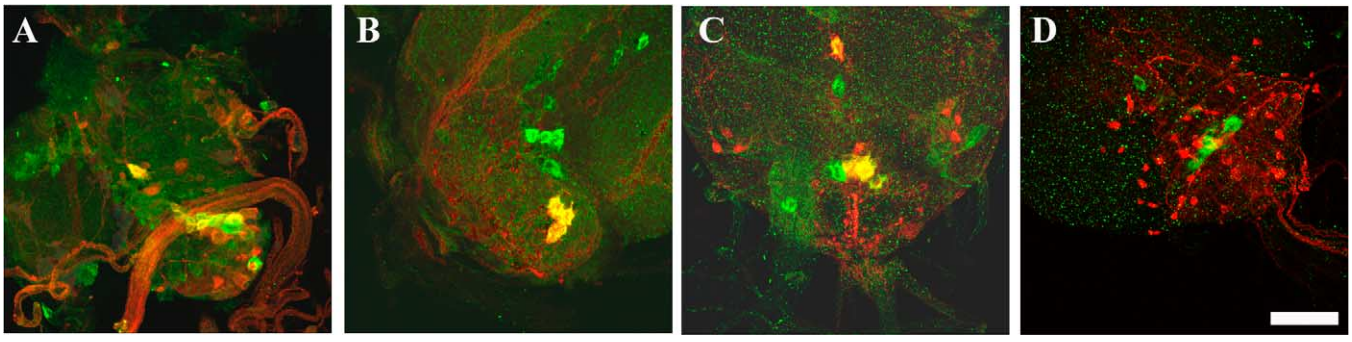

Fig. 9. Confocal images showing TBH-IRy (green) and β-galactosidase-IRy (red) in the fused abdominal ganglia of wild type females that carry different enhancer-GAL4 transgenes and a UAS-β-galactosidase transgene. Immunoreactive cells are visualized after immunofluorescent staining as described in Materials and methods. Note the collocalization (yellow) of the two antigens in neurons of *c164-GAL4* (A), *OK348-GAL4* (B), and *ptc-GAL4* (C) samples, but not in the *Ddc-GAL4* (D) sample. Scale bar, 50 μm.

tion step is a rather crucial one for reproduction as it triggers oocyte activation, stimulates oogenesis, and is affected by feedback mechanisms that regulate the production of mature eggs (Heifetz et al., 2001; Soller et al., 1997, 1999; reviewed in Bloch Qazi et al., 2003). In this study, examination of the sterility phenotype of the $T\beta h^{nM18}$ females that are depleted of octopamine suggested that this neuroactive substance is required for ovulation. The phenotype can be conditionally rescued by expressing the *Tβh* gene in the mutant females under the control of the hsp70-promoter, indicating that TBH expression is required for ovulation in the adult stage. When mature mated females do not ovulate, each ovariole accumulates more than two late stage oocytes, and this results in blockage of the maturation of additional oocytes. This is exactly the phenotype observed in the ovaries of $T\beta h^{nM18}$ mutant females where even four to five stage 14 oocytes reside within a single ovariole. The availability of mature oocytes points that oocyte development proceeds normally and also explains the quick initiation of egg laying and sterility rescue after the mutant females are fed on octopamine containing food (Monastirioti et al., 1996). Presumably, after octopamine is supplied in the system, the mature oocytes can be released from the ovaries, be fertilized, and normally develop into adults.

Ovulation process in *Drosophila* includes the release of the mature oocytes from the ovarioles and contractions of the ovary and oviduct musculature that push the eggs toward the uterus. Not much is known about the regulation of these two steps, but it is believed that they are under neurohormonal and neurotransmitter control, which may exert their action through hemolymph or by being locally released (Raabe, 1986). Gynandromorph studies suggested that the anatomical sites that control ovulation reside in the thoracicoabdominal ganglia of the CNS as correlation between the sex of the thoracic cuticle and the ovulation process was found (Szabad and Fajszi, 1982). In addition, decapitated females can deposit more than one egg, and these eggs must have ovulated after decapitation since the uterus can hold only one egg at a time. On the other hand, the ovarian and oviductal epithelium in *Drosophila* is highly innervated (Demerec, 1950; my upublished observations), presumably by neurons that are located in the fused abdominal ganglia of the thoracic CNS. The TBH-immunohistochemistry presented in this study shows that at least part of this innervation is octopaminergic and derives from the octopamine AC cells (Monastirioti et al., 1995) that are located in the ventral midline of the abdominal ganglion. Moreover, the ovulation defect of the $T\beta h^{nM18}$ females indicates that octopamine plays a crucial role in this process. Although some effect of octopamine in the reproductive tract via hemolymph cannot be excluded, the location of the target sites of the TBH immunoreactive fibers that nicely correlates with the mutant phenotype of the octopamine-less flies strongly suggests that the effect of Octopamine in the process is accomplished by its local release from these fibers.

In support of the hypothesis that ovulation is indeed controlled by the focus of TBH/octopamine cells in the abdominal ganglion is the restoration of fertility in the mutant females by the selective reestablishment of TBH expression in subsets of the normally TBH-expressing neurons of this region. The use of different GAL4 lines offers significant value in the selective rescue experiments. Although these drivers express GAL4 in other parts of the CNS as well, the overlap of expression of the ones giving rescue (*c164-GAL4, OK348-GAL4, ptc-GAL4*) is the abdominal ganglion. Moreover, the overlapping pattern of expression of the three GAL4 lines and TBH, as well as the negative result in the attempt to rescue $T\beta h^{nM18}$ sterility by the *Ddc-GAL4* driver show that selective TBH expression should be driven not only in the correct CNS region but in the correct neurons as well, in order to induce rescue. However, the possibility that octopamine cells, which are located in other CNS sites and could contribute in the ovulation control, remained undetermined in this study, cannot be excluded.

It is important to note at this point that there is no evidence about expression of other known chemical modulators in the specific TBH/octopamine cells. Of the three GAL4 lines used in the study, only *ptc-GAL4* relates to a known protein, Patched, that is involved in the Hedgehog signaling pathway and the transcriptional regulation of target genes (Ingham et al., 1991), while there is no information yet on the genes regulated by the *c164* and *OK348* enhancers. My experiments with the *Ddc-GAL4* line also led to the conclusion that neither dopamine nor serotonin is expressed in the AC cluster of TBH/octopamine cells. It is noteworthy that some of the neurons expressing these two substances in the abdominal ganglion region innervate parts of the reproductive tract (my unpublished observations), but any relation between the function of any of the two amines and the egg laying process is currently unknown.

The exact function of octopamine on the oviducts and ovaries of *Drosophila* remains to be investigated. Physiological evidence coming from experiments in other insects proposes that octopamine modulates the contractions of the oviductal muscles (Orchard and Lange, 1985; Kalogianni and Theophilidis, 1993) by being released from octopamine median unpaired neurons that are located in the abdominal ganglia of the CNS in these species and innervate the oviducts (Kalogianni and Pfluger, 1992; Kalogianni and Theophilidis, 1993). In Locusts, octopamine binds to octopamine 2B type receptors (Orchard and Lange, 1986) and triggers activation of adelylate cyclase and cAMP signal transduction pathway (reviewed in Lange and Nykamp, 1996). By analogy, an octopamine-specific receptor that stimulates cAMP and intracellular Ca^{2+} has been cloned from *Drosophila* (Han et al., 1998) but whether this receptor is expressed in the reproductive tract or mediates the action of octopamine in the ovulation process is currently unknown.

Besides octopamine, proctolin, glutamate, and SchistoFLRF-amide also regulate oviduct muscle contractions at

least in locusts (reviewed in Lange and Nykamp, 1996). Whether these or other neuroactive substances interact with octopamine and affect ovulation in *Drosophila* is an open question. In addition, it is well established that extrinsic factors can influence the overall rate of oogenesis and egg laying process in *Drosophila*. Ovulation and egg laying are stimulated upon mating by male accessory gland proteins (Acps) that are transferred to the female by the ejaculate together with sperm (reviewed in Wolfner, 1997). Acp70A (sex peptide) promotes vitellogenesis by stimulating JH synthesis in the corpus allatum (Moshitzky et al., 1996), while Acp26Aa (ovulin) stimulates release of stage 14 oocytes from the ovary (Heifetz et al., 2000). On the other hand, different environmental conditions such as day/light cycle, humidity, or nutrition appear to have effects in the female egg laying behavior. It is conceivable that these extrinsic factors/signals require an intrinsic physiological mechanism to target in order for the egg laying process to be enhanced or inhibited under stimulatory (mating) or inhibitory (hazardous environment) conditions. The octopamine system offers such an intrinsic female mechanism that is important for ovulation and can potentially integrate the different external stimuli so that the rate of this physiological process to be regulated.

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