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Role of the Median Neurosecretory Cells in the Ovarian Development of the Blow Fly *Protophormia terraenovae*

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ABSTRACT—Staining with paraldehyde-thionin-paraldehyde-fuchsin labeled 10-14 median neurosecretory cells (MNC) in each hemisphere of the brain of diapause and nondiapause adults of the blow fly, *Protophormia terraenovae* (Diptera: Calliphoridae). Various surgical operations were performed on the brain-retrocerebral complex of female adults to investigate the role of these cells in ovarian development and diapause. Under the diapause-averting conditions of LD 18:6 (18 h light and 6 h darkness) and 25°C, most of the intact and sham-operated females had vitellogenic ovaries, whereas all of the females with the MNC completely removed had immature ovaries. The corpus allatum (CA) was significantly smaller in the MNC-removed females than in the intact and sham-operated ones. After severance of the nervi corporis cardiaci, most of the females had immature ovaries. Even after severance of the cardiac recurrent nerve, there were vitellogenic ovaries in about 60 % of the females. However, after removal of the corpus cardiacum and hypocerebral ganglion complex (CCHG), no females had vitellogenic ovaries. Under the diapause-inducing conditions of LD 12:12 and 20°C, most females had immature ovaries and the CA was small irrespective of the surgical procedures. The results indicate that the MNC may secrete an allatotropic factor to stimulate vitellogenesis. The factor seems to be released to the hemolymph from the CCHG and a part of the aorta.

INTRODUCTION

Female adults of the blow fly, *Protophormia terraenovae* (Diptera: Calliphoridae), enter reproductive diapause with an arrest of vitellogenesis under short-day conditions and low temperature (Numata and Shiga, 1995). Removal of the corpus allatum (CA) under diapause-averting conditions and the application of a juvenile hormone (JH) analogue or implantation of the active CA under diapause-inducing conditions showed that a reduction of JH secretion from the CA causes adult diapause in this species (Matsuo *et al.*, 1997). Transection of the nervus corporis allati (NCA) induces vitellogenesis in blow flies under diapause-inducing conditions, and it was therefore proposed that the brain reduces the endocrine activity of the CA through the NCA in diapause adults (Matsuo *et al.*, 1997).

Similar results have been reported in several other species with adult diapause (*e.g.*, Hodková, 1976; Panov and Kryuchkova, 1977; Poras, 1982; Poras *et al.*, 1983; Kotaki and Yagi, 1989; Morita and Numata, 1997). In *Pyrrhocoris apterus* and *Tetrix undulata*, not only transection of the NCA but also surgical removal or electrocauterization of the median neurosecretory cells (MNC) in the pars intercerebralis (PI) induces ovarian development in diapause adults (Hodková, 1976; Poras, 1982). Therefore, the MNC are considered to be the inhibitory center for the activity of the CA in these species. In *P. terraenovae*, the MNC are a possible inhibitory center for CA activity in diapause. However, in a closely related species, *Calliphora vicina* (= *erythrocephala*), the stimulating effect of the MNC on ovarian development has been proposed for many years (Thomsen, 1952).

The aim of the present study was to examine the role of the MNC in ovarian development and diapause in *P. terraenovae*. First we observed the MNC with a staining method for neurosecretory cells, and then we examined the effect of surgical removal of these cells. The corpus cardiacum has long been known as the release site of the MNC, although the CA and aortal neurohemal organs were also shown to be release sites of the MNC in Diptera (Normann, 1983). Therefore, we removed the corpus cardiacum and hypocerebral ganglion complex (CCHG) and transected axons between the brain and the CCHG to identify the release sites of the MNC.

MATERIALS AND METHODS

Insects

Adults of *P. terraenovae* were collected at a swine farm in Obihiro City, Japan. Their progeny were maintained in laboratory cultures

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under a photoperiod of LD 18:6 (18 h light and 6 h darkness) at 25±1°C. Insects for the experiments were reared under the diapause-averting conditions of LD 18:6 and 25±1°C, or the diapause-inducing conditions of LD 12:12 and 20±1°C, as previously reported (Numata and Shiga, 1995). Only females were used in the present study. Female adults were collected on the day of emergence (day 0), and 30–50 individuals were reared without males in a plastic pot (15 cm diameter, 9 cm depth) covered with a nylon net. They were fed on sucrose, a piece of beef liver and water.

Staining of the neurosecretory cells

After cutting off the mouth part and removing the posterior cuticle plate of individual flies, the whole head was fixed in aqueous Bouin's solution for 24 h at room temperature, and then the brain was dissected out. The brain was dehydrated, then embedded in Paraplast® (Sherwood Medical, St. Louis, MO, USA) to make serial sections (10 μm thickness). These sections of the brain were stained with paraldehyde-thionin-paraldehyde-fuchsin (PTh-PF) (Panov, 1980).

Surgical procedures

The following surgical operations were performed on virgin females reared under LD 18:6 at 25°C on day 0 or day 1, and those reared under LD 12:12 at 20°C on day 5. Five days after the operation, the developmental stage of the ovaries was examined and the CA volume was measured.

Extirpation of the MNC—Flies were anesthetized with ethyl ether, and the head was bent forward into clay to expose the posterior surface. Under a stereoscopic microscope, a small square window was made with a razor blade in a central region of the posterior cuticle of the head to expose two groups of MNC, which were visible as bluish white. The bluish white regions were scratched off with a sharpened tungsten wire and taken out with forceps. After the operation, the removed piece of the cuticle was put onto its original position. For the sham operation, the fly was anesthetized and the MNC were exposed but not removed.

Severance of the nervi corporis cardiaci (*NCC*)—From the brain the bilateral NCC exit and join with the recurrent nerve to form a single nerve bundle, the cardiac recurrent nerve (CRN). The CRN is connected to the CCHG. Flies were mounted in clay without anesthesia, and the head was bent forward to expose the posterior surface. Under a stereoscopic microscope, the posterior part of the brain close to the esophagus foramen was exposed by removing a small piece of head cuticle as described above. Both NCC were transected with fine forceps near the brain.

Severance of the CRN and removal of the CCHG—Flies were mounted in clay without anesthetization, and the head was bent forward to expose the posterior surface. Under a stereoscopic microscope, the neck membrane was partially removed with a razor blade to expose the CCHG and CRN. The CRN was transected near the CCHG with fine scissors, or after cutting the NCA and the CRN, the CCHG was removed. For the sham operation, only the neck membrane was removed.

Developmental stage of ovaries

To examine the reproductive status, female adults were dissected, and the developmental stage of the ovaries was judged to be mature or immature, depending on the occurrence of vitellogenesis in the oocytes (Harlow, 1956).

Measurement of CA volume

The CA was dissected out and its length (L) and width (W) were measured using a video micrometer (VM-30, Olympus, Tokyo). From these values, the volume (V) was calculated assuming that the CA is an ellipsoid: $V = \pi LW^2/6$.

Statistical analysis

The proportion of females with vitellogenic ovaries and the volume of the CA were examined statistically by the Tukey-type multiple comparison test and the Tukey test, respectively (Zar, 1996, pp. 560-561, 212-217).

RESULTS

Histology

Histological observations were made in nondiapause female adults raised under LD 18:6 at 25°C and diapause female adults raised under LD 12:12 at 20°C on day 10. Two groups of PTh-PF-positive cells were located closely together in the PI of the brain (Fig. 1A). No other PTh-PF-positive cells

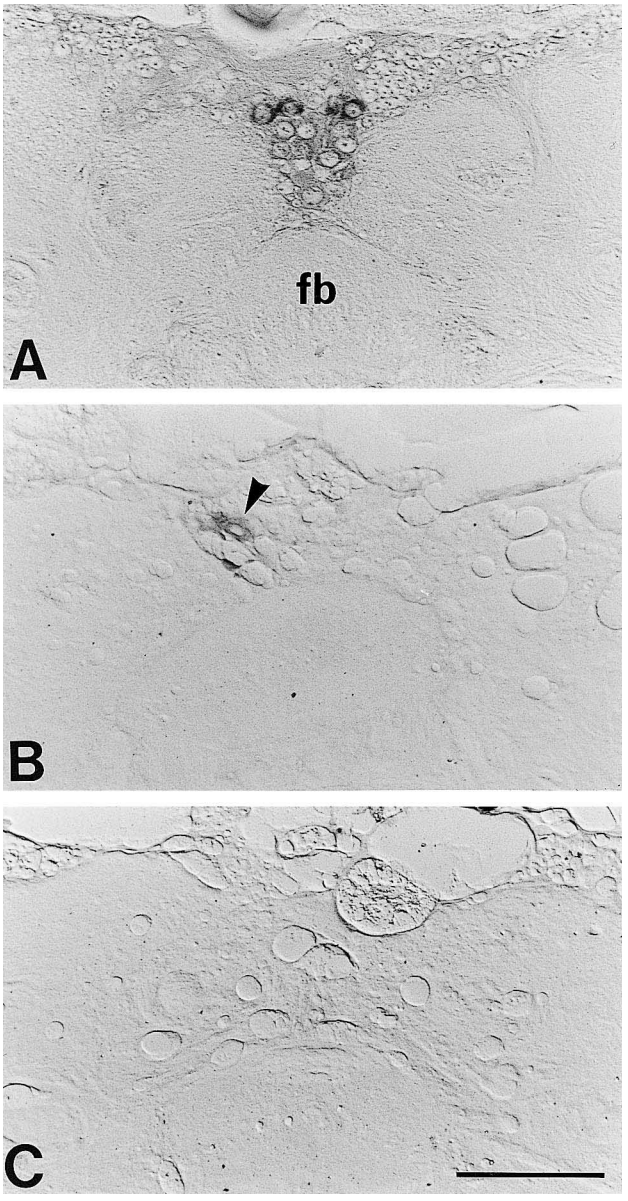


Fig. 1. Neurosecretory cells in the brain of adult female *Protophormia terraenovae*. Frontal sections of the intact brain (**A**) and the brain after removal of the median neurosecretory cells (MNC) (**B** and **C**) are shown after staining with paraldehyde-thionin-paraldehyde-fuchsin. In B, one of the MNC remained (arrow). In C, no MNC remained. fb, fan-shaped body. Scale bar, 100 μm.

were found in the brain. The numbers of PTh-PF-positive cells in each hemisphere of the brain were 12–14 (median = 12.5, n = 8) and 10–14 cells (median = 12, n = 10) in diapause and nondiapause adults, respectively, with no significant difference between them ($P > 0.05$, Mann-Whitney *U* test). In both groups of flies, three to four cells in the dorsal regions were stained more intensely than cells in the ventral regions (Fig. 1A). Sometimes the dorsal MNC of the nondiapause flies were stained deeply as compared to those of the diapause ones.

Effects of surgery

Under LD 18:6 at 25°C, most of the intact and sham-operated females had vitellogenic ovaries, whereas all of the females with the MNC completely removed (Fig. 1C) had immature ovaries (Fig. 2). The CA was significantly smaller in the MNC-removed flies than in the intact and sham-operated flies. When a few of the MNC remained (Fig. 1B), the ovaries had undergone vitellogenesis in 90% of the females. The CA were significantly larger than those of the females with the MNC completely removed (Fig. 2). Under LD 12:12 at 20°C, most of the intact, sham-operated and MNC-removed females had immature ovaries and small CA, with no significant differences (Fig. 2).

After the severance of bilateral NCC, most of the females had immature ovaries and small CA even under LD 18:6 at 25°C. By the severance of the CRN, females having vitellogenic ovaries reached about 60% under LD 18:6 at 25 °C, although they had small CA as was found in the NCC-severed ones. After the removal of the CCHG, however, no females had vitellogenic ovaries (Fig. 3). Even though we left the CA *in situ* by the removal of the CCHG, we could not find the CA at that place on dissection because the CA was pulled posteriorly into the thorax. Therefore we could not measure the volume of the CA after the removal of CCHG. Under LD 12:12 at 20°C, in most of the intact, sham-operated and nerve-severed females, the ovaries remained immature and their CA were small with no significant differences (Fig. 3).

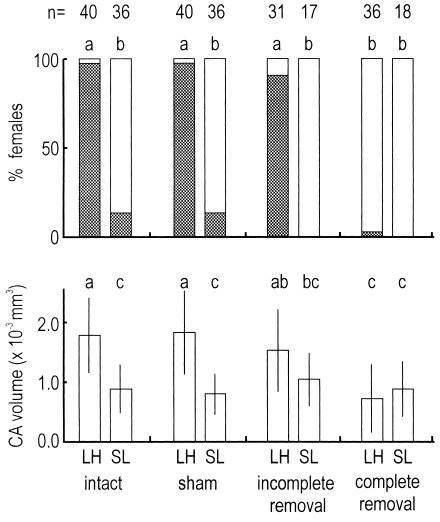


Fig. 2. Effect of the surgical removal of the median neurosecretory cells (MNC) on ovarian development and on the volume of the corpus allatum (CA) in adult female *Protophormia terraenovae*. Shaded columns, proportion of vitellogenic females; vertical bar, standard deviation. No MNC remained in complete removal (Fig. 1C), whereas in incomplete removal a few cells remained (Fig. 1B). LH, LD 18:6 at 25°C; SL, LD 12:12 at 20°C. The values marked with the same letter above the columns were not significantly different ($P > 0.05$).

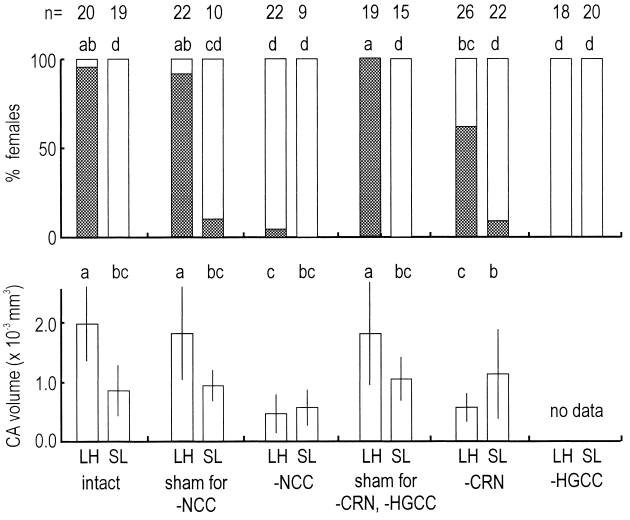


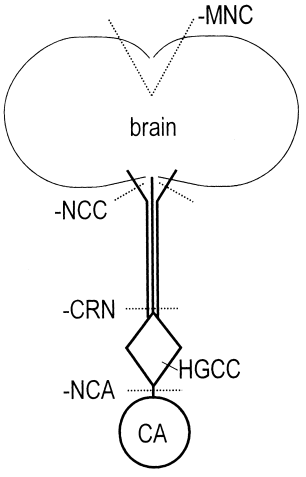
Fig. 3. Effects of severance of the nervi corporis cardiaci (–NCC) or the cardiac recurrent nerve (–CRN) and that of the removal of the hypocerebral ganglion and corpus cardiacum complex (–CCHG) on ovarian development and on the volume of the corpus allatum (CA) in adult female *Protophormia terraenovae*. Shaded columns, proportion of vitellogenic females; vertical bar, standard deviation. LH, LD 18:6 at 25°C; SL, LD 12:12 at 20°C. The values marked with the same letter above the columns were not significantly different ($P > 0.05$).

DISCUSSION

Staining with PTh-PF labeled 10–14 MNC in each hemisphere of the brain in *P. terraenovae*. No other cerebral neurosecretory cells, such as two groups of lateral neurosecretory cells and some other cells shown in each hemisphere of the brain in *C. vicina* and *Phormia regina* (Thomsen, 1965; Hsiao and Fraenkel, 1966), were stained by this staining. Histological differences have been reported between the neurosecretory cells of diapause and nondiapause adults in some insects. In many cases, the number of neurosecretory cells stained histologically was smaller in diapause adults than in reproductive ones (*e.g.*, Hoffmann, 1970; see Denlinger, 1985). The staining intensity of the MNC is higher in reproductive females than in diapause ones in *Pterostichus nigrita* (Hoffmann, 1970). In contrast, the staining intensity of MNC is higher at the onset of diapause in *Chilocorus bipustulatus* (Vagina, 1975). In *P. terraenovae*, however, there is no significant difference in the number of PTh-PF positive cells between the brains of diapause and nondiapause adults. The MNC of the nondiapause flies were stained more intensely than those of the diapause flies. However, we cannot attribute the difference only to the diapause status. In the present study temperature or age might cause difference in the staining in-

tensity. The neurosecretory cells show no apparent difference between diapause and nondiapause adults in *Nomadacris septemfasciata* (Strong, 1966).

Figure 4 summarizes the effects of surgical operations on vitellogenesis in *P. terraenovae* shown in the present and previous studies (Matsuo *et al.*, 1997). When the MNC were completely removed, no vitellogenesis was observed both under diapause-averting and diapause-inducing conditions, contrary to the results in *P. apterus* and *T. undulata* (Hodková, 1976; Poras, 1982). Consequently, these cells are essential for vitellogenesis in *P. terraenovae*. There are three possible explanations for this stimulating effect of the MNC on vitellogenesis: (1) The MNC stimulate the activity of the CA to secrete JH, which is necessary for vitellogenesis in *P. terraenovae* (Matsuo *et al.*, 1997). Such a mechanism has been shown in a strain of *Locusta migratoria* with adult diapause (Tobe *et al.*, 1982). The primary structure of allatotropin, i.e., a peptide stimulating the activity of the CA to secrete JH, was identified only in *Manduca sexta* (Kataoka *et al.*, 1989), but its presence has been shown in several other species (Tobe and Stay, 1985). In *Galleria mellonella*, allatotropin was partially purified and immunocytochemically localized in two pairs of MNC and the corpus cardiacum (Bogus and Scheller, 1994, 1996). (2) The MNC stimulate the ovaries to secrete ecdysteroids, which are necessary to vitellogenin synthesis in *P. terraenovae* (Huybrechts and De Loof, 1982). The primary structure of ovary ecdysteroidogenic hormone, which is a peptide that stimulates the ovaries to secrete ecdysteroids, was recently identified in *Aedes aegypti*, and localized immunocytochemically in two or three pairs of MNC (Brown *et al.*, 1998). (3) Neurosecretory materials from the MNC are necessary for vitellogenesis without the intervention of JH or ecdysteroids.



	vitellogenesis	
	LH	SL
Intact	+	–
-MNC	–	–
-NCC	–	–
-CRN	+/–	–
-HGCC	–	–
-NCA*	+	+
-CA*	–	

Fig. 4. Effects of various surgical operations on ovarian development in adult *Protophormia terraenovae*. –MNC, removal of the median neurosecretory cells; –NCC, transection of the nervi corporis cardiaci; –CRN, transection of the cardiac recurrent nerve; –CCHG, removal of the corpus cardiacum and hypocerebral ganglion complex; –NCA, transection of the nervus corporis allati; –CA, removal of the corpus allatum. LH, LD 18 : 6 at 25°C; SL, LD 12 : 12 at 20°C.
* After Matsuo *et al.* (1997).

In *P. terraenovae*, the CA is larger in nondiapause adults than in diapause ones (Matsuo *et al.*, 1997; present results), and after MNC removal in the nondiapause adults the CA volume was reduced as almost the same size as that of the intact diapause ones. These results would support the first hypothesis mentioned above, if there is a correlation between volume and secretory activity in the CA of *P. terraenovae*, as shown in many other insects (Tobe and Stay, 1985). In *C. vicina* also, the removal of MNC suppressed vitellogenesis with the reduction of the size of CA. However, the ovarian follicles were smaller in the MNC-removed flies than those of allatectomized flies, and implantation of the CA to the MNC-removed flies did not completely recover their ovarian development (Thomsen, 1952). In *P. regina*, the application of a JH analogue partially restored vitellogenesis that had been suppressed by the removal of MNC (Pappas and Fraenkel, 1978). No remarkable relation was found between the volume and secretory activity of the CA in *C. vicina* (Lea and Thomsen 1969). We should assume that the size does not always reflect the activity in the CA. We must measure the JH synthetic activity of the CA in MNC-removed flies to determine which hypotheses mentioned above is correct in *P. terraenovae*. In addition, we notice that the above three hypotheses are compatible and the MNC perform two or three functions in vitellogenesis.

Because transection of the NCA did not affect vitellogenesis under diapause-averting conditions (Matsuo *et al.*, 1997), the MNC have their terminals on anterior regions to the CA stimulating the vitellogenesis. Considering the fact that transection of the NCC resulted in no vitellogenesis but severance of the CRN did not inhibit vitellogenesis completely, the release sites of the MNC materials distribute in both of the CRN and the CCHG. In *P. terraenovae*, lateral neurosecretory cells releasing a neuropeptide, corazonin, have the releasing sites in the CRN and on the aorta anterior to the CCHG (Cantera *et al.*, 1994). The MNC may also deposit their terminals in the CRN or on the aorta or both on the way to the CCHG.

According to the above assumption, the removal of the CCHG should have a similar effect to that of the transection of the CRN near the CCHG. However, no vitellogenesis was observed after removal of the CCHG under diapause-averting conditions. Another factor from intrinsic neurosecretory cells in the CCHG may be involved in vitellogenesis in *P. terraenovae*.

Transection of NCA induces vitellogenesis in blow flies under diapause-inducing conditions (Matsuo *et al.*, 1997). This indicates that the brain inhibits the endocrine activity of the CA through the NCA in diapause adults. The inhibitory center of the brain during diapause is still unclear. Our recent results show that not only the MNC but also some neurons in the pars lateralis innervate the CA (Shiga *et al.*, submitted). Therefore, removal of these cells by methods such as those used in the present experiments can be the next step for determining the inhibitory center of the CA.

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