SHORT COMMUNICATION

The Role of Neurosecretory Neurons in the Pars Intercerebralis and Pars Lateralis in Reproductive Diapause of the Blowfly, *Protophormia terraenovae*

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Abstract Microlesions of the brain were made to examine the role of neurosecretory neurons in the pars intercerebralis (PI) and pars lateralis (PL) in the induction of reproductive diapause of the female blowfly *Protophormia terraenovae*. Under both diapause-inducing (LD 12:12, 20° C) and diapause-averting conditions (LD 18:6, 25° C), the ovaries invariably failed to develop when the PI was removed. When the PL was removed bilaterally, the ovaries developed in most of the females, irrespective of the rearing conditions. Removal of the PL prevented females from entering reproductive diapause. These results show that certain neurosecretory neurons in the PI are necessary for vitellogenesis, and that the PL contains inhibitory neurons which suppress vitellogenesis during reproductive diapause.

The hormonal basis for reproductive diapause has been examined in several orders of insects (Denlinger 1985). A key factor in reproductive diapause is the failure of the corpus allatum (CA) to release juvenile hormone (JH). Surgical manipulations have demonstrated that the brain exerts its control over the CA by both humoral and neural pathways (Denlinger 1985; Matsuo et al. 1997; Morita and Numata 1997). Cerebral neurons controlling the CA have terminals in the corpus cardiacum and the CA, and their anatomical arrangements suggest that they are neurosecretory neurons in the pars intercerebralis (PI) and pars lateralis (PL; Raabe 1989). Hence, understanding the functional roles of neurons in the PI and PL in controlling the CA is of great interest with regard to the physiological mechanism of reproductive diapause. The role of the PI neurons in reproductive diapause varies between species (Hodková 1976; Poras 1982; Poras et al. 1983; Toyoda et al. 1999). In the linden bug *Pyrrhocoris apterus* and the grasshopper *Tetrix undulata*, neurons in the PI inhibit ovarian development during diapause (Hodková 1976; Poras 1982), while in the migratory locust *Locusta migratoria* and the blowfly *Protophormia terraenovae*, neurons in the PI are necessary for ovarian development (Poras et al. 1983; Toyoda et al. 1999). In contrast, the role of neurons in the PL in reproductive diapause has been characterized so far only in *L. migratoria*. Electrical cauterization of the PL results in CA activation and vitellogenesis, even in diapause-inducing conditions, suggesting that neurons in the PL inhibit CA activity during diapause (Poras et al. 1983). In the cockroach *Diploptera punctata*, which does not have reproductive diapause, microlesions and immunohistochemical study have demonstrated that inhibition of the CA of virgin females is caused by allatostatin, a neuropeptide produced by neurosecretory neurons in the PL (Rüegg et al. 1983; Stay et al. 1992).

Most of these findings regarding the control of ovarian development have been obtained from cautery or surgical removal of the PI or PL. These two loci are located close to each other, and projections of their neurons cover the superior protocerebral neuropil. Consequently, distinguishing between functional roles of the PI and PL is sometimes difficult. For example, cauterization of the PI also causes interruption of the axonal pathways from the PL neurons to the CA (Poras et al. 1983; Rüegg et al. 1983). To distinguish between the function of neurons in the PI and PL the cautery must be restricted to a small area of the brain and should be accompanied by a histological examination.

The blowfly *P. terraenovae* has a reproductive diapause induced by short-day and low-temperature conditions (Numata and Shiga 1995). Matsuo et al. (1997) have proposed that cerebral neurons directly innervating the CA cause a reduction in JH secretion, resulting in reproductive diapause. Furthermore, nickel backfills reveal that most neurons in the PL and a few neurons in the PI innervate the CA in *P. terraenovae* (Shiga et al. 2000). These observations suggest that the PL, PI, or both contain inhibitory neurons that induce reproductive diapause in this fly. In the present study microlesions of the brain were made to examine the role of neurosecretory neurons in the PI and PL in the induction of reproductive diapause in *P. terraenovae*.

Flies were reared under diapause-inducing conditions (LD 12:12 and 20±1° C) or diapause-preventing conditions (LD 18:6 and 25±1° C). Larvae, pupae and adults of each group were reared in the same conditions. Only females were used for the experiments. Operations were made 1 day after adult emergence (day 1). The methods used for brain surgery are described by Toyoda et al. (1999). Bluish-white neurosecretory cells, visible in the PI or PL, were removed with a sharpened tungsten hook. After the operations 10–15 females were isolated in a plastic cage with sucrose, beef liver and water. On day 11 under LD 12:12 at 20° C or on day 6 under LD 18:6 at 25° C, flies were first subject to staining of the neurosecretory neurons, and then ovarian developmental stages were examined. For staining of the neurosecretory neurons $NiCl_2$ backfills were made through the cardiac recurrent nerve and followed by silver intensification (Shiga et al. 2000). Paraldehyde fuchsin stains the neurosecretory cells only in the PI, not in the PL, of *P. terraenovae* (Toyoda et al. 1999). Then we also used paraldehyde fuchsin for the PI removal group. Females were classified into three groups according to ovarian developmental stages: previtellogenic, vitellogenic but not fully matured, fully matured (Matsuo et al. 1997). Only females with previtellogenic ovaries were considered to be in diapause.

In the intact and sham-operated groups most females had previtellogenic ovaries under LD 12:12 at 20° C, and under LD 18:6 at 25° C most of the females had fully matured ovaries (Fig. 1). When the PI was removed, the ovaries invariably failed to develop, irrespective of the rearing conditions. Under LD 18:6 at 25° C the proportion of previtellogenic females was significantly higher in the PI-removal group than in the other three groups (intact, sham-operated, and PL-removal). These results confirmed the ablation experiments by Toyoda et al. (1999). When the PL were removed bilaterally, the ovaries developed in 76% of the females reared under LD 12:12 at 20° C and in 84% of those under LD 18:6 at 25° C (Fig. 1). Under LD 12:12 at 20° C the proportion of females with previtellogenic ovaries was significantly lower in the PL-removal group than in the other three groups (intact, sham-operated, and PI-removal). Removal of the PL prevented females from entering reproductive diapause. Removal of the PL did not affect ovarian development under LD 18:6 at 25° C.

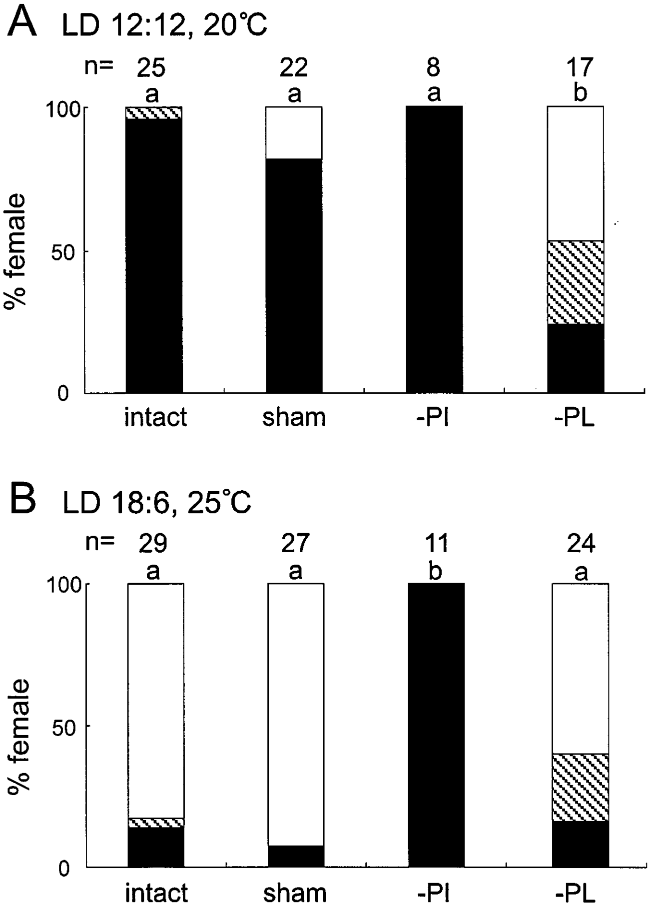

Fig. 1A,B. Effects of surgical removal of the pars intercerebralis (*PI*) or pars lateralis (*PL*) on ovarian development in *Protophormia terraenovae*. Operations were made on day 1 and ovarian stages were examined on day 11 under LD 12:12 at 20° C (A), and on day 6 under LD 18:6 at 25° C (B). *Open columns* Females with fully matured ovaries; *hatched columns* females with vitellogenic ovaries; *closed columns* females with previtellogenic ovaries. Removal of the PL prevented females from entering reproductive diapause. *Columns with the same letter* no significant difference in rate of females with previtellogenic ovaries in respective conditions (Tukey-type multiple comparison test for proportions, $P<0.05$; Zar 1999)

Figure 2 shows backfills of intact and experimental brains. In intact brains the backfill labeled a large paramedial cluster of somata in the PI and seven or eight pairs of somata in the PL (Fig. 2A, Shiga et al. 2000). Our previous study demonstrated that the PI neurons send axons to the nervi corporis cardiaci (NCC) via the median bundle (MB), and that the PL neurons send axons to the NCC through the posterior lateral tract (PLT; Shiga et al. 2000). When the PI was removed, no somata in the PI were labeled

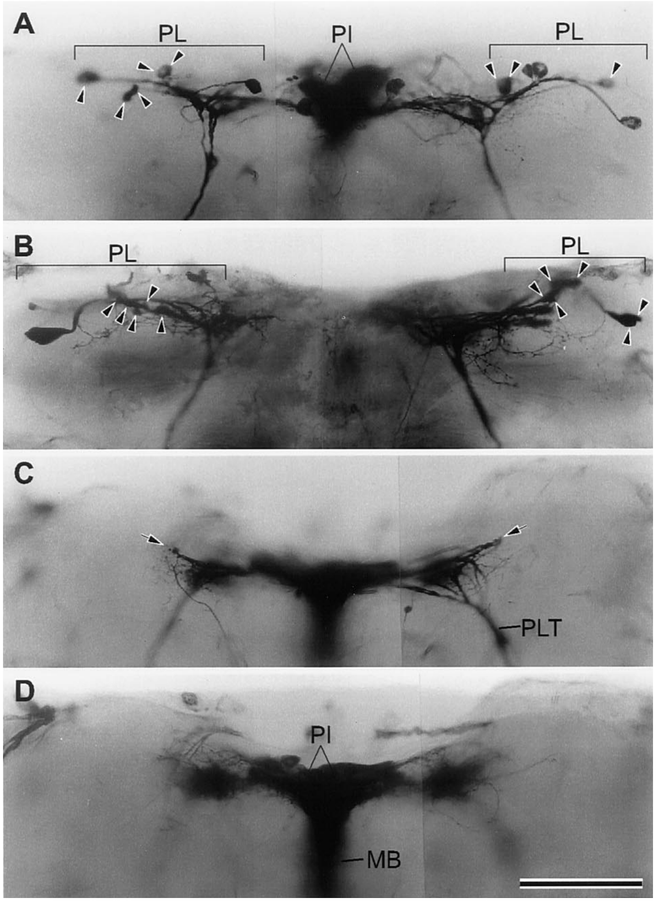

Fig. 2A–D. Photomicrographs of whole-mount preparations of brain neurons back-filled from the cardiac-recurrent nerve of the adult blowfly *Protophormia terraenovae* (dorsal part of the brain in frontal view). A) Intact brain. A paramedial cluster of somata in the pars intercerebralis (*PI*) and seven or eight pairs of somata in the pars lateralis (*PL*) are stained. *Arrowheads* out of focus somata. B) The brain with the PI removed. Note that only PL neurons were labeled. C) The brain with the PL removed. The PL somata were not labeled, but their fibers and axons in the superior protocerebrum (*arrows*) and in the posterior lateral tract (*PLT*) still remained even 10 days after the operation. D) The same preparation as C but in a different focal plane. Somata in the PI and axons in the median bundle (*MB*) were labeled as in the intact brain. The ratio of experimental flies checked by backfills or paraldehyde fuchsin was 8/8 for PI removal under LD 12:12 at 20° C, 7/17 for PL removal under LD 12:12 at 20° C, 8/11 for PI removal under LD 18:6 at 25° C, and 2/24 for PL removal under LD 18:6 at 25° C. The denominators show the number of flies in Fig. 1. When one or more somata were stained in the surgically ablated regions, these flies were excluded from the data. *Scale* 100 μm

(Fig. 2B), but their prominent fibers remained in the MB (not shown). There are two kinds of PL neurons in *P. terraenovae*. One type of neurons, PL-c, send axons to the contralateral NCC via the pathway adjacent to the PI, and the other type, PL-i, send axons to the ipsilateral NCC (Shiga et al. 2000). Consequently, in the present study axonal pathways of the PL-c neurons were interrupted by the PI removal operation. However, most of the PL-i neurons were labeled (Fig. 2B). When bilateral regions of the PL were removed, no somata in the PL were labeled, but their axons and fibers were stained in the PLT even 10 days after the operation (Fig. 2C). Somata in the PI and their fibers in the MB were stained as in intact brains (Fig. 2D).

Matsuo et al. (1997) reported that when the nervi corporis allati of *P. terraenovae* are severed, ovarian development is not inhibited in diapause-inducing conditions. In the present study removal of the PL also resulted in vitellogenesis in the same conditions. The known projection pattern of the PL neurons and data from the present study demonstrate that the PL contains neurons that inhibit ovarian development during diapause. Application of a JH analogue or transplantation of the CA induces complete ovarian development in adults in diapause (Matsuo et al. 1997), suggesting that the PL neurons inhibit ovarian development by preventing JH production by the CA. An inhibitory role of the PL neurons in the JH production by the CA also has been demonstrated in *L. migratoria* and *D. punctata* (Poras et al. 1983; Rüegg et al. 1983).

In contrast, the neurosecretory neurons in the PI are necessary to promote ovarian development in *P. terraenovae*, as shown by Toyoda et al. (1999). In flies this role of the PI is totally different from that in *P. apterus* and *T. undulata* in which the ovaries can develop without neurosecretory neurons in the PI (Hodková 1976; Poras 1982). Furthermore, when the PI somata were cauterized in *L. migratoria* and *D. punctata*, PL axons were also interrupted, and this treatment resulted in activation of the CA, showing that these insects can develop ovaries without the PI neurons (Poras et al. 1983; Rüegg et al. 1983; Stay et al. 1992). In *P. terraenovae*, however, when only the PI was removed, or when both PI and PL were disconnected from the CA by severing the NCC, the ovaries did not develop (Toyoda et al. 1999). The PI is necessary for ovarian development in *P. terraenovae*. To characterize the roles of the PI and PL neurons in JH biosynthesis by the CA of *P. terraenovae*, radiochemical assays are now underway.

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