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CONTROL OF DIAPAUSE-FACTOR SECRETION FROM THE SUBOESOPHAGEAL GANGLION IN THE SILKWORM, *BOMBYX MORI*: THE ROLES OF THE PROTOCEREBRUM AND TRITOCEREBRUM*

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Abstract—To localize any functional areas in the brain which control the secretion of the diapause factor from the suboesophageal ganglion in the *Bombyx* silkworm, various parts of the brain were excised or transected surgically. It was demonstrated that cortices slightly lateral to the median line on the dorsal side of the protocerebrum (Lmdp) are closely related to the inhibitory mechanism in the pupa producing non-diapause eggs. On the other hand, cortices of anterolateral areas on the ventral side of the protocerebrum (Alvp) participate in the stimulatory mechanism in the pupa producing diapause eggs. Moreover, it is suggested that the tritocerebrum is competent to stimulate secretion of the diapause factor from the suboesophageal ganglion not only in the pupae producing diapause eggs but also in the pupae producing non-diapause eggs. From these results, we propose a possible relationship between the Alvp, Lmdp and tritocerebrum concerning regulation of diapause-factor secretion from the suboesophageal ganglion.

Key Word Index: Diapause-factor secretion, cerebral control, embryonic diapause, *Bombyx* silkworm

INTRODUCTION

The silkworm, *Bombyx mori*, enters diapause at the stage of the late gastrula (Toyama, 1902; Sonobe *et al.*, 1986). Embryonic diapause is predetermined at the period of ovarian development by a neurohormone (Sonobe and Ohnishi, 1971; Sonobe, 1974; Kubota *et al.*, 1979; Sonobe and Odake, 1986), called the diapause factor or diapause hormone, which is secreted from the suboesophageal ganglion during pupal–adult development (Fukuda, 1951; Hasegawa, 1951). According to Fukuda and Takeuchi (1967), the diapause factor is secreted from a pair of neurosecretory cells, the diapause-factor cells, lying on the ventral side of the suboesophageal ganglion. That is to say, in the non-diapause-egg-producing-pupa, diapause-factor cells accumulate a large amount of neurosecretory material staining with azocarmine, suggesting that the diapause factor is stored but not secreted. By contrast, the diapause-factor cells of the pupa producing diapause eggs are hardly visible, possibly owing to a lack of neurosecretory material in the cytoplasm, suggesting that the diapause factor is probably released without storage.

By transplantation experiments with the brain and the suboesophageal ganglion, and transection experiments of the circum-oesophageal connectives, Fukuda (1951, 1953, 1962) has shown that the secretion of diapause factor from the suboesophageal ganglion is inhibited by the brain through the circum-oesophageal connectives in the non-diapause-egg-producing-pupa, whereas it is stimulated by the brain through the connectives in the pupa producing diapause eggs. However, no attempt has been made to locate the functional areas in the brain which exert the stimulatory or inhibitory effect upon the suboesophageal ganglion. We therefore initiated this study to examine this point.

MATERIALS AND METHODS

The bivoltine hybrid race between Taizo (Daizo) and Nichi-106 of *Bombyx* was used in this study. In the bivoltine race, the voltinism is determined by the environmental stimuli experienced by the mother moth (pupa) when she was in the embryonic stage (Kogure, 1933). To obtain non-diapause-egg-producing-pupae and diapause-egg-producing-pupae exclusively, eggs were incubated in the dark at 15°C, and under continuous illumination at 26°C, respectively. The larvae were reared in a 12-h light–12-h dark cycle at 25°C.

Female pupae within 1 h or at 14 h after larval–pupal ecdysis were used for surgical operations on the brain. The pupa anaesthetized with ethyl ether was mounted on an operating block. Local operations on the brain were performed with a pair of fine forceps and microsurgery scissors through a square window made by first removing a piece of cuticle above the brain. After the operation the cuticle was replaced and cut edges sealed with paraffin wax. The detailed locations of the surgical operations on the brain are specified in Fig. 1. Azan-stained sections of the brain–suboesophageal ganglion complex served to confirm the extent of the lesions (Matsutani, unpublished work).

Most of the operated pupae emerged 8–9 days after larval–pupal ecdysis at 25°C as did normal individuals. After mating the moths were allowed to lay eggs.

*Dedicated to the memory of Dr Soichi Fukuda (1907–1984).

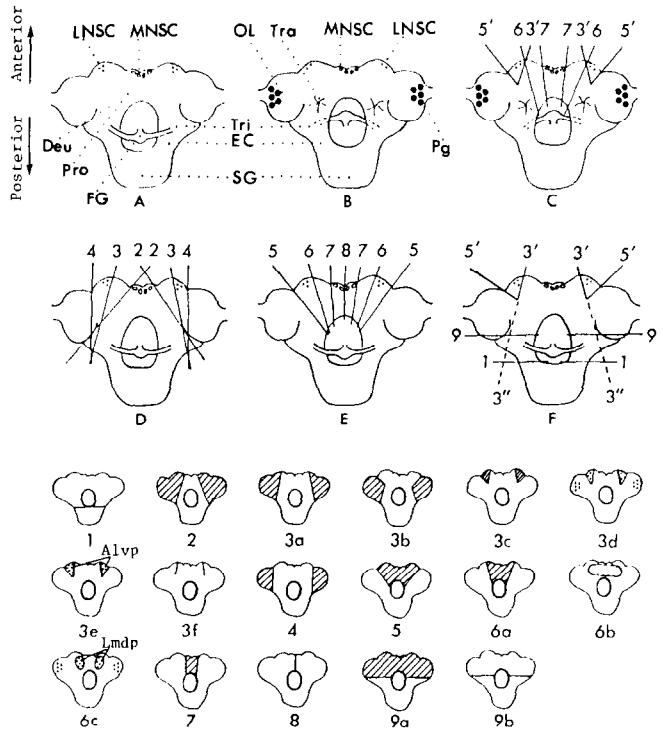


Fig. 1. Semi-schematic drawing of the complex of brain and suboesophageal ganglion to show the locations of surgical operations (experimental series 1–9b). A, D, E, F: ventral view, B, C: dorsal view. Deu, deutocerebrum; EC, circum-oesophageal connective; FG, frontal ganglion; LNSC, lateral neurosecretory cells; MNSC, medial neurosecretory cells; OL, optic lobe; Pg, pigment; Pro, protocerebrum; Sg, subocsohageal ganglion; Tra, trachea; Tri, tritocerebrum.
1, Bilateral transection circum-oesophageal connectives; severed at line 1 in F.
2, Excision of the shaded portion; severed at line 2 in D.
3a, Excision of the shaded portion; severed at line 3 in D.
3b, Excision of the shaded portion; severed at line 3" and 5' in F.
3c, Excision of the shaded portion; severed at line 3' and 5' in F.
3d, Excision of the cortex in stippled areas between line 3' and 5' in C.
3e, Excision of the cortex in stippled areas between line 3' and 5' in F; Alvp, cortex of anterolateral areas on the ventral side of the protocerebrum.
3f. Bilateral section; severed at line 3' in F.
4, Excision of the shaded portion (optic lobes); severed at line 4 in D.
5, Excision of the shaded portion; severed at line 5 in E.
6a, Excision of the shaded portion, severed at line 6 in E.
6b, Excision of the cortex in stippled area between the two line 6's in E.
6c, Excision of the cortex in stippled areas between line 6 and 7 in C; Lmdp, cortex slightly lateral to the median line on the dorsal side of the protocerebrum.
7, Excision of the shaded portion; severed at line 7 in E.
8, Midsagittal bisection of the protocerebrum; severed at line 8 in E.
9a, Excision of complex of protocerebrum, deutocerebrum and optic lobes; severed at line 9 in F.
9b, Bilateral section; severed at line 9 in F.

The diapause status of the eggs laid was examined 11 days after oviposition, since larvae hatched completely from non-diapause eggs 8–9 days after oviposition at 25°C.

RESULTS

Localization of effective areas in the brain of pupae producing non-diapause eggs

To confirm the inhibitory effect by the brain upon diapause-factor secretion from the suboesophageal ganglion, circum-oesophageal connectives of the non-diapause-egg-producing-pupa were transected bilaterally. As shown in Table 1 (series 1, upper), when the pupae were operated on within 1 h after larval–pupal ecdysis, about two-thirds of the resulting moths failed to produce non-diapause eggs exclusively; they laid either diapause eggs only or mixed populations of diapause and non-diapause eggs. This agrees with Fukuda's result (1953) showing that the pupal brain in the non-diapause-egg producer inhibits the secretory function of the suboesophageal ganglion through the circum-oesophageal connectives. However, when the operation was performed at 14 h after larval–pupal ecdysis, most of the resulting moths laid non-diapause eggs only (series 1, lower). These results

suggest that the function of the suboesophageal ganglion has been inhibited more rigidly in the older non-diapause-egg-producing-pupa than in the younger individual: the timing of operation is critical for examining the effect of the brain upon the suboesophageal ganglion. On the basis of the above results, the following 10 series of operations were performed to locate the inhibitory areas in the brain: they were carried out mainly within 1 h after larval–pupal ecdysis. The results are summarized in Table 1.

When the optic lobes were excised, all resulting moths laid non-diapause eggs (series 4). This suggests that the optic lobes do not participate in the control mechanism of secretion of the diapause factor.

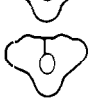
When the medial part of the protocerebrum was excised, the production of non-diapause eggs was interrupted significantly (series 5, 6a). However, the excision of small part of the protocerebrum containing the medial neurosecretory cells hardly interfered with the production of non-diapause eggs (series 7). These results therefore suggest that the parts slightly lateral to the median line are related to the inhibitory effect upon the suboesophageal ganglion.

Next, to examine whether the effective region is localized on the ventral or dorsal side of the brain, an area of cortex on the ventromedial region (series 6b) or areas on the dorsal region of the protocerebrum (series 6c) were stripped off. The excision of the former did not show an appreciable effect on changing the voltinism of the eggs. Whereas the excision of the latter within 1 h of larva–papal ecdysis, which removed the cortical areas slightly lateral to the median line on the dorsal side of the protocerebrum (Lmdp), altered significantly the eggs to the diapause type (series 6c, upper). Therefore, these results suggest that the cortical areas of the Lmdp are closely related to the inhibitory mechanism over the secretory function in the suboesophageal ganglion. On the other hand, when the cortices of the Lmdp were excised at 14 h after larval–pupal ecdysis, the voltinism was not interfered so effectively (series 6c, lower). These results suggest again that the timing of the operation is critical for changing the secretory function of the suboesophageal ganglion, as also shown in the transection experiment of circumoesophageal connectives (series 1).

Mid-sagittal bisection of the protocerebrum did not affect the non-diapause property of the eggs (series 8), which suggests that the inhibitory effect from the Lmdp arrives at the suboesophageal ganglion without passing through the contralateral hemisphere.

The complex of optic lobes, protocerebrum, and

Table 1. Effect of surgical operations on the brain of pupae producing non-diapause eggs on the production of non-diapause eggs

Experimental series	Operations	Operation time (hours after larval–pupal ecdysis)	No. of moths laying eggs	No. of moths laying diapause eggs only	No. of moths laying mixed diapause eggs and non-diapause eggs	No. of moths laying non-diapause eggs only
1		0–1	36	4	18	14
		14	34	1	4	29
4		0–1	15	0	0	15
5		0–1	22	12	6	4
6a		0–1	19	12	6	1
6b		0–1	15	0	0	15
6c		0–1	27	7	12	8
		14	27	1	2	24
7		0–1	13	0	3	10
8		0–1	17	0	0	17
9a		0–1	12	9	3	0
		14	27	26	1	0
9b		0–1	15	14	1	0
		14	17	17	0	0
10	Sham operation	0–1	28	0	0	28
		14	32	0	0	32

The details of the cutting planes in operations for each experiment are shown in Fig. 1.

most of deutocerebrum was excised within 1 h or at 14 h after larval–pupal ecdysis. Most of the eggs laid were of the diapause type, regardless of the timing of the operation (series 9a). An almost similar effect was observed when the brain was severed at the level between the deutocerebrum and the tritocerebrum (series 9b).

Localization of effective areas in the brain of the pupae producing diapause eggs

To ascertain the stimulatory effect by the brain upon the secretory function of the suboesophageal ganglion, the circum-oesophageal connectives of diapause-egg-producing-pupae were transected bilaterally within 1 h or at 14 h after larval–pupal ecdysis. As shown in Table 2 (series 1), most of the resulting moths laid non-diapause eggs only or mixed populations of non-diapause and diapause eggs, irrespective of the time at which the operations were carried out. This agrees with Fukuda's finding (1951, 1953); i.e. the brain in the diapause-egg-producing-pupa stimulates the secretion of the diapause factor from the suboesophageal ganglion through the circum-oesophageal connectives. This also suggests that in the diapause-egg-producing-pupa, the timing of the operation is not so critical as in the case of the pupae producing non-diapause eggs, at least up to 14 h after larval–pupal ecdysis. Consequently, we carried out 13 series of experiments to locate stimulatory areas in the brain, the operations being carried out within 14 h after larval–pupal ecdysis. The results are summarized in Table 2.

When the lateral part of the protocerebrum together with the optic lobes were excised, the effect was similar to that of the transection experiments of the circum-oesophageal connectives (series 2, 3a). How-

Table 2. Effect of surgical operations on the brain of pupae producing diapause eggs on the production of diapause eggs

Experimental series	Operations	Operation time (hours after larval–pupal ecdysis)	No. of moths laying eggs	No. of moths laying diapause eggs only	No. of moths laying mixed diapause eggs and non-diapause eggs	No. of moths laying non-diapause eggs only
1		0–1	26	2	10	14
		14	36	7	9	20
2		14	10	1	2	7
3a		14	22	1	7	14
3b		14	32	20	5	7
3c		14	15	0	9	6
3d		14	19	17	1	1
3e		14	21	0	3	18
3f		14	19	2	4	13
4		14	10	10	0	0
5		14	28	21	7	0
8		14	18	14	2	2
9a		14	36	35	1	0
9b		14	18	17	1	0
10	Sham operation	0–1	29	29	0	0
		14	62	62	0	0

The details of the cutting planes in operations for each experiment are shown in Fig. 1.

ever, when only the optic lobes were excised, the operation did not show the appreciable effect on the production of the diapause eggs (series 4). From these results, it is suggested that the lateral parts of the protocerebrum contain the areas which are competent for stimulating the suboesophageal ganglion.

Subsequently, to examine whether the area related to stimulation is localized in the anterior part or the posterior part of the lateral protocerebrum, the anterior parts (series 3c) and the posterior parts together with optic lobes (series 3b) were excised separately. As shown in each experimental series, the excision of the anterior parts affected the production of the diapause eggs more than that of posterior parts with the optic lobes. To restrict the effective area, the cortical areas of the dorsal side (series 3d) or corresponding areas on the ventral side (series 3e) in the anterior parts of the lateral protocerebrum were stripped off. The excision of the dorsal side scarcely interfered with the diapause property of the eggs laid. On the other hand, when the anterolateral areas on the ventral side of the protocerebrum (Alvp) were stripped off, most of the resulting moths laid non-diapause eggs only (series 3e). These results suggest that the areas related to the stimulatory effect upon the suboesophageal ganglion are localized in the cortical areas of the ventral side, designated as the Alvp, but not in the dorsal side. However, the excision of a more extensive part of the protocerebrum including the Alvp scarcely interfered with the production of diapause eggs (series 5). The discrepancy between these results will be discussed later. Almost the same result as that achieved by removal of the cortical areas of the Alvp was also obtained when the protocerebrum was severed obliquely at the level between the medial part and Alvp (series 3f).

Mid-sagittal bisection of the protocerebrum scarcely affected the production of the diapause eggs (series 8), suggesting that the stimulatory effect from the Alvp arrives at the suboesophageal ganglion without passing through the contralateral hemisphere.

When the complex of optic lobes, protocerebrum and most of deutocerebrum was excised, many of the eggs entered diapause (series 9a). A comparable effect on the voltinism of the eggs was observed by the bilateral section at the level between the deutocerebrum and the tritocerebrum (series 9b).

DISCUSSION

Fukuda (1951, 1952, 1953) was the first to show that the secretion of diapause factor is controlled by the brain through the circum-oesophageal connectives. This finding was confirmed and extended to include the control mechanism of secretion not only from the female suboesophageal ganglion, but also from the male suboesophageal ganglion (Fukuda and Sonobe, 1973; Sonobe and Keino, 1975; Sonobe *et al.*, 1977). As shown in experimental series 1 in Tables 1 and 2, we also confirmed the stimulatory effect and inhibitory effect of the brain of the diapause-egg-producing-pupa and the non-diapause-egg-producing-pupa, respectively, as described by Fukuda. We further tried to clarify the functional localization in the brain related to controlling secretion of the diapause factor from the suboesophageal ganglion. In this study we used surgery exclusively, including transection and partial excision, since it is known that these techniques are useful for limiting any functional areas in the physiologically active organ [see, for example, Nishiitsutsuji-Uwo and Pittendrigh (1968), and Rüegg *et al.* (1983)].

In the non-diapause-egg-producing-pupa, the excision of the cortex in the Lmdp regions brought about a significant production of diapause eggs (Table 1, series 6c). From this result it is suggested that the Lmdp area may function *in situ* as an inhibitory source upon the secretory function of the suboesophageal ganglion (Fig. 2A). The excision of these putative inhibitory areas was more effective within 1 h after larval–pupal ecdysis than at 14 h after larval–pupal ecdysis (Table 1, series 6c). This finding agrees with the result of the transection experiment of the circum-oesophageal connectives (Table 1, series 1), which suggests therefore that the inhibitory effect must have already reached to the suboesophageal ganglion in the 14-h old pupa: the fact that all the resulting eggs did not become the diapause type, even after removal within 1 h after larval–pupal ecdysis, suggests that the Lmdp cortex has started to inhibit the function of the suboesophageal ganglion before larval–pupal ecdysis. We could not test this hypothesis, because it was difficult to operate surgically before larval–pupal ecdysis. Transection at the level between the tritocerebrum and the deutocerebrum was more effective than bilateral transection of the circum-oesophageal connectives (compare series 9b with series 1 in Table 1). Moreover, when the complex of protocerebrum with optic lobes and deutocerebrum was excised, the resulting eggs almost exclusively developed as the diapause type (Table 1, series 9a). These results suggest that the tritocerebrum is involved in stimulatory function upon the suboesophageal ganglion, and that normally its

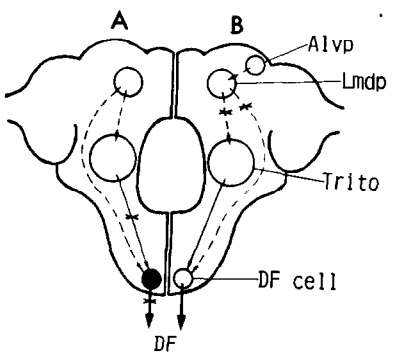


Fig. 2. Possible relationship between the putative stimulatory pathway and inhibitory pathway in the brain–suboesophageal ganglion system. A, brain–suboesophageal ganglion complex in pupae producing non-diapause eggs; B, brain–suboesophageal ganglion complex in pupae producing diapause eggs. ——, stimulatory effect; – – –, inhibitory effect; x, pathway depressed normally; DF-cell, diapause factor cell which may accumulate secretory material (A) and liberate it (B); Alvp, cortex of anterolateral area on the ventral side of the protocerebrum; Lmdp, cortex slightly lateral to the median line on the dorsal side of the protocerebrum; Trito, tritocerebrum. For further details, see text.

function may be suppressed by another area in the protocerebrum or the deutocerebrum, possibly by the cortex of the Lmdp, so long as the nervous connections between the protocerebrum and the tritocerebrum are intact. The cortex of the Lmdp may depress the tritocerebrum in addition to the suboesophageal ganglion (Fig. 2A).

In the pupae producing diapause eggs, excision of the cortices of the Alvp interfered with the production of such eggs (Table 2, series 3e), suggesting that these areas are related to the stimulatory mechanism in the brain–suboesophageal ganglion system. However, excision of more extensive area of the protocerebrum including the Alvp was less effective than excision of the only Alvp (compare 5 with 3e in Table 2). These results lead us to surmise that the medial part of the protocerebrum participates in the inhibitory mechanism in the brain–suboesophageal ganglion system, and that these areas may correspond with the Lmdp on the basis of the case of the non-diapause-egg-producing-pupa (Fig. 2, A, B). Subsequently, to examine the relationship between the Alvp and the putative areas related to the inhibitory effect in the protocerebrum: that is, the Lmdp, the brain was severed bilaterally at the level between the Alvp and the Lmdp. The bilateral severances were effective on changing the voltinism of the eggs (Table 2, series 3f). This result suggests that the cortical areas of the Alvp contribute to the stimulatory mechanism in the brain–suboesophageal ganglion system by depressing the inhibitory effect in the Lmdp cortical areas rather than by stimulating directly the secretory function in the suboesophageal ganglion (Fig. 2B): the suboesophageal ganglion must be freed from the inhibitory effect of the Lmdp by the inhibitory action of the Alvp.

An additional pathway of the inhibitory and stimulatory effects is suggested from the operations on the protocerebrum in the diapause-egg-producing-pupa (series 3e, 5 in Table 2). That is to say, at least another area except for the protocerebrum must stimulate the secretory function of the suboesophageal ganglion in the diapause-egg-producing-pupa, since the excision of the protocerebrum brings about positively the production of the diapause eggs (Table 2, series 5, 9a). Thus, to examine the source of the stimulatory effect in the brain, it was transected at the level between the tritocerebrum and the deutocerebrum (Table 2, series 9b). The production of the diapause eggs was stimulated by the transection of this position as well as by the excision of the protocerebrum (compare 9b with 5, 9a in Table 2). This suggests that the tritocerebrum exerts a stimulatory effect upon the suboesophageal ganglion in the same way as in the non-diapause-egg-producing-pupa. Therefore, it is surmised that normally the Lmdp cannot exert its inhibitory effect on the tritocerebrum owing to inhibition from the Alvp, and consequently the tritocerebrum can stimulate the secretion of diapause factor from the suboesophageal ganglion (Fig. 2B): the Alvp contributes to the stimulatory mechanism in the brain–suboesophageal ganglion system by depressing the inhibitory effect of the Lmdp toward the tritocerebrum.

As shown in Fig. 2, we propose that the difference in the cerebral control of the suboesophageal ganglion in the non-diapause-egg-producing-pupa and the diapause-egg-producing-pupa is due to the functional differences in the Alvp between the two types of pupae. The cause which induces this difference is obscure, but it is conceivable that environmental factors, such as temperature and light conditions, might predetermine the function of the Alvp during embryonic development. It has been suggested that restricted areas of the protocerebrum function as a photoreceptor in several insects (Williams, 1969; Steel and Lees, 1977; Kono *et al.*, 1983). On this basis, it would be interesting to see whether the cortex of the Alvp functions as the receptor of environmental factor in *Bombyx* silkworm, as well.

In our present experiments, it is not possible to limit the specific cells which participate in the putative Alvp–Lmdp–tritocerebrum–suboesophageal ganglion system and to determine the neurochemical nature of the control in the system. However, on the basis of the process of inhibition and stimulation in the brain which we proposed in Fig. 2, it is surmised that nervous impulses presumably participate in the system. On the other hand, the possibility that neurosecretory axons may arrive directly to the suboesophageal ganglion from some centre in the brain cannot be ruled out, since a substance having diapause-factor activity has been found in the brain (Sonobe and Keino, 1975). To understand more precisely the mechanism by which the brain controls the secretion of diapause factor from the suboesophageal ganglion, further analysis is required.

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