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Immunocytochemical localization of dromyosuppressin (DMS) in Phormia regina (Meigen) and effect of DMS and benzethonium chloride on crop muscle contractions.

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IMMUNOCYTOCHEMICAL LOCALIZATION OF DROMYOSUPPRESSIN (DMS) IN *PHORMIA REGINA* (MEIGEN) AND EFFECT OF DMS AND BENZETHONIUM CHLORIDE ON CROP MUSCLE CONTRACTIONS

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by

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ABSTRACT

IMMUNOCYTOCHEMICAL LOCALIZATION OF DROMYOSUPPRESSIN (DMS) IN *PHORMIA REGINA* (MEIGEN) AND EFFECT OF DMS AND BENZETHONIUM CHLORIDE ON CROP MUSCLE CONTRACTIONS

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Dromyosuppressin (DMS) has been recently isolated from the adult dipteran, *Drosophila melanogaster*. The myosuppressin family of peptides is known to suppress spontaneous muscle contractions in several other insect species. The myosuppressin family of peptides is also unique in having a nonpeptidal agonist, benzethonium chloride (Bztc). Dromyosuppressin has not yet been isolated or localized in *Phormia regina*, an important model system for feeding behavior and reproductive biology.

Immunocytochemistry has shown DMS immunoreactivity in the central nervous system and crop of *P. regina*. Immunoreactive cells and nerve fibers were located throughout the brain, subesophageal ganglion, optic lobes, and thoracico-

abdominal ganglion. Immunoreactive fibers were also located in the hypocerebral ganglion/corpora cardiaca complex, cardiac recurrent nerve, on the crop duct, and were widespread over the surface of the crop.

Previous studies on feeding behavior in *P. regina* have concluded that the crop muscle is not under nervous control, however, the extensive network of nerve fibers immunoreactive to DMS on the surface of the crop muscle, indicates that DMS may play a role in crop functioning. Since DMS is known to be a myoinhibitory peptide, DMS may be active in suppressing spontaneous crop muscle contractions in *P. regina.*

An *in vitro* bioassay allowing for direct application of DMS and Bztc to the crop muscle demonstrated that DMS and Bztc are both significantly effective at slowing crop contractions. The average rate of crop muscle contraction with no treatment was 48.2 contractions per minute. After applications of 1 μl of 10^{-6} M DMS or 1 μl of 10^{-3} M Bztc, the contraction rate decreased to 2.2 ± 0.2 contractions per minute and 6.1 ± 0.7 contractions per minute, respectively. These studies demonstrate that in *P. regina*, DMS and its nonpeptidal agonist, Bztc are effective in slowing spontaneous crop contraction, *in vitro*.

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CHAPTER I

LITERATURE REVIEW

Introduction

Many different neuropeptides have been isolated in a wide variety of insect species, but little is known about the specific functions of the majority of these peptides. Neuropeptide research is a vast and important area of study because neuropeptides "control life processes involved in development, behavior, metabolism, and reproduction, and do so at exceedingly low levels (Masler *et al.*, 1993)". Understanding the roles of neuropeptides is essential to understanding how insect systems work at a more detailed level and may contribute to the development of new and more sophisticated control agents.

The primary focus of this laboratory has been on the interactions among nutrition, endocrines, and oogenesis in the black blow fly, *Phormia regina* (Yin and Stoffolano, 1990; Yin *et al.*, 1994). Neuropeptides are involved in the regulation of these processes, so it was reasonable to consider that newly isolated peptides may also be involved in feeding and reproduction. Myosuppressins are known to suppress spontaneous muscle contractions in both the hindgut, oviduct, and heart of other insect species (Holman *et al.*, 1986; Lange *et al.*, 1991; Robb *et al.*, 1989). Dromyosuppressin (DMS) has recently been isolated from the dipteran, *Drosophila melanogaster* (Nichols, 1992). It has not yet been tested in the black blow fly, *P. regina*, an important model system for feeding behavior (Dethier, 1976) and reproductive biology (Yin and Stoffolano, 1990).

Neuropeptides in Insects

The nervous system was first reported to be a source for endocrine agents when Kopèc (1917) discovered that the brain controlled molting and metamorphosis in the gypsy moth. Scharrer (1941) first identified and described neurosecretory cells and the neuroendocrine process in insects (Keeley and Hayes, 1987). The first two insect peptides characterized structurally were proctolin, an inhibitor of both visceral and somatic muscle (Starratt and Brown, 1975), followed by the discovery of adipokinetic hormone (AKH) (Stone *et al.*, 1976).

Until recently, little research had been done on insect neurohormones for several reasons; they are difficult to isolate and define, sometimes unstable, and often present in individual insects in very small amounts (Keeley and Hayes, 1987). Several years after the isolation of proctolin and adipokinetic hormone, many advances in techniques for isolation, purification and structural characterization of peptides were made such as; reverse-phase high-performance liquid chromatography, fast-atom bombardment mass spectrometry, and gas-phase sequencing (Holman *et al.*, 1990). The techniques available now make it less expensive to obtain large quantities of pure synthetic peptides and analogs for physiological and immunocytochemical studies. As a result, a large number of new peptides have been isolated and characterized in a wide variety of insect species.

Most of the known insect neuropeptides are members of a few large families of peptides, which are grouped by structural similarities or functions. In a 1990 review of insect neuropeptides, Holman *et al.* (1990) describes the following eight peptide families. The AKH/RPCH (red pigment concentrating hormone) family is

made up of peptides similar in structure to locust adipokinetic hormone. The myotropins are composed of subgroups consisting of proctolin, leucokinins, sulfakinins, and pyrokinins with structural differences between the groups. The next family, which includes the myosuppressins, is the FMRFamide-related peptides. Another family is made up of the diuretic and antidiuretic peptides, which control water balance, ion balance, and waste removal. The remaining families are eclosion hormones and steroidogenic hormones, which stimulate the production of steroid hormones such as ecdysone, and allatotropins, which stimulate the release of juvenile hormone from the corpora allatum.

Neuropeptides in Diptera

A considerable amount of work has been done on neuropeptides in Diptera. Recently, allatostatins, myosuppressins, tachykinins, and sulfakinins have all been immunocytochemically localized in dipterans (Table 1.1). Though many peptides have already been isolated in dipteran species, little is known about their respective functions. Allatostatins are known to inhibit juvenile hormone production in other orders of insects, but have not been shown to do so in the dipterans tested (Yoon and Stay, 1995). This suggests that the same peptide may have different roles in different species. Tachykinin and allatostatin immunoreactivity is found throughout the brain and gut tissues of *Calliphora vomitoria* and both peptides are suggested to have roles in controlling contraction of the gut (Duve and Thorpe, 1994; Lundquist *et al.*, 1994; Nässel *et al.*, 1995). No functions of myosuppressin or sulfakinin are known in Diptera, but in other species they have been shown to be myoinhibitory and myostimulatory, respectively (Nachman *et al.*, 1993).

Myosuppressin Family of Peptides

Myosuppressins are part of the FMRFamide family which was first identified with the isolation of FMRFamide from the clam, *Macrocallistica nimbosa*, by Price and Greenberg (1977). FMRFamide is the primary member of an interphyletic group of structurally related peptides which have been isolated from a wide range of vertebrate and invertebrate species (Greenberg and Price, 1992; Raffa, 1989). The myosuppressins are the first and only myoinhibitory peptides isolated from this peptide family thus far (Fonagy *et al.*, 1992).

The first myosuppressin to be isolated and structurally identified was leucomyosuppressin (LMS) from the cockroach, *Leucophaea maderae,* which inhibits spontaneous contraction of the adult cockroach hindgut (Holman *et al.*, 1986). It was later shown that LMS also inhibits evoked transmitter release at the neuromuscular junction of the mealworm, *Tenebrio molitor* (Yamamoto *et al.*, 1988).

The isolation of LMS was followed by SchistoFLRFamide, which was isolated from the adult locust, *Schistocerca gregaria* (Robb *et al.*, 1989). SchistoFLRFamide was not named as a myosuppressin based on sequence similarities only because, in addition to suppressing spontaneous heart contractions, it also has a strong potentiating effect on the extensor tibiae muscle and appears to have long term potentiating effects on the heart (Robb *et al.*, 1989).

SchistoFLRFamide was later shown to also be involved in suppressing contractions of the locust oviduct (Lange *et al.*, 1991). Myosuppressin was then

isolated in two dipteran species; dromyosuppressin from *Drosophila melanogaster* (Nichols, 1992) and the structurally identical neomyosuppressin from the grey flesh fly, *Neobellieria bullata* (Fonagy *et al.*, 1992). Myosuppressins have since been immunocytochemically localized in two adult dipterans; LMS in a blood-feeding dipteran, *Stomoxys calcitrans* (Meola *et al.*, 1991), and DMS in *D. melanogaster* (McCormick and Nichols, 1993).

The structures of the myosuppressins (Table 1.3) isolated from the cockroach, locust, and fruit fly are highly identical, differing only in the N-terminal amino acid residue. Neomyosuppressin, isolated from the flesh fly, is structurally identical to dromyosuppressin.

Nonpeptidal Mimics

In addition to being important in understanding physiological systems of insects, neuropeptides may also offer new approaches to insect control as novel insecticides. Unfortunately, peptides are not good candidates for control agents for several reasons. Peptides are slow and expensive to synthesize and their chemistry makes them susceptible to degradation under field conditions (Holman *et al.*, 1990). Application would also be difficult because their polarity would make uptake through the cuticle difficult; and, if fed, they may be easily digested (Masler *et al.*, 1993).

Nonpeptidal mimics offer a better source for the development of insect control agents and are useful research tools. Nonpeptidal mimics are better suited for field application because their chemistry makes them more resistant than neuro-

peptides to degradation in the insect gut or under field conditions (Lange *et al.*, 1995; Nachman *et al.*, 1996). They are also generally more abundant, easy to produce in large quantities, and often commercially available.

Benzethonium chloride (Bztc), an agonist to the myosuppressins, is one of the first nonpeptidal agonist reported for an insect neuropeptide (Nachman *et al.*, 1996; Nachman *et al*, 1999). Bztc effectively mimics the effect of leucomyosuppression on the inhibition of cockroach hindgut contractions, suppression of mealworm neuromuscular junction, and suppression of locust oviduct contractions (Nachman *et al*, 1996). Structurally, Bztc shares several specific functional groups with the myosuppressins (Fig.1.1) and can mimic both the binding and activation regions (Nachman *et al.*, 1996).

The present study was undertaken to determine if dromyosuppressin immunoreactivity could be localized in *P. regina*; and, if present, to begin to investigate what functions it might have in this species. The purpose of the following chapters is to describe the distribution of dromyosuppressin in *P. regina* and then to determine if DMS and Bztc play a role in the control of crop contractions. With this information known, later studies can expand on the functions of the myosuppressins in *P. regina*.

Table 1.1 A survey of neuropeptides present in adult Diptera

Peptide	Species	Localization	Function	References
Allatostatin	*D.melanogaster*	Several pairs of cells in the brain, optic lobes, SOG, and TAG. Many cells in posterior midgut and fibers extending to hindgut.	Suggested to inhibit contractions of hindgut and act as a neuromodulator. Does not act as a JH inhibitor in Diptera, but does inhibit JH in cockroach.	Yoon and Stay, 1995
Callatostatin	*C.vomitoria*	Fibers in TAG with axons reaching hindgut, rectum, rectal papillae, and oviduct. Many cells in brain, SOG, and posterior midgut.	Suggests that callatostatins may be involved in regulating JHB_3 production in other stages than adult. Also suggest control of gut musculature.	Duve *et al.*, 1993 and Duve and Thorpe, 1994
Callitachykinin	*C.vomitoria*	Brain, SOG, TAG, and endocrine cells in the gut.	No functions identified, but known to stimulate hindgut contractions in the cockroach.	Nässel *et al.*, 1995
Dromyosuppressin	*D.melanogaster*	Two cells near rectum, brain, optic lobes, crop.	Suggests multiple functions	McCormick and Nichols,1993
Locustatachykinin	*C.vomitoria*	Many distinct cells and fibers in brain, TAG, SOG, and cells in the foregut and midgut.	Suggests that it may have a role in intestinal function.	Lundquist *et al.*, 1994
Sulfakinin	*C.vomitoria*	Four pairs of neurons in the brain with extensive projections.	Suggests that sulfakinins may act as neurotransmitters or neuromodulators in Diptera.	Duve *et al.*, 1994

SOG= subesophageal ganglion; TAG= thoracico-abdominal ganglion; JH= juvenile hormone

Table 1.2 Distribution of the myosuppressin family of peptides in Diptera and other species

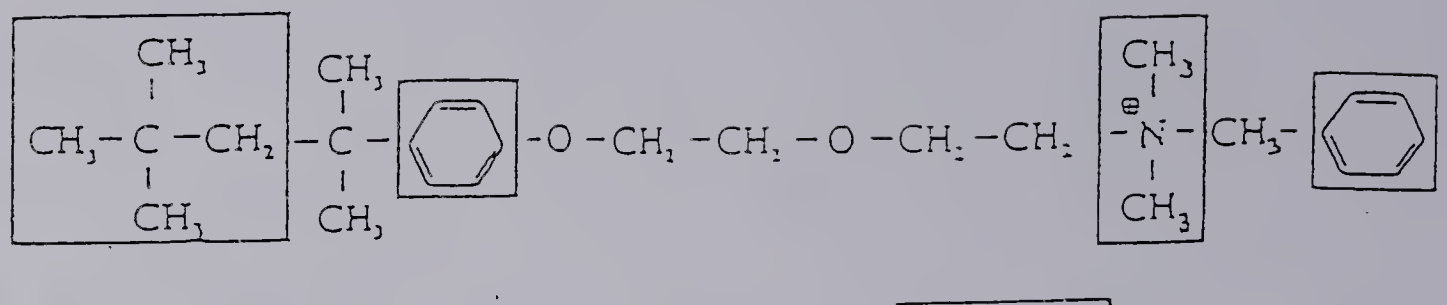
Myosuppressin	Species	Localization	Function	References
IN THE DIPTERA				
Dromyosuppressin	*D.melanogaster*	Two cells near rectum, brain, optic lobes, crop.	Suggests several functions.	McCormick and Nichols, 1993
Dromyosuppressin, Leucomyosuppressin	*S.calcitrans*, *H.irritans*	Hypocerebral ganglion, surface of proventriculus.	No functions suggested.	Meola *et al.*, 1996
Leucomyosuppressin	*S.calcitrans*	Brain, thoracic abdominal ganglion, nervi corpus cardiacum entering corpus cardicum and wall of aorta extending to proventriculus.	Knowledge of function limited to inhibition of muscle activity.	Meola *et al.*, 1991
Neomyosuppressin	*N.bullata*	Not isolated or localized in this paper.	Inhibition of muscle activity.	Fonagay *et al.*, 1992
IN OTHER SPECIES				
Leucomyosuppressin	*L.maderae*	Isolated from head extracts of *L.maderae*. Also found in hindgut.	Functions as an inhibitor of visceral muscle.	Holman *et al.*, 1986
Leucomyosuppressin	*T.molitor*	Not localized or isolated in this paper.	Inhibits evoked transmitter release from presynaptic membrane of excitatory motor neurons terminating in skeletal muscle.	Yamamoto *et al.*, 1988
SchistoFLRFamide	*S.gregaria*	Not localized or isolated in this paper.	Potentiating effect on spontaneous heart contraction and extensor tibiae muscle.	Robb *et al.*, 1989

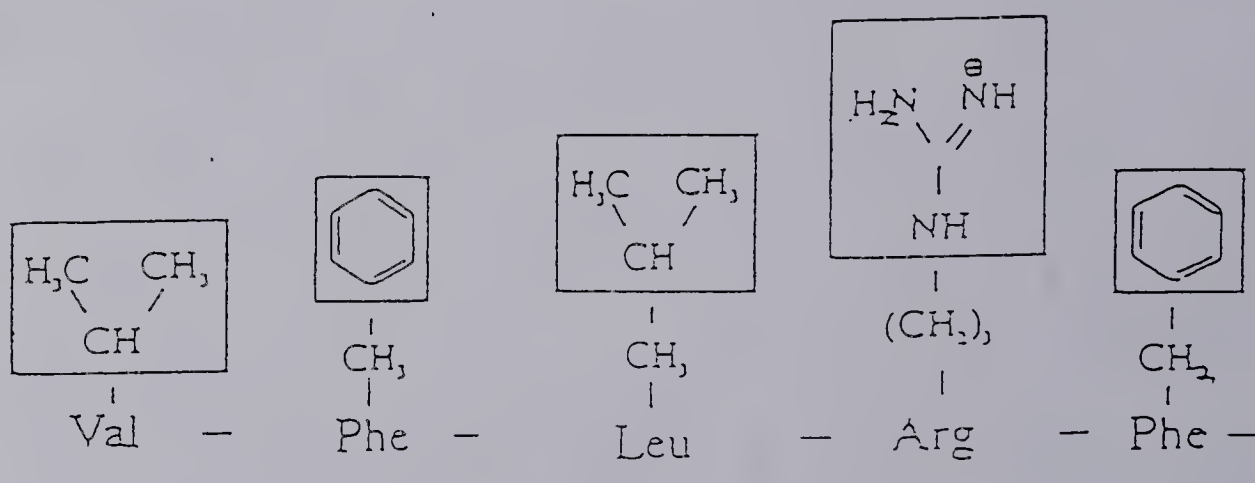
Table 1.3 A comparison of the structures of myosuppressins isolated from the fruit fly *Drosophila*, the flesh fly *Neobellieria*, the cockroach *Leucophaea*, and the locust *Schistocerca*.

Fruit fly	Thr	Asp	Val	Asp	His	Val	Phe	Leu	Arg	Phe	NH_2
Flesh fly	Thr	Asp	Val	Asp	His	Val	Phe	Leu	Arg	Phe	NH_2
Cockroach	pGlu	Asp	Val	Asp	His	Val	Phe	Leu	Arg	Phe	NH_2
Locust	Pro	Asp	Val	Asp	His	Val	Phe	Leu	Arg	Phe	NH_2

Figure 1.1 Comparative structures of benzethonuim chloride (Bztc) (Upper) and the C-terminal portion of the myosuppressins (Lower). Both have two phenyl rings, a basic, positively charged group, and at least one branch-chained, hydrophobic group (from Nachman *et al.*, 1996).

Benzethonium chloride





Val – Phe – Leu – Arg – Phe – NH_2

C-terminal myosuppressin

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CHAPTER II

IMMUNOCYTOCHEMICAL LOCALIZATION OF DROMYOSUPPRESSIN IN ADULT, FEMALE *PHORMIA REGINA*

Introduction

In spite of their importance, few studies of the myosuppressin peptides have been undertaken in the Diptera. To date, myosuppressins have been chemically isolated in *Drosophila melanogaster* (Nichols, 1992), and the flesh fly, *Neobelleria bullata* (Fonagy *et al.*, 1992) and reported in the stable fly, *Stomoxys calicitrans* (Meola *et al.*, 1991) and the horn fly, *Hematobia irritans* (Meola *et al.*, 1996). The majority of work on myosuppressins has been done in the locust, *Schistocerca gregaria* (Robb *et al.*, 1989), the cockroach, *Leucophaea maderae* (Yamamoto *et al.*, 1988; Holman *et al.*, 1986), and the mealworm, *Tenebrio molitor* (Yamamoto *et al.*, 1988). The presence of myosuppressin has not yet been reported in the black blow fly, *Phormia regina*, despite the importance of this fly as a model system for feeding behavior (Dethier, 1976) and reproductive biology (Yin and Stoffolano, 1990).

Myosuppressin is considered a myoinhibitory peptide and is known to suppress spontaneous heart, hindgut, and oviduct contractions in non-dipteran species (Holman *et al.*, 1986; Lange *et al.*, 1991; Robb *et al.*, 1989). No specific functions of this peptide have yet been determined in the Diptera. In dipterans, the myosuppressions isolated are structurally identical (see Table 1.3). Based on the widespread distribution of DMS in the CNS, crop, and hindgut of *D. melanogaster*

(McCormick and Nichols, 1993) and because all myosuppressins isolated from Diptera to date have the same structure, DMS is likely to have a similar distribution and function in *P. regina*.

The purpose of this study was to determine if dromyosuppressin was also a brain-gut peptide in the blow fly, *Phormia regina*. DMS immunoreactive tissues were identified in the central nervous system and digestive tract of *P. regina* using immunocytochemical techniques.

Materials and Methods

Maintaining Flies

Flies used in these experiments were reared using the procedures outlined by Stoffolano (1974). Flies were reared under a 16:8 LD photoperiod at 28° ± 2° C, and 50% relative humidity. Flies were grouped by time of emergence and all individuals that emerged in a 10-h time period were fed at the same time and considered the same cohort. Only females were used because preliminary immunostaining results showed no difference between the sexes. After emergence, all flies were placed in a 23 x 23 x 23 cm^3 cage and (except when starved) provided with 4.3% sugar solution from a white plastic cup measuring 8 cm high and 11.5 cm in diameter with an Absorbal® feeding wick placed through the cover.

Sugar-fed flies were fed three days or longer on 4.3% sugar solution and then used for experiments. Liver-fed flies were fed on 4.3% sugar solution for 3 days. At the end of day 3, the sugar solution was removed and only water was

provided overnight. On day 4, they were given a meal of chopped beef liver for 120 minutes. The liver was provided on a clear plastic, 11.5 cm diameter lid and was chopped with a knife until it was soft and almost of a liquid consistency. Immediately following this feeding period, the flies were weighed and only females weighing 54 mg or more were used in experiments. This standardization of weight assured that each consumed a large enough protein meal to ensure that completion of the neuroendocrine and physiological cascade leading to oogenesis would occur (Yin and Stoffolano, 1990; Yin *et al.*, 1994).

Dissection Techniques

Techniques varied depending on both the type of tissue being removed for study and the diet. Before any dissections, flies were removed from the cage, anesthetized with CO_2, and placed on ice.

For the basic gut dissection, flies were pinned to a wax bottom dissecting dish, dorsal side up, with one pin slightly off center (to avoid pinning part of the gut tissue down) in the meso-thorax. Wings were removed for greater accessibility to the abdomen. The tip of the proboscis was then cut off to allow for easier removal of the attached foregut. The cuticle on the abdomen, close to where it joins the thorax, was gently pulled away with fine forceps. Using forceps, the midgut and crop duct could be grasped and the midgut, proventriculus, foregut, and attached crop duct pulled out of the thorax. The rest of the gut could then be grasped and removed. This usually results in the removal of the entire gut up to portions of the hindgut past the Malpighian tubules, but not including the posterior hindgut. The gut was then placed in *Phormia regina* saline (Chen and Friedman, 1975) in a

black, three welled, ceramic dish for better visibility. Trachea and any other attached tissues were cleared away and the clean gut was then transferred to 4% paraformaldehyde.

The normal dissection described above was used for liver-fed flies with slight modification because the gut of the liver-fed fly becomes distended and is extremely fragile. Also, the crop is greatly distended with the liver meal and must be cleaned of the ingested liver. To clean the crop, a small tear was made on one of the crop lobes and it was rinsed in saline until the contents were completely emptied. The normal dissection technique also results in the removal of the hypocerebral ganglion/corpora cardiaca, and corpus allatum/cardiac recurrent nerve complex, which are attached to the proventriculus and foregut. This complex is visible under the dissecting scope as a light blue triangle between the foregut and proventriculus.

In dissections where the whole gut, including the complete hindgut was required, great care was taken. The midgut and foregut were removed as described above; but, instead of pulling the remaining gut tissue from the fly, the whole abdomen was removed along with the midgut and foregut. After removal, the abdomen was placed in cold saline in a black, three-welled, ceramic dish and dissected accordingly. The cuticle, ovaries, fat body, and other surrounding tissues were dissected away under saline to allow for careful removal of the hindgut.

For brain dissection, anesthetized flies were injected in the meso-thorax with about 1.5 ml of 4% paraformaldehyde with a plastic, 10 ml syringe until the ovipositor and proboscis protruded. This was done to begin fixing the brain tissues,

which made it easier for the complete removal of the brain and surrounding tissues. The rest of the dissection was done in paraformaldehyde instead of saline. After injection of fixative, the abdomen was cut off at a slight angle just before the thorax begins. The thorax was then pinned down ventral side up in a dissecting dish, the antennae removed, and the proboscis cut off. A fine pin was inserted into the opening in the head where the proboscis was cut away. Next, the cuticle and muscles of the thorax were carefully pulled away starting with the outside edges of the thorax while carefully leaving the thoracico-abdominal ganglion attached. Once the thorax tissues had been completely removed, the pin in the head capsule was removed and the head capsule was carefully peeled away from the brain with a forcep. This was accomplished by carefully inserting one tip of the forcep under the cuticle and peeling the lifted cuticle away with the other forcep. Once the cuticle had been completely removed, leaving the brain and attached thoracico-abdominal ganglion, the complex was moved to a black, three-welled, ceramic dish and cleaned of trachea plus any attached tissues under fresh cold paraformaldehyde.

Immunocytochemical Protocol

The immunocytochemical protocol followed was described by Davis (1987), with the following modifications. Before the application of the primary antibody, preparations were soaked in 10% nonimmune goat serum (NGS) in phosphate buffered saline with Triton X100 (PBST) for one hour instead of the 4 hours described by Davis. In addition, the preparations were also left in the primary antibody, dromyosuppressin, at a 1:500 dilution, for 36 hours instead of 18 hours. After primary incubation, the preparations were washed 5 times in PBST at 1 hour

each wash and then incubated for 1 hour in NGS before the application of the secondary rhodamine-labeled antibody. The addition of the flourescently labeled secondary antibody was done in the dark and preparations were kept in the dark for the duration of the protocol to ensure that the light would not effect the fluorescence of the secondary antibody.

Further modifications were made after the secondary antibody incubation period. Tissues were washed only 3 times at 20 minute intervals in PBST with gentle agitation and then cleared in a glycerin series of 40%, 60%, and 80% glycerin in 10% NGS/PBST at 30 minutes each. At the end of the last glycerin wash, preparations were then mounted on slides using Vectashield® mounting medium, observed, and photographed using both the fluorescent light microscope and confocal laser scanning microscope. For the control, the same methods were used substituting nonimmune rabbit serum for the dromyosuppressin antibody.

The DMS antisera used in these experiments was generously provided by Nichols and was a polyclonal antisera raised in rabbits to the N-terminal amino acid residues of DMS, thus avoiding the common C-terminal sequence -RFamide (McCormick and Nichols, 1993).

Results

Cells and nerve fibers showing positive immunoreactivity for DMS were widespread in the brain, hypocerebral ganglion complex, thoracico-abdominal ganglion, and crop when compared with the controls. No cells immunoreactive to DMS were found in the gut except for the possibility of 2 cells in the hindgut.

Immunoreactive cells in the brain are widespread (Fig 2.1). In the brain and subesophageal ganglion there are at least six sets of immunoreactive cells. Figure 2.1A, shows the brain and subesophageal ganglion. In the protocerebrum, there are two clusters of cells in the midline and four more clusters of 1-3 cells each surrounding those (Fig 2.1A, C, and D). The medial group of midline cells consists of 18-20 small cells, while the anterior group is made up of 4 larger cells and 6 or more smaller cells (Fig. 2.1C). In the subesophageal ganglion, three groups of cells are present (Fig 2.1A, E, and, F). In the optic lobes, two distinct cells and their axons are seen (Fig 2.1B).

Immunoreactivity in the hypocerebral ganglion/corpora cardiaca complex consisted of a layered network of many immunoreactive nerve fibers, but no immunoreactivity was observed in the corpus allatum (Fig. 2.2). In Figure 2.2A, fibers from the hypocerebral ganglion can be seen extending towards and onto the surface of the proventriculus. The cardiac recurrent nerve, running along the foregut to the hypocerebral ganglion also shows immunoreactivity (Fig. 2.2B). Four immunoreactive cells and associated fibers were also observed in the posterior thoracico-abdominal ganglion (Fig. 2.2C).

Immunoreactive fibers and two fibers running the length of the crop duct of sugar-fed, liver-fed, and starved flies (Fig 2.3) form an extensive network on the surface of the crop (Fig. 2.3 A-D). Figure 2.4 is a composite drawing to scale showing the distribution of immunoreactivity to DMS in adult, female *P. regina*.

Discussion

The distribution of DMS immunoreactivity in *P. regina* is similar to that of other dipterans (See Table 1.2). In *D. melanogaster*, DMS has been localized throughout the brain, subseophageal ganglion and central nervous system, two cells and several nerve fibers in the hindgut, and nerve fibers on the surface of the crop and crop duct (McCormick and Nichols, 1993). In the stable fly, *S. calcitrans*, gut tissues were not tested but, immunoreactivity was present in the brain, subseophageal ganglion, thoracico-abdominal ganglion, and nerve fibers within the corpora cardiaca, which extended to the aorta (Meola *et al.*, 1991).

Myosuppressins are known to suppress spontaneous muscle contractions of the heart, oviduct, and hindgut in non-dipteran species (Holman *et al.*, 1986; Robb *et al.*, 1989; Lange *et al.*, 1991). Based on the widespread distribution of DMS immunoreactive fibers on the crop in *P. regina* and *D. melanogaster*, it is possible that the peptide may also affect crop muscle contractions in the Diptera.

Little is known about the regulatory control of crop muscle contractions in Diptera, but gut and crop contractions are not considered to be under nervous control (Knight, 1962). Thomson (1975) suggests that contractions in the gut and crop occur because of interactions between stretch-sensitive muscles and the hydrostatic pressure of the crop contents. Previous studies have also shown that severing all connections to the central and stomatogastric nervous systems does not interfere with crop emptying (Gelperin, 1966). The presence of a network of DMS immunoreactive fibers on the crop, however, indicates that DMS may have an effect on crop functioning. A myoinhibitory peptide, like DMS, may have a function in

suppressing crop contractions during feeding, thus allowing the crop to expand and fill. This hypothesis can be tested, *in vitro*, by applying DMS to the isolated crop and measuring its effect on contraction rate.

The widespread distribution of immunoreactivty in the brain, subesophageal ganglion, cardiac recurent nerve, thoracico-abdominal ganglion, hypocerebral ganglion/corpora cardiaca complex and crop (Fig. 2.4), demonstrates that dromyosuppressin is also a brain-gut peptide in *P. regina*. As of this time, no function of DMS has been determined in *P. regina* or in any other species of Dipterans. Myosuppressins are known to suppress spontaneous muscle contractions in other insect species, but future studies will be needed to determine if it has similar effects on the immunopositive tissues shown here for *P. regina*.

Figure 2.1 Distribution of dromyosuppressin immunoreactive cells and nerve fibers in the brain, optic lobes, and subesophageal ganglion of adult, female *Phormia regina*. Figure A shows immunoreactive cells in the brain (BR) and subesophageal ganglion (SOE). Figure B shows two immunoreactive cells in the optic lobe (OL). Figures C and D, show the distribution of immunorective cells in the protocerebrum. In Figure C, the cluster of 18-20 small medial cells is shown. In Figure D, the medial cells are obscured by the esophagus, while the 4 surrounding cell clusters are clearly visible. Figures E and F, show the distribution of immunoreactive cells in the SOE, with arrows pointing to three distinct groups of cells.

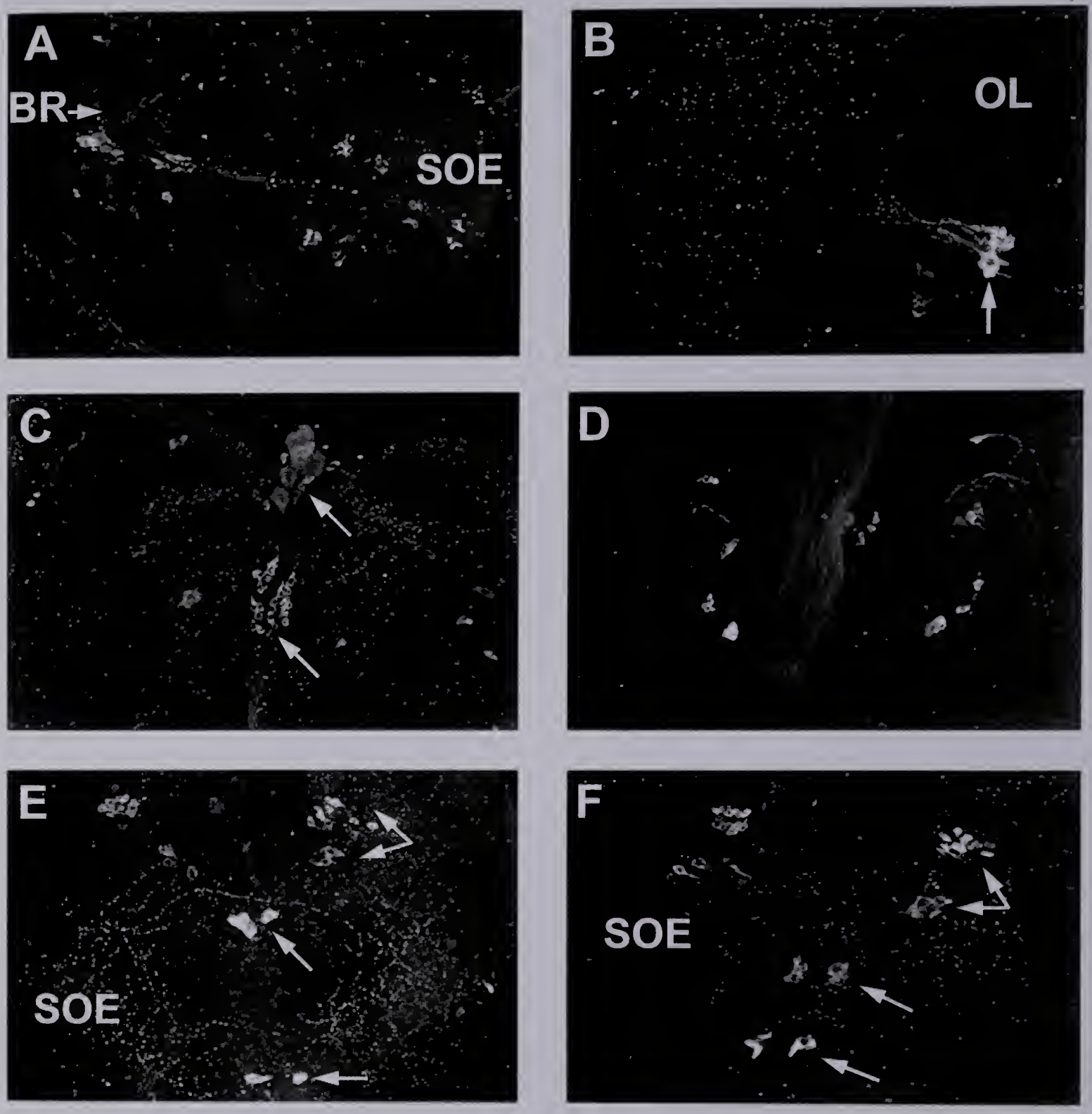
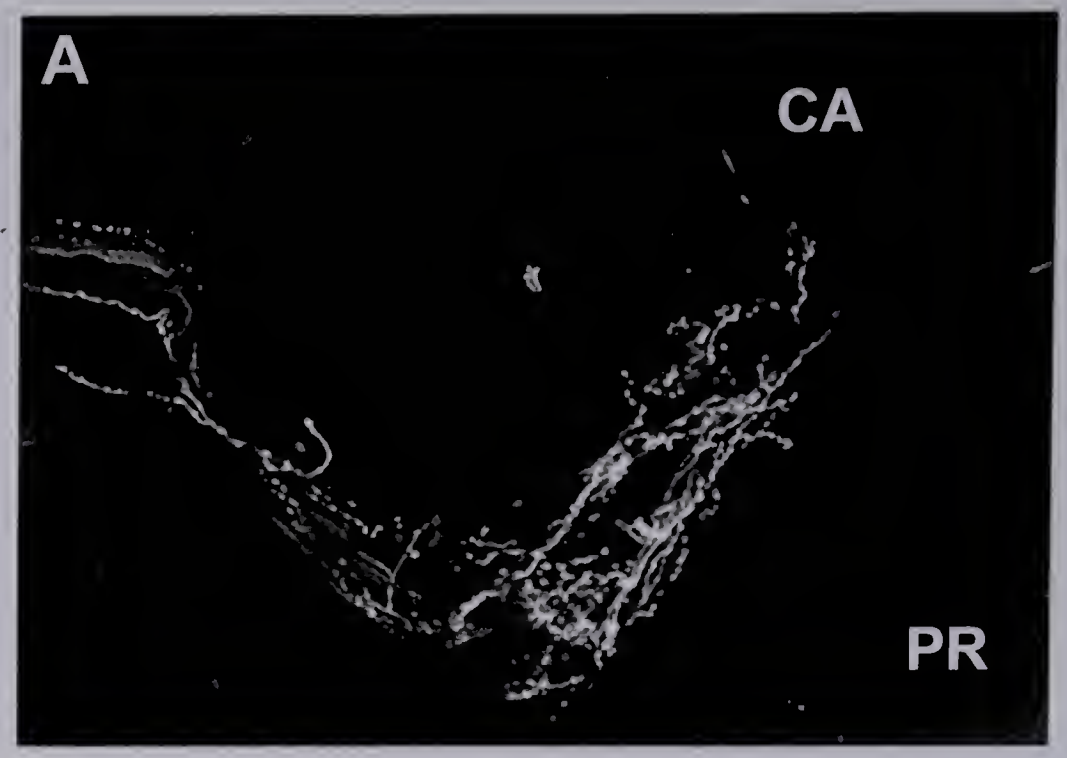
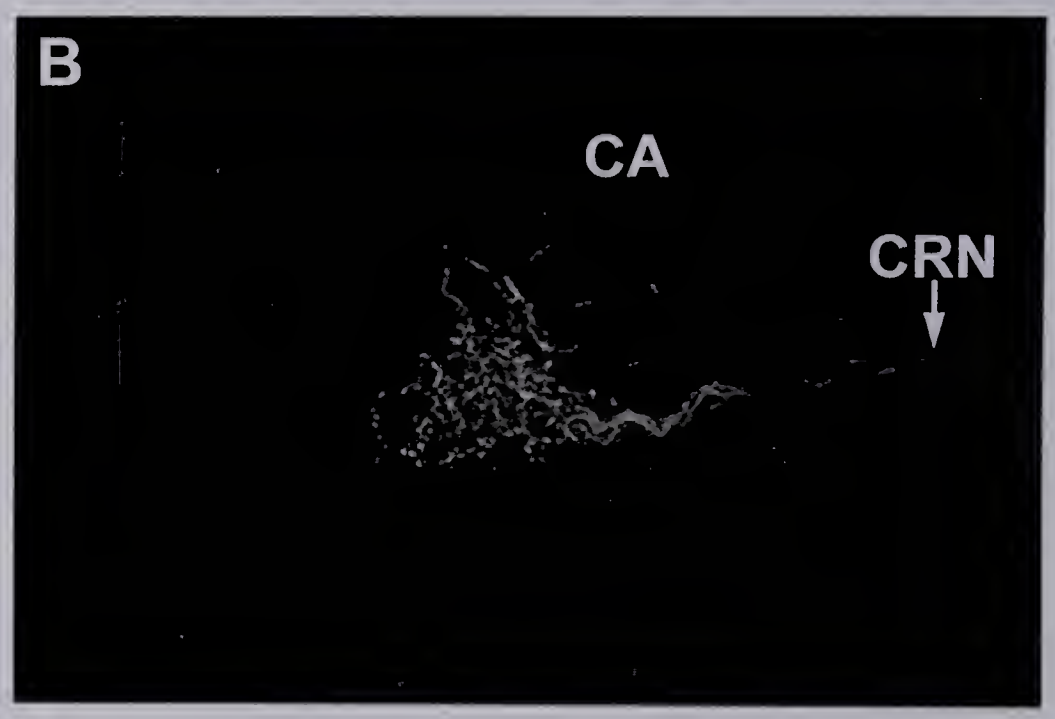

A
BR
SOE
B
OL
C
D
E
SOE
F
SOE

Figure 2.2 Distribution of dromyosuppressin immunoreactive cells and nerve fibers in the hypocerebral ganglion/corpora cardiaca complex (HG/CC), cardiac recurrent nerve, and thoracico-abdominal ganglion of adult, female *Phormia regina*. In Figure A, nerve fibers in the hypocerebral ganglion/corpora cardiaca complex are shown extending towards the proventriculus (PR). In Figure B, which also shows the HG/CC, the cardiac recurrent nerve (CRN) shows immunoreactivity, and Figure C shows four immunoreactive cells in the thoracico-abdominal ganglion (TAG).


A
CA
PR


B
CA
CRN

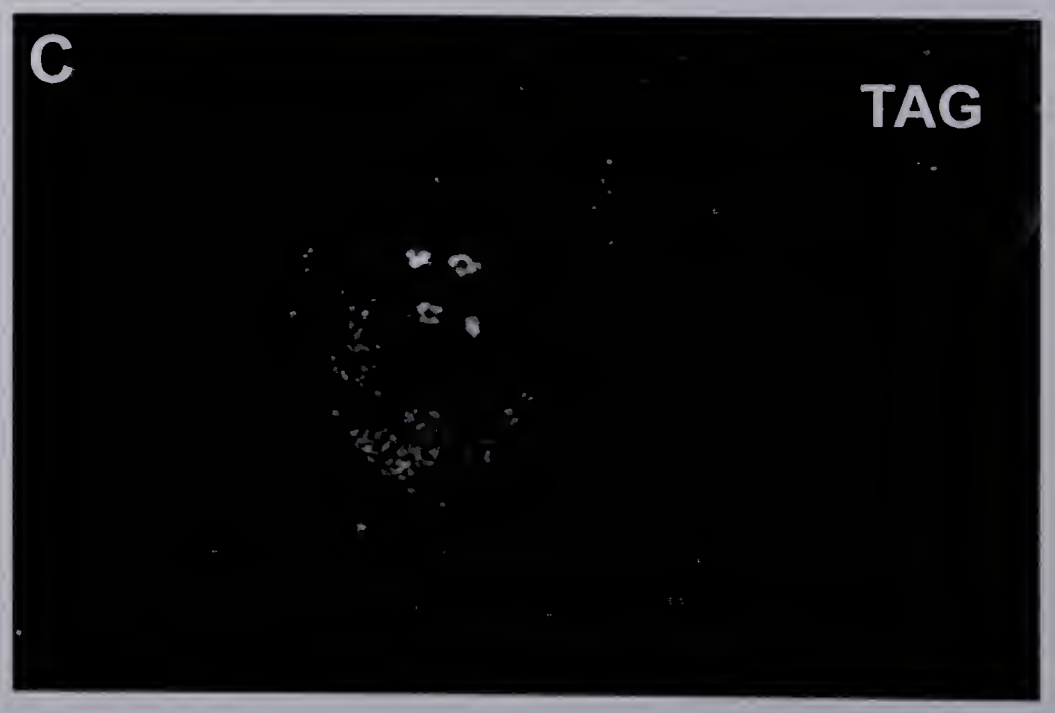

C
TAG

Figure 2.3 Distribution of dromyosuppressin immunoreactive nerve fibers on the crop and crop duct of adult, female *Phormia regina* (A-D). Crop duct (CD); arrows indicate nerve fiber network.

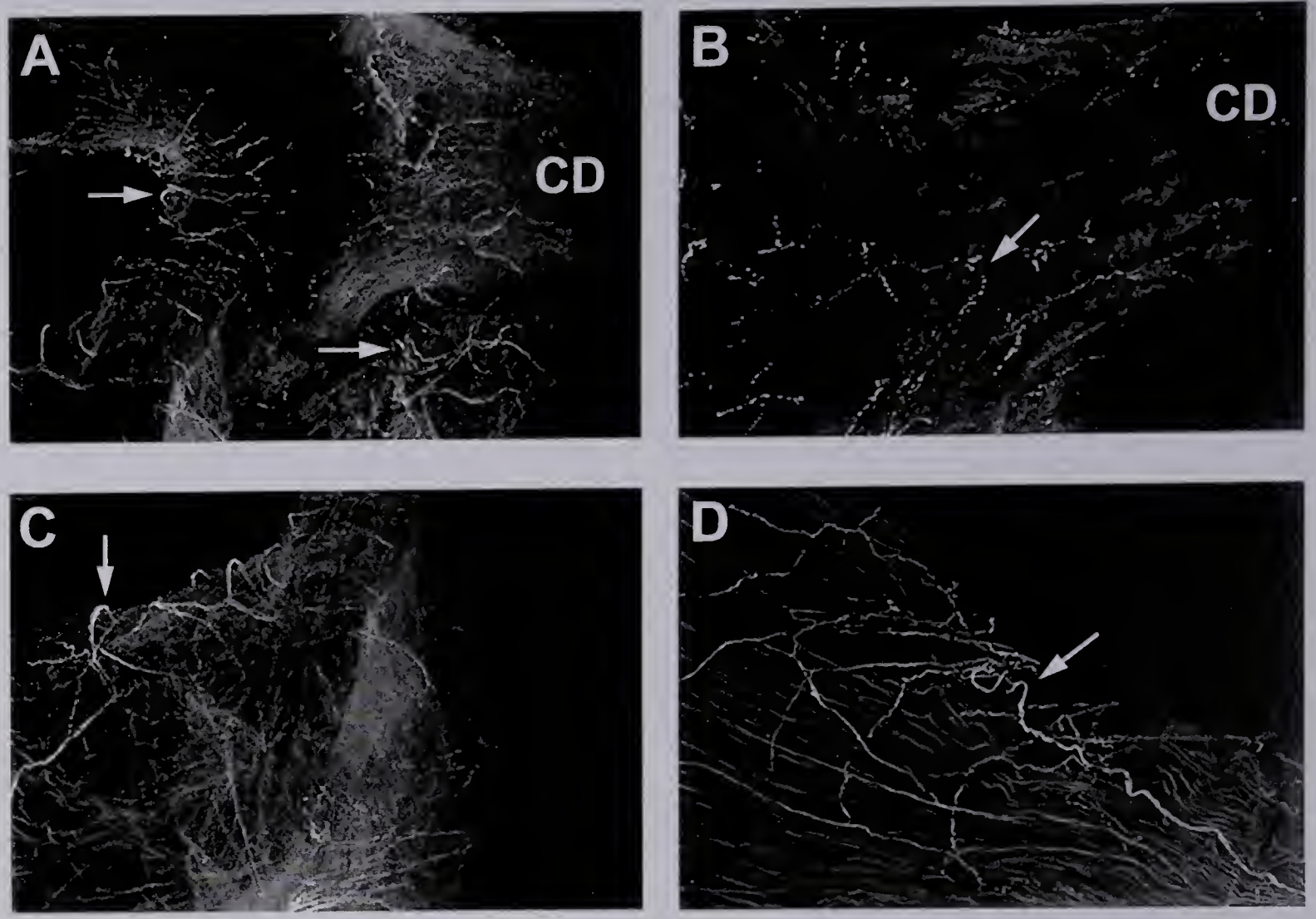
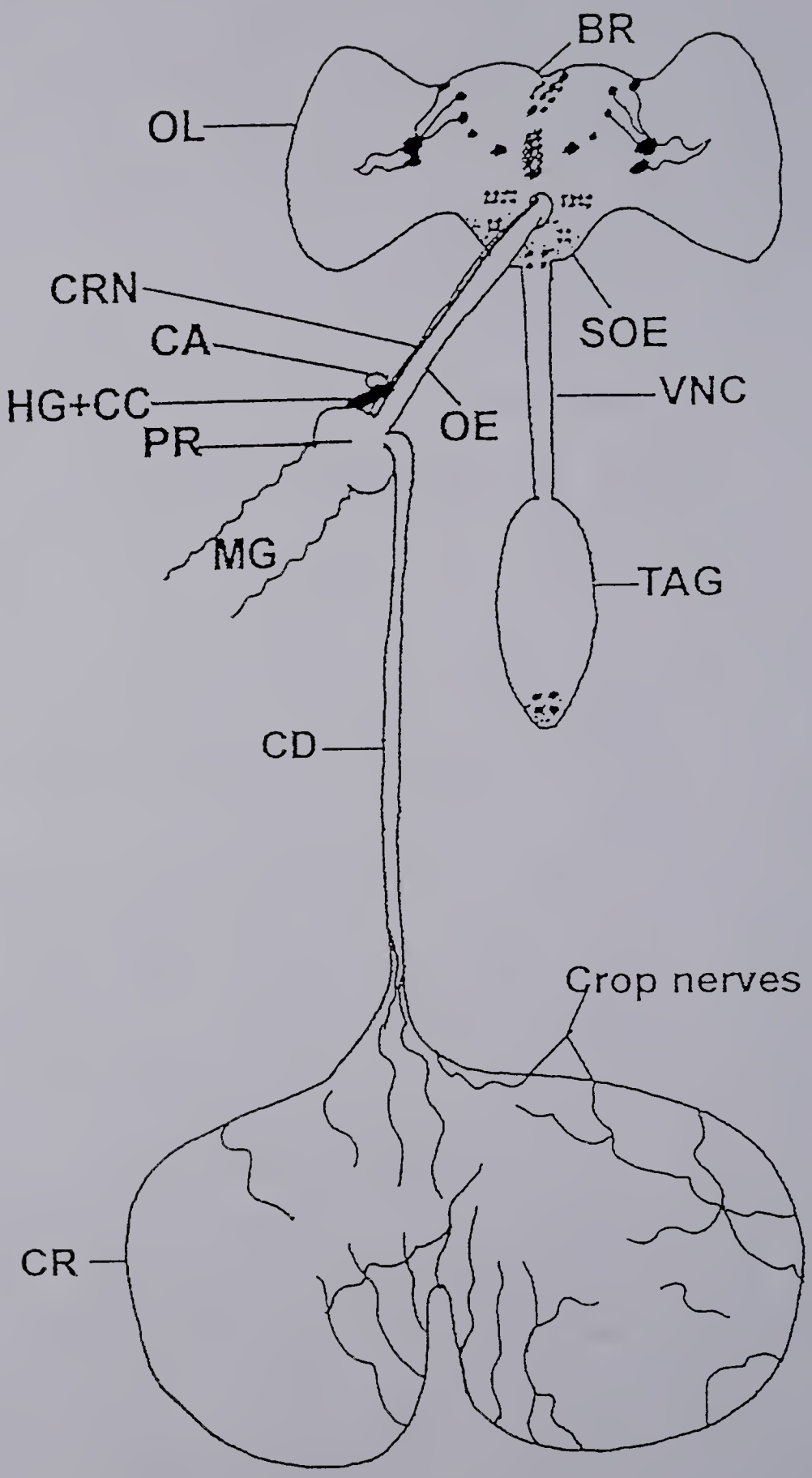

A
CD
B
CD
C
D

Figure 2.4 Distribution of dromyosuppressin immunoreactive cells and fibers in adult, female *Phormia regina*. Immunoreactive cells are widespread in the brain (BR), optic lobes (OL), and subesophageal ganglion (SOE). Four cells are present in the posterior thoracico-abdominal ganglion (TAG) and numerous immunoreactive fibers are present on the crop (CR), in the hypocerebral ganglion/corpora cardiaca (HG+CC), cardiac recurrent nerve (CRN), and brain. Corpus allatum (CA), crop duct (CD), and midgut (MG).


BR
OL
CRN
CA
HG+CC
PR
OE
SOE
VNC
MG
TAG
CD
Crop nerves
CR

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CHAPTER III

IN VITRO EFFECTS OF DROMYOSUPPRESSIN ON CONTRACTIONS IN THE CROP OF ADULT, FEMALE *PHORMIA REGINA*

Introduction

Feeding behavior in *Phormia regina* has been studied intensively (Dethier, 1976), while extensive work on various factors influencing feeding behavior has also been done (e.g., feeding rate, crop emptying, and peristalsis of the alimentary canal) (Knight, 1962; Gelperin, 1966; Thomson and Holling, 1974, 1975; Thomson, 1975a). Whether these factors are under neural, hormonal, or both types of control has been investigated to varying degrees. Nerve fibers were observed on the adult dipteran crop (Graham-Smith, 1934), but previous studies have all concluded that the crop is not likely to be under nervous control. Knight (1962) tested this hypothesis by severing all connections to the central and stomatogastric nervous system and was still able to observe the gut and crop functioning normally. Gelperin (1966) also ruled out nervous and hormonal control of crop emptying. In the cockroach, however, crop emptying is known to be under nervous control. Davey and Treherne (1963) showed that in the cockroach, crop emptying is controlled by the stomatogastric nervous system.

The presence of dromyosuppressin (DMS) immunoreactive nerve fibers on the crop and crop duct of *P. regina* (see chapter II), suggests that DMS may play a role in crop function. DMS, a myoinhibitory peptide, may be partially responsible for regulating or suppressing spontaneous crop contractions in this blow fly. DMS may be more active in crop filling than crop emptying, by suppressing contractions

and allowing the small empty crop to expand and fill. To date, most studies on control of crop functioning in *P. regina* have focused on crop emptying and not crop filling (Gelperin, 1966; Thomson, 1975b; Thomson and Holling, 1976a,b).

The hypothesis to be tested in this study is that DMS acts to inhibit spontaneous contractions of the crop of *Phormia.* The crop is known to continue contracting when removed from the fly and placed in saline (Knight, 1962). An *in vitro* study allows for easy application of the peptide to the crop and permits direct observation of the effect of these substances on the normal rate of crop contractions.

Materials and Methods

Maintaining Flies

The stock colony was maintained as previously described (page 16) according to the protocol of Stoffolano (1974). Flies were prepared according to Thomson and Holling (1974), so that all had an empty crop at the start of each experiment. In order to achieve this basal threshold, they were provided at time of emergence with 4.3% sugar solution and water for 3 days. On the 4th day, the 4.3% sugar solution was removed and both 0.01 M sucrose solution and water were provided separately for 2 days. After the 0.01 M sucrose solution was removed on day 6, flies were given only water for 24 hours before experimentation. Females used for experiments were 7 days post-emergence and all weighed between 30 mg and 34 mg. This weight range was chosen based on an average weight of 32 mg for starved flies and which is essential for flies to complete oogenesis after feeding to repletion on one 22 mg meal of liver.

Bioassay Procedure

Flies were anesthetized with CO_2, immediately put on ice, and individually placed dorsal side down by attaching their wings to an applicator stick covered with double sided sticky tape. Each fly was given 2-4 minutes to recover from the effects of anesthetization. Immediately following recovery, flies were fed individually by placing the labellum on the measured drop of 1.0 M sucrose solution colored with 10 mM Amaranth measured out in individual droplets of 4.5 µl, placed on a clear plastic lid. This volume (i.e., 4.5 µl) was used because Thomson (1975a) showed that it provided the highest crop contraction rate. Flies were dissected immediately after taking in the entire 4.5 µl of solution. Each dissection was completed in less than 2 minutes following feeding. The crop was removed and placed in 40 µl of *Phormia* saline in a glass depression slide. Each trial of 20 flies, 10 experimental and 10 control, was completed within 3.5 hours.

In order to determine the number of contractions per minute without any treatment, the excised crop was placed in the *Phormia* saline for a one minute adjustment period. Following adjustment, the number of contractions for one minute was determined. Based on the results of these trials, crops contracting over 40 beats per minute were considered normal, while crops contracting less than 40 times in the first minute after the adjustment period were discarded from the proceeding experiments.

Contractions were measured by visually picking a similar point on each crop, close to where the lobes branch and the crop duct begins, and then counting

each wave passing through this point. It was essential to choose a single point for recording contractions because the crop tends to contract in waves. Thus, the same wave could be counted more than once at different points on the crop. Crops that contracted only on the tips of the lobes were not used.

A sham control was also done before applications of the peptide to determine if the addition of *Phormia* saline would have any effect on the crop contraction rate. One µl of *Phormia* saline was applied with a 10 µl glass syringe onto the center of the droplet of saline without the tip of the syringe touching the droplet. In these trials, and in the following DMS trials, crops were given a one minute adjustment period, and contractions were counted for 1 minute. If the number of contractions in this 1 minute period was 40 or greater, various treatments were applied, and contractions again counted for one minute after application.

To measure the effects of the DMS peptide on crop contractions, the same preparation as described above was used. After counting the crop contraction rate for one minute, 1 µl of 10^{-6} M DMS, dissolved in *Phormia* saline, was carefully placed onto the center of the droplet of saline using a 10 µl glass syringe and the contraction rate per minute, after the treatment, was counted as described above.

Dissection Techniques

Immediately after completion of feeding on the provided 4.5 µl of 1.0 M sucrose-Amaranth solution, flies were removed from the applicator stick by inserting a dissecting pin at an angle into one side of the thorax and pulling the fly off the tape. The fly was then pinned down, ventral side up, in a wax bottom

dissecting dish, and a second pin placed on the opposite side into the thorax to firmly hold the fly down. The legs were then removed to facilitate dissections by allowing greater accessibility to the tissues using forceps.

The cuticle was then removed from the thorax where the forelegs were attached. This exposes the proventriculus, crop duct, and foregut. The crop duct is easily distinguished because it is colored by the sucrose/Amaranth solution. At the junction of the foregut and midgut (i.e., just anterior to the proventriculus) and where the crop duct joins the foregut, the crop duct was pinched with the forceps, lifted up, snipped with scissors close to the junction of the proventriculus, and then released.

Next, the ventral abdomen was opened for removal of the crop at the junction of the thorax and the abdomen. This was done by gently pulling and carefully lifting up the cuticle on the first three abdominal sclerites so as not to puncture the crop. Once the crop and crop duct are exposed, the duct can be grasped with the forceps and the crop and duct removed and placed immediately in a clear depression slide containing 40 µl of *Phormia* saline. The crop itself was never grabbed or touched by the forceps.

Results

The average number of crop contraction per minute for 3 trials of 30 crops each from flies fed 4.5 µl of 1.0 M sucrose, was 50.4 ± 1.5 (Table 3.1). The sham treatment value of 46.1 ± 1.1 (Table 3.2), which was observed after the application of 1 µl of *Phormia* saline to the crop, when compared to the sham control value of

50.8 ± 1.5, was not significantly different ($p > 0.05$). The addition of DMS, in most cases, stopped all contractions immediately after its application (Table 3.3). Experiments using DMS showed that the application of 10^{-6} M DMS to the crop had a significant suppressing effect, lowering the contraction rate from an average of 46.2 ± 5.0 to 2.2 ± 0.2 (Table 3.4) or a 95% reduction ($p < 0.05$). If contractions did not stop immediately, 2 or 3 slow contractions occurred before crop contractions completely stopped for the duration of the time period.

Discussion

Results (Table 3.1) to establish the normal, *in vitro,* crop contraction rate without the addition of any treatment, were consistent with the results of Thomson (1975a). He showed that the highest crop contraction rate, 50 contractions per minute, occurred at a crop volume of 4.5 µl while this study showed average values of 50.4 ± 1.5 (control) and 50.8 ± 1.5 (sham control). This is the volume at which the crop, *in vitro*, is most active and was therefore chosen for further experiments to best demonstrate the effects of DMS on the contraction rate of an active crop. It was also necessary to show that the addition of more *Phormia* saline to the medium containing the crop would not affect its normal, *in vitro*, functioning. There was no significant difference between the number of contractions in the sham treatment and sham control trials. Thus, any effect of the treatment would be due to the treatment alone.

Results show that the application of 10^{-6} M DMS to the medium containing the crop, produced a significant reduction in the number of contractions per minute.

DMS suppresses crop contractions almost immediately after it is applied and continues to inhibit contractions for at least one minute after its application.

Previous work (Knight, 1962; Gelperin, 1966) on crop function has shown no evidence of the crop being under neural or hormonal control. In Chapter II of this thesis, the crop of *Phormia* was shown to have a neural network of DMS immunoreactive fibers on its surface. In this chapter, experiments clearly demonstrate that when applied, *in vitro*, at a low concentration, DMS effectively shuts down crop contractions. These results demonstrate that crop muscle contraction, at least its suppression, in *Phormia regina* is controlled, in part, by the DMS peptide.

Further experiments must be done on the effects of DMS on the crop muscle, *in vivo*, and in regards to crop filling and emptying to better understand the role of this peptide in crop function. Also, the effects of DMS on a protein meal in the crop, should be investigated. In addition to better understanding the physiology and feeding behavior of this model fly system, DMS may also prove useful as a control agent. Applications of DMS may disrupt feeding just enough to prevent normal growth and development of the fly. Though peptides are usually not effective in field applications because they are quickly degraded in the field and in the insect gut, benzethonium chloride is a nonpeptidal myosuppressin agonist, which may be effective under these conditions.

Table 3.1 Average rate of crop contraction in adult, female *Phormia regina* with no treatment. [1,2,3]

Trial 1	Trial 2	Trial 3	
64	57	52	
45	45	61	
56	43	40	
47	41	62	
50	44	43	
63	43	47	
43	48	55	
53	47	42	
71	56	42	
42	61	49	
Mean ± SD 53.4 ± 9.9	48.5 ± 7.0	49.3 ± 8.0	50.4 ± 1.5

[1] Females with empty crops were fed 4.5 µl of 1 M sucrose solution colored with 10 mM Amaranth.

[2] Crops were removed after feeding and placed in 40 µl of *Phormia* saline for a 1 min. adjustment period before recording the contraction rates.

[3] Number of contractions counted in 60 seconds.

Table 3.2 Average rate of crop contraction in adult, female *Phormia regina* after the application of 1 µl of *Phormia* saline. [1,2,3,4]

	Trial 1		Trial 2		Trial 3		
	Control	Treatment	Control	Treatment	Control	Treatment	
	57	50	48	48	47	43	
	49	47	42	40	54	45	
	60	37	46	42	51	44	
	53	46	50	38	49	40	
	47	40	56	55	61	55	
	52	47	48	44	53	52	
	54	52	56	52	48	46	
	48	40	61	59	47	42	
	45	39	49	46	60	59	
	46	44	43	42	46	48	
Mean	51.1	44.2	49.2	46.6	52.2	47.4	46.1 ± 1.1
± SD	± 5.0	± 5.0	± 6.4	± 6.9	± 5.5	± 6.1	

[1] Females with empty crops were fed 4.5 µl of 1 M sucrose solution colored with 10 mM Amaranth.

[2] Crops were removed after feeding and placed in 40 µl of *Phormia* saline for a 1 min. adjustment period before recording the contraction rates.

[3] Number of contractions counted in 60 seconds.

[4] 1 µl of *Phormia* saline was applied directly to the droplet containing the crop.

Table 3.3 The effect of dromyosuppressin on contractions of the crop in adult, female *Phormia regina*. [1,2,3,4,5]

	Trial 1		Trial 2		Trial 3		
	Control	Treatment	Control	Treatment	Control	Treatment	
	48	4	51	1	49	3	
	39	3	52	4	44	2	
	47	2	49	2	38	3	
	41	1	40	1	52	4	
	41	2	41	2	43	1	
	52	4	46	2	45	2	
	48	2	57	1	49	2	
	50	1	52	3	44	2	
	46	1	42	1	40	4	
	39	3	47	3	53	1	
Mean	45.1	2.3	47.7	2.0	45.7	2.4	2.2 ± 0.2
± SD	± 4.7	± 1.2	± 5.5	± 1.1	± 4.9	± 1.1	

[1] Females with empty crops were fed 4.5 µl of 1 M sucrose solution colored with 10 mM Amaranth.

[2] Crops were removed after feeding and placed in 40 µl *Phormia* saline for a 1 min. adjustment period before recording the contraction rates.

[3] Number of contractions counted in 60 seconds.

[4] Only crops with a contraction rate of over 40 contractions were used.

[5] 1 µl of 10^{-6} M DMS dissolved in *Phormia* saline was applied directly to the droplet of saline containing the crop.

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CHAPTER IV

IN VITRO EFFECTS OF BENZETHONIUM CHLORIDE ON CROP CONTRACTIONS OF ADULT, FEMALE *PHORMIA REGINA*

Introduction

Benzethonuim chloride is a nonpeptidal mimic of the myosuppressin family of peptides, and has been shown to act as an agonist for several known functions of myosuppressin in some insect species (See Fig. 1.1). This compound mimics the inhibitory activity of leucomyosuppressin in both the cockroach hindgut and mealworm neuromuscular junction, and of SchistoFLRF-amide on the locust oviduct (Lange *et al.*, 1995; Nachman *et al.*, 1996). Nonpeptidal agonists are important because they are more resistant to degradation by peptidase enzymes in the insect gut or hemolymph and thus allow lower doses to be effective. The nonpeptide is also more abundant and easily available commercially (Lange *et al.*, 1995).

In Chapter III, it was shown that dromyosuppressin (DMS) suppresses, *in vitro*, crop muscle contractions of adult *Phormia regina*. Benzethonuim chloride (Bztc), a nonpeptide which mimics other functions of myosuppressins, may also mimic the effect of DMS on crop muscle contractions without confounding effects of any hemolymph borne chemicals.

The hypothesis to be tested is that Bztc may act to inhibit spontaneous contractions of the crop muscle of adult *Phormia*. The crop muscles are known to continue contracting when removed from the fly and placed in *Phormia regina* saline (Knight, 1962). An *in vitro* study allows for easy application of the agonist to

the crop and permits direct observation of the effect of this substance on the normal rate of crop contractions without confounding effects of any hemolymph borne chemicals.

Materials and Methods

Maintaining Flies

The stock colony of flies was maintained as previously described (page 34) according to Stoffolano (1974). Flies were prepared for these experiments according to the methods of Thomson and Holling (1974) to ensure the crops would be empty .

Bioassay Procedure

To measure the effects of Bztc on the rate of crop muscle contractions, the same bioassay preparation was used as described in the previous chapter (pages 35-36).

Crop muscle contraction rate was counted for one minute and any crops with less than 40 contractions per minute were discarded. One µl of 10^{-3} M Bztc, dissolved in *Phormia* saline, was carefully placed onto the center of the droplet of saline using a glass, 10 µl syringe and crop contractions were counted for one minute immediately after the treatment was applied.

Dissection Techniques

Dissection techniques used in this experiment were those previously described in Chapter 3.

Results

The application of Bztc to the crop resulted in a significant reduction of contractions per minute (Table 4.1). Untreated crops contracted an average of 48.1 ± 1.4 contractions per minute while the Bztc treated crops contracted at an average of 6.1 ± 0.7 contractions per minute (Table 4.2) ($p < 0.05$). This represents an 87% reduction in the number of contractions per minute. Before Bztc was applied, the lobes of the crop were wrinkled and irregular. After application of the agonist, the lobes of the crop began to smooth out and muscle contractions slowed until stopping completely.

Discussion

Benzethonium chloride has been the first nonpeptidal agonist reported for an insect neuropeptide. Nonpeptidal agonists are important to neuropeptide research because they are easier to synthesize and less susceptible to degradation in either the insect gut or under field conditions (Holman *et al.*, 1990). These qualities make nonpeptidal mimics useful research tools and as possible insect control agents.

The results of this *in vitro* bioassay demonstrate that Bztc is effective at suppressing spontaneous crop muscle contractions in *P. regina*. Applications of 10 µl of 10^{-3} M Bztc effectively shut down crop muscle contractions, but did not appear to act as quickly as the DMS peptide (see chapter III). In addition to mimicking the effect of the myosuppressins on the cockroach hindgut, the mealworm neuromuscular junction, and the locust oviduct (Holman *et al.*, 1986; Yamamoto *et al.*, 1988), Bztc can also now be listed as suppressing spontaneous crop muscle

contractions in female, *P. regina*. Further research is needed to determine if Bztc can mimic the effect of myosuppressin on the crop, *in vivo*, and how it effects crop filling and emptying.

If Bztc can also suppress crop contractions, *in vivo*, it may have potential uses as a both to further our understanding of physiology and feeding behavior of this model system, and as a potential control agent. Nonpeptidal mimics are important to neuropeptide research since they can be used in ways neuropeptides cannot.

Table 4.1 The effect of benzethonium chloride on contractions of the crop in adult, female *Phormia regina*. [1,2,3,4,5]

Trial 1		Trial 2		Trial 3	
Control	Treatment	Control	Treatment	Control	Treatment
59	3	50	3	38	12
46	4	32	7	46	6
60	10	41	2	49	6
59	9	52	2	51	8
41	4	58	2	54	2
47	7	52	5	57	5
39	4	47	2	58	9
43	13	54	14	44	2
48	6	56	10	40	3
37	15	41	3	43	4
Mean 47.9	7.5	48.3	5.0	48	5.7
± SD ± 8.6	± 4.1	± 8.1	± 4.1	± 7.0	± 3.2

[1] Females with empty crops were fed 4.5 µl of 1 M sucrose solution colored with 10 mM Amaranth.

[2] Crops were removed after feeding and placed in 40 µl *Phormia* saline.

[3] Number of contractions counted in 60 seconds.

[4] Only crops with a contraction rate of over 40 beats were used.

[5] 1 µl of 10^{-3} M Bztc dissolved in *Phormia* saline was applied directly to the crop.

Table 4.2 The effects of applications of a sham treatment, dromyosuppressin, and benzethonium chloride on contractions of the crop in adult, female *Phormia regina*.[1,2,3,4,8]

Treatment applied	Control	Treatment
Sham[5] ± SD	50.8 ± 1.5	46.1 ± 1.1
DMS[6] ± SD	46.2 ± 5.0	2.2 ± 0.2
Bztc[7] ± SD	48.1 ± 1.4	6.1 ± 0.7

[1] Females with empty crops were fed 4.5 µl of 1 M sucrose solution colored with 10 mM Amaranth.

[2] Crops were removed after feeding and placed in 40 µl *Phormia* saline.

[3] Number of contractions counted in 60 seconds.

[4] Only crops with a contraction rate of over 40 beats were used.

[5] 1 µl of *Phormia* saline was applied directly to the droplet containing the crop.

[6] 1 µl of 10^{-6} M DMS dissolved in *Phormia* saline was applied directly to the droplet of saline containing the crop.

[7] 1 µl of 10^{-3} M Bztc dissolved in *Phormia* saline was applied directly to the crop.

[8] Results are based on 3 trials of 20 flies each.

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CHAPTER V

GENERAL DISCUSSION

In the previous chapters it has been established that dromyosuppressin immunoreactivity is widespread in the central nervous system and crop of *P. regina*. Because this peptide is known to suppress spontaneous muscle contractions in other insect species (Holman *et al.*, 1986; Lange *et al.*, 1991; Robb *et al.*, 1989; Yamamoto *et al.*, 1988), its abundance in nerve fibers on the crop suggest that it may act in suppressing spontaneous crop muscle contractions. Though nerve fibers were known to be present on the surface of the crop (Graham-Smith, 1934), crop functioning was not considered to be under nervous or hormonal control (Knight, 1962; Gelperin, 1966).

In order to determine if DMS could be involved in controlling crop muscle contractions, an *in vitro*, bioassay was developed. DMS or its nonpeptidal agonist, benzethonuim chloride (Bztc) was applied directly to the *Phormia* saline surrounding a contracting crop. The result of these applications was a significant decrease in the number of crop muscle contractions per minute. With applications of 1 μl of 10^{-6} M DMS, the rate of contraction per minute decreased by 95% and by 87% for applications of 1 μl of 10^{-3} M Bztc. DMS and Bztc significantly decrease the number of crop muscle contractions at low doses, *in vitro*.

Further experiments must be conducted to evaluate the effects of DMS and Bztc on crop muscle contractions, *in vivo*, and in regards to crop filling and

emptying. If DMS suppresses crop muscle contraction, it may be imperative to crop filling by allowing the crop to stretch and fill instead of continually contracting and expelling ingested food. Low DMS levels could initiate crop emptying, by allowing crop contractions to occur and push food through the proventricular valves into the midgut.

In addition to better understanding the physiology and feeding behavior of this model system, DMS and Bztc may prove useful as potential control agents. The myosuppressins are unique, in being the first family of insect neuropeptides to date with an established nonpeptidal mimic. New research has resulted in the development of pseudotetrapeptide allatostatin analogs. This represents the first design and synthesis of a natural insect neuropeptide and is an important step in making theses compounds a potentially new form of insect control (Nachman *et al.*, 1999). Since peptides can be difficult to isolate and highly degradable, a commercially available, more stable compound can allow for many other testing options. Applications of this peptide or its nonpeptidal agonist may be able to disrupt the feeding process enough to prevent normal growth and reproductive development of the fly.

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