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# *N*-β-ALANYLDOPAMINE AND *N*-ACETYLDOPAMINE OCCURRENCE AND SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA* (L.)

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**Abstract**—*N*-β-Alanyldopamine (NBAD), *N*-acetyldopamine (NADA), dopamine (DA), and 3,4-dihydroxyphenylalanine (DOPA) were detected in the brain and ganglia of the central nervous system of larval and adult tobacco hornworms, *Manduca sexta*, by reversed phase HPLC with electrochemical detection. NBAD predominated in larval neural tissue during development of the fifth instar and increased to peak concentrations of 936, 650 and 263 nmol $g^{-1}$ in the abdominal ganglia, subesophageal plus thoracic ganglia and brain, respectively, at the wandering stage of development. Concentrations of all catecholamines decreased in the pharate pupa and were generally lowest in the adult nerve cord. DA was the second most abundant catecholamine in larval ganglia, but the primary catecholamine in adult ganglia. Relatively high levels of DOPA also occurred in the ganglia of wandering larvae but not at other times. NADA was detected only in the abdominal ganglia of day 3 larvae. *N*-Acyltransferases that catalyze synthesis of NBAD and NADA from DA also were present in abdominal ganglia, as demonstrated by analysis of *in vitro* cultures in which exogenous DA stimulated synthesis of both *N*-acylated catecholamines. Maximal NBAD synthesis occurred in ganglia removed from wandering stage larvae (9.3 nmol $g^{-1}$ $day^{-1}$), whereas NADA synthesis was highest in ganglia from pharate pupae (7.3 nmol $g^{-1}$ $day^{-1}$). Thus, *N*-β-alanylation and *N*-acetylation are competing metabolic reactions for DA in the hornworm's nervous system. The role played by the *N*-acylated catecholamines in *M. sexta* neurophysiology is unknown, but these compounds may be storage or inactive forms of the putative neurotransmitter DA.

*Key Word Index*: *N*-β-alanyldopamine, *N*-acetyldopamine, dopamine, catecholamines, neurotransmitter, insect, nervous system, tobacco hornworm, *Manduca sexta*, *N*-acyltransferase, cuticle

## INTRODUCTION

Biogenic amines, including dopamine (DA), norepinephrine (NE), octopamine, 5-hydroxytryptamine and tyramine have been localized in the nervous system of several insect species and other invertebrates (reviewed by Evans, 1980; Brown and Nestler, 1985; Pitman, 1985). Dopamine is assumed to function as a neurotransmitter, and it may also modulate certain insect behaviors (Kostowski *et al.*, 1966; House and Ginsborg, 1982; Mercer and Flanagan, 1988). *N*-Acylated derivatives of biogenic amines may also be present in the insect nervous system, but their physiological role(s) remains unknown. *N*-Acetylation may be a major pathway for inactivation, removal or storage of the biogenic amines (Dewhurst *et al.*, 1972; Nishimura *et al.*, 1975; Vaughn and Neuhoff, 1976; Evans *et al.*, 1980; Hayashi *et al.*, 1977; Maranda and Hodgetts, 1977; Evans, 1980; Murdock and Omar, 1981; Mir and Vaughn, 1981a; Downer and Martin, 1987; Martin and Downer, 1989a,b; Martin *et al.*, 1989; Dubbels and Elofsson, 1989).

In addition to *N*-acetylation, conjugation of biogenic amines with β-alanine or sulfate also may occur, but these types of metabolites in the invertebrate nervous system are less well documented. Maxwell *et al.* (1980) provided evidence for regional localization of *N*-β-alanyltyramine in the nervous system of adult *Manduca sexta*, and Kennedy (1977, 1978) reported the presence of *N*-β-alanyloctopamine as well as sulfated conjugates of octopamine, dopamine and serotonin in lobster neurons.

*N*-β-Alanyldopamine (NBAD) is a major precursor of tanning quinonoid compounds in certain types of insect cuticle and occurs in high concentrations as a glucoside in hemolymph during pupal cuticle tanning of *M. sexta* (Hopkins *et al.*, 1982, 1984; Kramer and Hopkins, 1987; Krueger *et al.*, 1989). Recently, we reported on finding NBAD localized in neural tissue of *M. sexta*, when analyzing other tissues for catecholamines (Krueger *et al.*, 1989). However, to the best of our knowledge, this biogenic amine had not been detected previously in the nervous system of other insects (Brown and Nestler, 1985; Nagao and Tanimura, 1988). We present here the results of analytical studies on the localization and concentration of dopamine and its two *N*-acylated

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derivatives, NBAD and $N$-acetyldopamine (NADA), in the central nervous system (CNS) of the tobacco hornworm, *M. sexta*, during development and metamorphosis. We also have analyzed primary *in vitro* cultures of larval abdominal ganglia to assess the capacity of nervous tissue for $N$-acylation of exogenous DA. Patterns of stage- and region-dependent concentrations within the nervous system of *Manduca sexta*, as well as synthesis of both NBAD and NADA, indicate physiological roles for these catecholamines.

## MATERIALS AND METHODS

### *Insects*

*M. sexta* was reared as described by Bell and Joachim (1976) at $27 \pm 1$°C and a nondiapausing photoperiod of 16L:8D. To obtain a synchronous population, insects were developmentally staged by selecting larvae at ecdysis to fourth instars (Wielgus *et al.*, 1979). Larvae were selected again at day 0 following ecdysis to fifth instars. Wandering behavior, which was identified by dorsal heart exposure, was initiated on day 5, whereas day 9 was marked by the appearance of brown metathoracic bars on pharate pupal cuticle. Adults were analyzed that had eclosed within the previous 24 h.

### *Chemicals*

Chemicals were obtained from Sigma Chemical Co., St Louis, Mo. or Aldrich Chemical Co., Milwaukee, Wis. NBAD was synthesized by the procedure of Kramer *et al.* (1983).

### *Tissue collection*

Nervous tissue was obtained from insects that were immobilized on ice. The integument was opened dorsally, the digestive system was removed, and the brain and nerve cord were excised. Tissues were dissected in Ringer's solution containing 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM phenylthiocarbamide. The brain, entire abdominal nerve cord (AG), and combined subesophageal ganglion plus thoracic ganglia (STG) from 3 to 5 insects were pooled separately. All tissues were rinsed, blotted on absorbent paper and weighed immediately. Tissues were homogenized in ground glass grinders at 4°C in 1.2 M HCl containing 10 $\mu$g ml$^{-1}$ $\alpha$-methyl DOPA (AMD) as an internal standard.

### *Tissue culture*

The abdominal ganglia from animals of three stages of development were cultured *in vitro*. Larvae were rinsed in deionized water and surface sterilized in 70% ethanol for 2 min followed by two rinses in sterile deionized water. The ventral abdominal segments, with attached ganglia, muscles and fat body, were removed and immediately placed in ice-cold Grace's tissue culture medium. The nerve cord was dissected from the body wall and cleaned of extraneous tissues. After an additional rinse in culture medium, the nervous tissue was placed in wells of a tissue culture plate and cultured in Grace's medium that was saturated with phenylthiourea and contained 35 units of streptomycin and 80 units penicillin. Half of the cultures were supplemented with 1.4 mM dopamine. Cultures were maintained for 2 days at 26°C in an atmosphere of 95% $O_2$–5% $CO_2$. At the end of the incubation period, the tissue was weighed and catecholamines in either tissue or culture medium were extracted and analyzed.

### *Catecholamine analysis*

Catecholamines present in the supernatant (0.1 ml) from tissue homogenates or culture medium were extracted and recovered according to the procedure of Hopkins *et al.* (1984), and then analyzed by HPLC with electrochemical detection at a potential of +0.72 V (Fig. 1). A 5 $\mu$m particle reversed phase C-18 column (25 cm × 4.6 mm) was used at 30°C with a mobile phase of 10–12% methanol, 0.8% $H_3PO_4$, 0.6–0.8 g l$^{-1}$ sodium octyl sulfate and 0.04 g l$^{-1}$ ethylenediaminetetraacetic acid (EDTA) at pH 2.6. To confirm the identification of the catecholamines, a second mobile phase of 20% acetonitrile, 1–2% $H_3PO_4$, 0.16 g l$^{-1}$ sodium dodecyl sulfate and 0.04 g l$^{-1}$ EDTA, pH 3, also was used to resolve the compounds. The retention times of unknown catecholamines were compared to those of standard compounds and quantitated by comparison of integrated areas with that of the internal standard AMD. NBAD, NADA, DA and DOPA coeluted exactly with the unknown compounds using both mobile phases. Aliquots of some homogenates were hydrolyzed in 1.2 M HCl for 1 h at 100°C to determine that dopamine is a high yield hydrolytic product of the compounds identified as NBAD and NADA. The detection limit for NBAD, DA and NADA was 1 nmol g$^{-1}$. Statistical differences between means were tested using the least-squares differences (LSD) procedure of a Statistical Analysis Systems program (SAS Institute Inc., 1982).

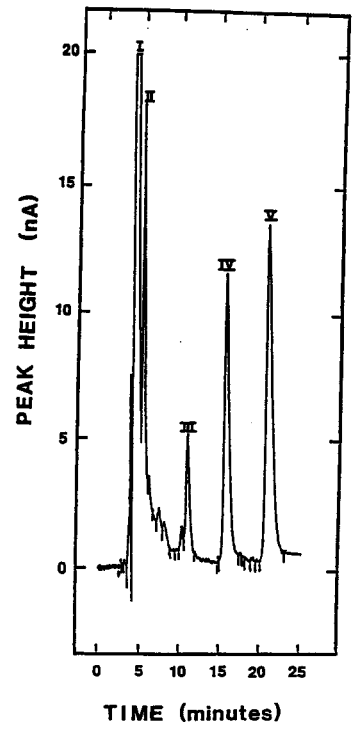


Fig. 1. HPLC–EC chromatogram of abdominal ganglia extract from *M. sexta* fifth larval instars. **I**, Ascorbic acid; **II**, unknown; **III**, dopamine; **IV**, $\alpha$-methyl-DOPA; **V**, $N$-$\beta$-alanyldopamine. NADA, not present in this sample, had a retention time of 18.0 min. See Materials and Methods for chromatographic conditions.

## RESULTS AND DISCUSSION

The concentrations of NBAD, NADA, DA and DOPA in ganglia of the central nervous system from *M. sexta* during development are shown in Table 1. DA was the major catecholamine in larval and adult ganglia during the first day after ecdysis and generally ranged from 20 to 40 nmol g$^{-1}$, whereas NBAD concentrations were second in relative concentration

Table 1. Catecholamine and DOPA concentrations (nmol $g^{-1}$ wet wt) in the central nervous system of *Manduca sexta* during development*

| Stage | Days after ecdysis† | Brain | | | | Subesophageal + thoracic ganglia | | | | Abdominal ganglia | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NBAD | NADA | DA | DOPA | NBAD | NADA | DA | DOPA | NBAD | NADA | DA | DOPA |
| Larva (5th) | 0 | $5^{a}$ | $<1^{a}$ | $40^{ab}$ | $<1^{a}$ | $3^{a}$ | $<1^{a}$ | $11^{a}$ | $<1^{a}$ | $10^{a}$ | $2^{a}$ | $35^{ab}$ | $<1^{a}$ |
| | 3 | $6^{a}$ | $<1^{a}$ | $14^{c}$ | $<1^{a}$ | $114^{b}$ | $1^{a}$ | $38^{a}$ | $<1^{a}$ | $293^{bc}$ | $29^{b}$ | $110^{c}$ | $<1^{a}$ |
| | 5 | $263^{b}$ | $2^{a}$ | $100^{a}$ | $30^{b}$ | $650^{c}$ | $<1^{a}$ | $48^{a}$ | $35^{b}$ | $936^{b}$ | $<1^{a}$ | $92^{bc}$ | $50^{b}$ |
| | 9 | $42^{c}$ | $<1^{a}$ | $18^{c}$ | $<1^{a}$ | $58^{b}$ | $1^{a}$ | $42^{a}$ | $<1^{a}$ | $129^{c}$ | $<1^{a}$ | $22^{a}$ | $<1^{a}$ |
| Adult | 1 | $2^{a}$ | $1^{a}$ | $30^{bc}$ | $<1^{a}$ | $5^{a}$ | $5^{a}$ | $22^{a}$ | $<1^{a}$ | $5^{a}$ | $1^{a}$ | $26^{a}$ | $<1^{a}$ |

*Means with the same superscript letter in the vertical columns are not significantly different ($P \leqslant 0.05$, $n = 3$).
†Day 3 = feeding, day 5 = wandering and day 9 = pharate pupa.

(2–10 nmol $g^{-1}$). DOPA and NADA were detected at low to trace levels. NBAD exhibited the greatest increases during the fifth instar of about 50-, 200- and 100-fold in the brain, subesophageal plus thoracic ganglia and abdominal ganglia, respectively. The highest NBAD concentration (approx. 900 nmol $g^{-1}$) occurred in abdominal ganglia of wandering larvae at day 5. DA was the second most abundant catecholamine on day 5, but it occurred at 3- to 10-fold lower levels than NBAD. DOPA and NADA were present at trace amounts, except when significant increases were exhibited by DOPA in all ganglia of day 5 larvae and by NADA in day 3 abdominal ganglia. Catecholamine levels were much less in day 9 pharate pupal and in adult ganglia than in day 5 larval ganglia. For example, nearly 200-fold lower NBAD was present in adult abdominal ganglia. DOPA, NADA and DA concentrations only varied about 50-, 30- and 7-fold, respectively, during the stages examined here.

Because of the relatively high levels of NBAD in the CNS, we wished to examine whether the abdominal ganglia were capable of conjugating DA with $\beta$-alanine or whether NBAD was accumulated from other sources. The results of analyses for NBAD and NADA in tissues and supernatants of cultured abdominal ganglia are shown on Table 2. Without DA supplementation, both catecholamines were synthesized, but 10–30 times more NBAD than NADA accumulated in the culture medium. When DA was added to the medium, NBAD synthesis was increased 2- to 4-fold, to as high as 9.3 nmol $g^{-1}$ per day of culture. NADA synthesis without exogenous DA did not exceed 0.1 nmol $g^{-1}$ per day, but with supplementation, NADA increased as much as 70-fold. Maximal synthesis of NBAD occurred in cultures of day 5 ganglia, whereas peak NADA synthesis was obtained with day 9 ganglia. Day 5 ganglia also contained the highest concentration of NBAD *in vivo*. Very little NBAD or NADA was sequestered in the tissue during *in vitro* culture (<2 nmol $g^{-1}$).

Table 2. *N*-Acylation of dopamine with $\beta$-alanine or acetate (nmol $g^{-1}$ $day^{-1}$) by abdominal ganglia from fifth larval instars of *Manduca sexta* cultured in Grace's medium *in vitro* with and without dopamine supplementation*

| Day after ecdysis† | NBAD | | NADA | |
|---|---|---|---|---|
| | +DA | −DA | +DA | −DA |
| 3 | $3.2^{a,1}$ | $1.5^{a,1}$ | $3.5^{a,1}$ | $0.1^{b,1}$ |
| 5 | $9.3^{a,2}$ | $2.7^{b,1}$ | $3.4^{a,1}$ | $0.1^{b,1}$ |
| 9 | $5.1^{a,1,2}$ | $1.3^{b,1}$ | $7.3^{a,2}$ | $<0.1^{b,1}$ |

*Means with the same superscript letters in the horizontal rows are not significantly different with respect to analysis with or without DA supplementation. Means with same superscript numbers in vertical columns are not significantly different with respect to stage of development ($P \leqslant 0.05$, $n = 6$–$8$).
†Day 3 = feeding, day 5 = wandering and day 9 = pharate pupa.

The *N*-acylcatecholamines, NBAD and NADA, as well as their precursors DA and DOPA, are localized within the CNS of larval, pharate pupal and adult *M. sexta*. NBAD has not been identified previously in the neural tissues, although it has been found in fifth larval instar integument and pharate pupal hemolymph of *M. sexta* (Hopkins *et al.*, 1982, 1984; Krueger *et al.*, 1989). NADA and DOPA are relatively minor in the CNS of *M. sexta*. The former catecholamine has been reported to occur in the CNS of *Periplaneta americana* and several other species (Murdock and Omar, 1981). The related monophenols, *N*-$\beta$-alanyltyramine (Maxwell *et al.*, 1980) and *N*-$\beta$-alanyloctopamine (Kennedy, 1977), were found in *M. sexta* and lobster neural tissue, respectively.

We also have measured catecholamine levels in the corpora allata and corpora cardiaca of *M. sexta*. Although NBAD and NADA were not detected (<1 nmol $g^{-1}$), DA was present at a concentration of $77.0 \pm 1.6$ nmol $g^{-1}$ (mean ± SE) or about 211 pg per complex in day 5 fifth larval instars. Sloley *et al.* (1986) measured DA at about one-half that amount in the *P. americana* corpora cardiaca/corpora allata complex.

In addition to the CNS and cuticle, NBAD also is found in tracheal samples and hemolymph of *M. sexta* (Hopkins *et al.*, 1984; Krueger *et al.*, 1989). However, we believe that ganglionic NBAD represents a separate pool from that of these tissues since (1) contamination of nervous tissue by trachea and hemolymph in our samples was quite low (<10% by weight); (2) trachea alone contain substantially less NBAD than the nervous tissue, and part of this may be localized in tracheal cuticle (Krueger *et al.*, 1989); (3) other heavily tracheated tissues, such as those of the digestive system, do not exhibit substantial NBAD levels (Krueger *et al.*, unpublished data); (4) hemolymph titers of NBAD are low when nervous tissue levels are at their highest (Krueger *et al.*, 1989); and (5) hemolymph NBAD is sequestered as a glucoside conjugate (Hopkins *et al.*, 1984). In *P. americana*, NADA was present in tracheal trunks at levels substantially higher than those found in nervous tissue (Murdock and Omar, 1981).

Using liquid chromatography with electrochemical detection, Murdock and Omar (1981) found nearly equal concentrations of DA and NADA in various ganglia of adult *P. americana*, whereas in this study, DA was considerably higher than NBAD in adult *M. sexta* ganglia. However, the relative concentrations of

NBAD and DA were reversed in ganglia of wandering larvae. Murdock and Omar (1981) did not analyze tissues from immature cockroaches. Surprisingly, NBAD accumulates to very high concentrations in ganglia of the entire CNS in *M. sexta* wandering larvae (day 5). Its precursor, DA, predominates in the CNS of newly ecdysed larvae and adults, but it also attains peak concentrations when NBAD levels are the highest. DA has been measured previously in the nervous system of *M. sexta* at the wandering stage of development (day 5 in this study) by Taylor and Newburgh (1979), who used a fluorescence based method of analysis. Those authors reported concentrations that were 2- to 3-fold higher than our values, but a similar regional distribution was observed. We found lower concentrations of NBAD in anterior ganglia than in the posterior ganglia. The significance of this regional distribution is unknown. Sloley and Downer (1987) reported that nervous tissue of *P. americana* contains DA at levels comparable to those of *M. sexta*. Levels of DA in mammalian dopaminergic neurons ($\sim$1–80 nmol $g^{-1}$, Stulik and Pacakova, 1986) are also similar to those in the *M. sexta* CNS.

Depending on the concentration tested, bursts of electrical activity or even synaptic blockage have been caused by DA in isolated insect ganglia of the CNS (Pitman, 1985). Orchard *et al.* (1986) reported that DA is present in the glandular lobe of *Locusta* corpus cardiacum, where it may serve as a neuromodulatory agent. Thus, it appears that DA has distinct neurotransmitter/neuromodulator functions in the insect nervous system, although more definitive studies of its roles are needed.

The significance of *N*-acylated catecholamines in the insect CNS is less clear. *N*-Acetylation generally is regarded as an inactivating pathway for insect monoamines. Little monoamine oxidase or catechol-*O*-methyl transferase activity has been found in invertebrates, whereas those enzymes play major roles in biogenic amine inactivation in vertebrates (Sharman, 1973; Evans and Fox, 1975a,b; Evans 1980; Brown and Nestler, 1985). Thus, *N*-acetylation may serve as a major inactivating mechanism for biogenic amines in insects, because other catabolic reactions appear to be lacking. NBAD and its precursors DA and DOPA all increased in the CNS during larval development, with NBAD accumulating to much higher levels, suggesting that sequestration of DA may occur as an inactive $\beta$-alanyl conjugate. Later during development, all of the catecholamines decreased to much lower concentrations, perhaps because of metabolism or transport.

NBAD may be an inactive derivative of DA or even $\beta$-alanine, the latter having also been detected in insect nervous tissue (Holman and Cook, 1982). $\beta$-Alanine has been implicated in neuroinhibitory mechanisms (Grossfeld, 1975). The pattern of regional and temporal localization suggests that NBAD may serve functions such as (1) storage of not only DA, but also of $\beta$-alanine as a readily utilizable conjugate; (2) neurotransmission or neuromodulation; (3) inactivation of either DA or $\beta$-alanine where the released neurotransmitter is conjugated to stop stimulation at the synapse; and (4) a structural role in the neuron or in the non-neuronal sheath.

NBAD rather than NADA is preferentially synthesized in the CNS of *M. sexta*, in contrast to *P. americana* and several other insects so far examined. NADA is derived from DA in most species, although in *Schistocerca gregaria*, NADA may be synthesized primarily from *N*-acetyltyramine rather than DA (Mir and Vaughn, 1981b). Although we have demonstrated that DA stimulates biosynthesis of both NBAD and NADA by ganglia cultured *in vitro*, the occurrence of *N*-$\beta$-alanyltyramine in ganglia of *M. sexta* (Maxwell *et al.*, 1980) also may indicate that another pathway for NBAD synthesis exists, involving *o*-hydroxylation of this compound. Thus, two reactions may lead to NBAD synthesis in *M. sexta* neural tissue, *N*-$\beta$-alanylation of DA and *o*-hydroxylation of *N*-$\beta$-alanyltyramine.

The only significant amount of DOPA in *M. sexta* nervous tissue was in ganglia from larvae exhibiting wandering behavior (Table 1). This may indicate that the *o*-hydroxylation of tyrosine is increased to a peak at that stage of development. Tyrosine monooxygenase has not been reported from insect nervous tissue, although we have observed this activity in preliminary experiments when tyrosine was incubated with homogenates of *M. sexta* nerve cords in the presence of 6-methyl-5,6,7,8-tetrahydropterin (Aso, Kramer and Hopkins, unpublished data). This enzyme may also catalyze the hydroxylation of *N*-$\beta$-alanyltyramine to NBAD.

Cultured abdominal ganglia of *M. sexta* were capable of synthesizing NBAD and NADA from exogenous DA in a stage specific manner. NBAD synthesis may be initiated in both larval ganglia as well as integument during the feeding period of the fifth instar (Krueger *et al.*, 1989). Both NBAD pools largely disappear during pharate pupal development, as the hemolymph NBAD glucoside pool increases to maximal levels. Integumental NBAD may be released during larval cuticle digestion by molting fluid and used for sclerotization of the pupal cuticle (Krueger *et al.*, 1989). Owen and Foster (1988) showed that DA levels in cerebral ganglia of *P. americana* cycle in phase with ootheca formation and suggested that neural DA metabolism is under the same regulation as that associated with oothecal tanning. However, the fate of NBAD sequestered in the ganglia is unknown. The fact that the *N*-acylated catecholamines were not sequestered within the ganglia *in vitro* to the same degree as observed *in vivo* indicates either that these metabolites are not stored within secretory vesicles, that a stimulus for release is contained in the culture medium, or that the catecholamines diffuse from the cut nerve endings.

In conclusion, we have demonstrated that NBAD is synthesized from DA and is sequestered in high concentrations within the ganglia of *M. sexta* at the end of larval development. Concentrations varied in both a temporal and regional manner, with the abdominal ganglia containing the highest concentrations. The function of the *N*-acylated catecholamines in the CNS remains unknown, but inactivation of DA and $\beta$-alanine entering the nervous system from the hemolymph remains a possibility. NBAD concentrations then become very low in pharate pupae suggesting its movement out of the nervous system, perhaps as part of the large

hemolymph pool of NBAD glucoside which serves as a diphenol and carbohydrate precursor for pupal cuticle tanning. Thus, it appears that a major metabolic route for DA in the hornworm's integument and nervous sytem is through $N$-$\beta$-alanylation, a situation not paralleled in vertebrates (Brown and Nestler, 1985; Kramer and Hopkins, 1987). Further research, including the subcellular localization and possible electrophysiological activity of these compounds, is needed to clarify their roles in the insect CNS.

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