Localization of the Pupal Melanization Reducing Factor of *Inachis io* (L.) and Comparison with Melanization and Reddish Coloration Hormone

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In *Inachis io,* a pupal melanization reducing factor (PMRF) which controls morphological color adaptation is located in the brain, suboesophageal ganglion, thoracic ganglia, and all abdominal ganglia. Higher PMRF amounts were extracted from abdominal ganglia than from the anterior ganglia. No PMRF activity could be found in the *Corpora cardiaca-Corpora allata* complex, in segmentally branching nerves of abdominal ganglia and their connectives.

Extracts from brain-thoracic ganglia and abdominal ganglia complex of *I. io* contained also a factor with melanization and reddish coloration hormone (MRCH) activity in *Pseudaletia separata* and with pheromone biosynthesis activating neuropeptide (PBAN) activity in *Bombyx mori.* However, injection of synthetic *Pseudaletia* pheromonotropin (Pss-PT) (= Pss-MRCH) into prepupae of *I. io* did not yield a melanization reducing effect. Therefore, PMRF and the PBAN/MRCH related neuropeptides seem to be different molecules. The PBAN-like factor from *I. io* is possibly related to the myotropins and pyrokinins of insects.

Introduction

In numerous nymphalid butterfly pupae morphological color adaptation to the background color (Poulton, 1887; Bückmann, 1960, 1974; Koch and Bückmann, 1984) is controlled by a factor from the anterior part of the prepupa, which gradually inhibits melanization (Bückmann, 1960, 1969). This factor was extracted from head-prothorax fragments of *Inachis io* and was named pupal melanization reducing factor (PMRF) (Bückmann and Maisch, 1987). PMRF activity has also been extracted from head-prothorax fragments of different other lepidopteran families including such without pupal color adaptation (Koch *et al.,* 1990) and even from crustacean sinus glands (Bückmann *et al.,* 1990). Enzymatic digestion demonstrates the peptide nature of PMRF (Bückmann and Maisch, 1987). Its apparent molecular weight is between 1000 and 6000 Da (Starnecker *et al.,* 1990). Purification and identification of the peptide are in progress.

One neuropeptide controlling pigmentation in preadult Lepidoptera has already been isolated and sequenced: The melanization and reddish coloration hormone (MRCH) of the armyworm larva, *Pseudaletia (= Leucania) separata.* It was extracted from brain and suboesophageal ganglion (SOG) and is effective in several armyworm species (Matsumoto *et al.,* 1981). It represents an 18 amino acid peptide having a C-terminal FXPRL-amide sequence which is essential for both MRCH and pheromone biosynthesis activating neuropeptide (PBAN) activities (Raina and Kempe, 1990; Matsumoto *et al.,* 1992 a, b).

In the present paper, we report on the neural origin and localization of PMRF in different parts of the nervous system of *I. io.* In addition, its possible functional relation to MRCH/PBAN was determined by reciprocal injection experiments. Various ganglion extracts from *I. io* as well as synthetic *Pseudaletia* pheromonotropin (Pss-PT) (= Pss-MRCH) were tested for melanization reducing activity in *I. io,* and extracts from *I. io* were tested for pheromonotropic activity in *Bombyx mori,* which would indicate at the same time the presence of MRCH.

Materials and Methods

Animals

Inachis io L. (Lepidoptera, Nymphalidae) were reared in a permanent stock colony according to Maisch and Bückmann (1987). Last instar larvae

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were kept in 30×35×35 cm boxes covered with a 30 cm high gauze dome, placed at a window under natural light conditions and fed with nettle (*Urtica dioica* L.). Mature larvae of the wandering stage crowd at the top of the gauze dome to become prepupae which are easily to be recognized by their hanging position. These prepupae were collected.

Bombyx mori (Kinshu and Showa) larvae were reared on an artificial diet as described previously (Fonagy *et al.*, 1992a).

Dissections and sample preparations

The following parts of the nervous system of 1 h old prepupae were dissected in lepidopteran ringer (KCl, NaCl, 150 mM each; $CaCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, KH_2PO_4, K_2HPO_4, 100 mM each; glucose 300 mM) (K. Endo, personal communication) and immediately transferred into ice-cold acetone: brain (Br) with retrocerebral complex [*Corpora cardiaca* (CC) and *Corpora allata* (CA)], suboesophageal ganglion (SOG), pro-, meso-, and metathoracic ganglia in connection ($TG_{1,2,3}$), and all abdominal ganglia (AG_{1-6} and the fused terminal ganglion AG_7). Further samples consisted of CC-CA complexes, the 6 abdominal connectives without the ganglia, the two pairs of nerves branching from AG_{1-6} taken all together, and the nerves fanning from the terminal abdominal ganglion (AG_7). The latter two samples were dissected in connection with adhering tissues. Salivary glands and parts of the fat body were used as controls.

Extraction of PMRF and bioassay

The tissues were sonicated twice in ice-cold acetone. After centrifugation (10 min, 15,000×*g*), the supernatant was discarded and the pellet extracted three times with ice-cold 80% (v/v) ethanol in water. Combined ethanolic extracts were dried in a high speed vacuum concentrator and redissolved in water. 10 µl of this solution were injected into each test animal. For some preliminary tests Br+SOG+TG complexes were homogenized and extracted in methanol–water–acetic acid (100:10:1, v/v). After injection of 20 equivalents per animal the resulting pupae showed a mean melanization score of 4.2, while injection of 20 equivalents from an 80% ethanol extraction resulted in a score of 1.8, which means a much higher yield in PMRF activity (see below). Lipids were removed from fat body as well as from samples with adhering fragments of fat body with diethylether and *n*-hexane before extraction of PMRF with 80% ethanol.

Samples were tested for their PMRF activity in *I. io* using the bioassay of Maisch and Bückmann (1987). Injection was carried out into prepupae kept on a black background which normally develop into strongly melanized pupae. The degree of melanization of the resulting pupae is determined by a scoring system of 5 classes, where class 5 represents the most intense and class 1 the least intense melanization. A mean melanization score of 1.0 corresponds to a 100% reduction of melanization.

The pheromonotropic activity of ganglia extracts from *I. io* was tested according to Matsumoto *et al.* (1990). Newly emerged females of *B. mori* were decapitated within 3 h and kept for 24 h at 25 °C. Samples of 10 µl were injected into abdomina and after 90 min pheromone glands were dissected and extracted with 100 µl of *n*-hexane and subjected to HPLC to measure the amount of bombykol as reported previously (Fonagy *et al.*, 1992a).

Wilcoxon's rank test was used for comparison of values in Table II.

Results

Distribution of PMRF activity in I. io

To reveal the distribution of PMRF within the central nervous system, brains and ventral nerve cords of 120 prepupae were dissected, divided into different parts and tested for PMRF activity (Table I). Extracts from the whole anterior part of the nervous system, consisting of Br+SOG+TG_{1-3} complexes, injected in a dosage of 20 equivalents per test animal, yielded melanization scores between 2 and 3, corresponding to a reduction of melanization ranging from 50% to 75%. Subdividing this anterior complex into 4 different parts (Br, SOG+TG_{1-3}, Br+SOG and TG_{1-3}) resulted in PMRF activities with melanization scores of about 2 for each complex (Table I). This indicates that PMRF is equally distributed in the anterior part of the nervous system.

Extracts from the complete abdominal nerve cord (AG_{1-7}) contained high PMRF activity, even

Table I. PMRF activity of extracts from different parts of the nervous system of *I. io*, scored in 5 melanization classes from 1 = light to 5 = dark. Mean melanization score is given with standard error of mean (±S.E.M.) of 6 animals (n = 6) injected with 20 equivalents each.

Tissue	Melanization score in sample No. 1	2	3
Br+SOG+TG_{1-3}	2.1 ± 0.1	3.0 ± 0.52	2.4 ± 0.19
Br	2.1 ± 0.1	2.1 ± 0.1	
SOG+TG_{1-3}	1.9 ± 0.1	1.7 ± 0.3	
Br+SOG	2.9 ± 0.56	2.0 ± 0	
TG_{1-3}	2.1 ± 0.12	2.0 ± 0.16	
AG_{1-7}	1.0 ± 0	1.0 ± 0	1.2 ± 0.12
AG_{1-4}	1.0 ± 0		
AG_{5-7}	1.0 ± 0		
Water injection	4.8 ± 0.11		
Untreated animals on black background	4.95 ± 0.05 (n = 10)		
Untreated animals on yellow background	1.1 ± 0.06 (n = 10)		

Br, brain; SOG, suboesophageal ganglion; TG_{1-3}, thoracic ganglia 1–3; AG_{1-7}, abdominal ganglia 1–7. Samples 1–3 are from three different independent stocks of animal material.

Table II. PMRF activity of extracts from abdominal ganglia of *I. io* with and without segmental branching nerves. Mean melanization score is given ±S.E.M. of n animals injected with 25 ganglia equivalents each.

Tissue	Melanization score	n
Abdominal ganglia without nerves		
AG_{1+2}	1.6 ± 0.1	6
AG_{3+4}	2.2 ± 0.34	6
AG_{5+6}	2.7 ± 0.37	6
AG_7	2.8 ± 0.46	6
Abdominal ganglia with nerves		
AG_{1+2}	2.3 ± 0.4	6
AG_{3+4}	1.6 ± 0.1	6
AG_{5+6}	1.7 ± 0.12	6
AG_7	2.6 ± 0.19	6
Water injection	4.8 ± 0.12	9

AG_{1+2}, abdominal ganglia 1 and 2 ...; AG_7, fused terminal abdominal ganglion. Differences between AG with and without nerves each are not significant ($p > 0.05$).

more than the anterior part (Table I). This can not only be an effect of the larger number of AG compared to the 5 ganglia of the Br+SOG+TG complex, because even after separation of the abdominal nerve cord into two parts (AG_{1-4} and AG_{5-7}) each of them yielded a melanization score of 1, which means the maximum melanization reducing effect.

After a further subdivision of the abdominal nerve cord into three parts of 2 ganglia each and the separate AG_7 a lower PMRF activity was expected than from larger number of AG. Therefore, this time 25 equivalents per test animals were used. Resulting pupae showed rather uniformous melanization scores ranging from 1.6 to 2.8 (Table II). This demonstrates that PMRF is equally distributed among all seven AG.

Extracts of the AG including their segmental branching nerves and associated neurohemal areas did not exhibit significantly more PMRF activity than those of the ganglia alone, indicating that these nerves do not contain substantial amounts of PMRF (Table II). This result was confirmed by testing extracts of pooled branching nerves from AG where no PMRF activity was measurable (Table III).

Neither the combined connectives between AG nor the CC–CA complexes contained PMRF activity. The same was the case in control tissues of salivary glands and fat body (Table III).

Table III. PMRF activity of extracts from nervous system, retrocerebral complex, nerves and control tissues of *I. io*. Mean melanization score is given ±S.E.M. of n animals injected with 20 equivalents each.

Tissue	Melanization score	n
Br+SOG+TG_{1-3}	2.1 ± 0.13	4
CC-CA complex	4.8 ± 0.11	6
Connectives of AG_{1-7}*	4.5 ± 0.2	4
Segmental nerves of AG_{1-6}**	5.0 ± 0.0	4
Nerves branching $AG7_{\#}$	4.5 ± 0.35	4
Salivary glands	5.0 ± 0.0	5
Fat body$^{\#\#}$	4.6 ± 0.11	5
Water injections	4.6 ± 0.2	7
Untreated animals on black background	4.9 ± 0.09	11
Untreated animals on yellow background	1.2 ± 0.1	11

Br+SOG+TG_{1-3}, brain-suboesophageal ganglion-thoracic ganglia 1–3 complex; CC–CA, *Corpora cardiaca-Corpora allata* complex; AG, abdominal ganglia; *, all 6 connectives between AG; **, segmental nerves consisting of two paired nerves branching from each of the AG_{1-6}; $^{\#}$, nerves branching AG_7 consisting of the nerves fanning from terminal abdominal ganglion; $^{\#\#}$, fat body from prepupae.

MRCH activity in the central nervous system of I. io

The melanization and reddish coloration hormone (MRCH) of *P. separata* can be assayed by its effect as pheromone biosynthesis activating neuropeptide (PBAN) in *B. mori*. In preliminary experiments, PBAN activity tested in *Bombyx* moths as well as MRCH activity tested in *Pseudaletia* larvae could be extracted from head-prothorax fragments of *I. io* with 80% ethanol (data not shown). To screen for pheromonotropic activity within the central nervous system of *I. io*, ganglia were separated into two parts consisting of nerve cords from Br+SOG+TG and of the 7 abdominal ganglia (AG_{1-7}). Both extracts were equally capable of stimulating the pheromone glands of *Bombyx* females to produce bombykol (76.8 to 84.8 ng at 2 equivalents and 62.4 to 72.0 ng at 20 equivalents, respectively). This shows that *I. io* prepupae contain a PBAN-like factor in the anterior as well as in the posterior part of central nervous system. In this very sensitive bioassay a dose of 2 equivalents was sufficient to effect maximum pheromone production (Table IV).

Genuine MRCH did not show any PMRF activity in *I. io*. Injection of 3 synthetic MRCHs, at that time named *Pseudaletia* pheromonotropins (Pss-$PT_{1-18}NH_2$, Pss-$PT_{2-18}NH_2$ and Pss-$PT_{7-18}NH_2$) in doses between 15 and 500 pmol into *I. io* prepupae also had no melanization reducing effect (Table V). 1 pmol of this hormone and its fragments tested in the *Bombyx* PBAN bioassay, and 10 pmol tested in the *Pseudaletia* MRCH bioassay showed high activities (Matsumoto *et al.*, 1992 a, b).

Table IV. PBAN and PMRF activities of extracts from anterior and posterior parts of the central nervous system of *I. io* prepupae. Mean values ±S.E.M.

Tissue	PBAN activity in *Bombyx mori* Production of ng bombykol/PG	Eq.	*n*	PMRF activity in *Inachis io* Melanization score	Eq.	*n*
Br+SOG+TG	76.8 ± 10.0	2	3	2.7 ± 0.4	20	5
	62.4 ± 11.1	20	3			
AG_{1-7}	84.8 ± 14.2	2	3	1.7 ± 0.2	20	5
	72.0 ± 20.9	20	3			
Water injection	4.8 ± 1.4		3	4.7 ± 0.21		10

PG, pheromone gland; Eq., equivalents of ganglion complexes injected; for further abbreviations see Table I.

Table V. Effect of synthetic *Pseudaletia* pheromonotropin (Pss-PT) and two of its fragments on melanization of *I. io* pupae. Mean melanization score ± S.E.M. of 5 test animals.

	Dose injected [pmol]	Melanization score
Pss-$PT_{(1-18)NH_2}$	500	4.3 ± 0.2
	250	4.5 ± 0.31
	125	4.7 ± 0.2
	60	5.0 ± 0.0
	30	4.3 ± 0.34
	15	4.5 ± 0.28
Pss-$PT_{(2-18)NH_2}$	500	4.6 ± 0.29
	250	4.4 ± 0.29
	125	4.6 ± 0.19
	60	4.4 ± 0.29
	30	4.6 ± 0.4
	15	4.7 ± 0.2
Pss-$PT_{(7-18)NH_2}$	500	4.4 ± 0.19
	250	4.4 ± 0.19
	125	4.3 ± 0.3
	60	4.6 ± 0.19
	30	4.5 ± 0.22
	15	4.7 ± 0.2

From the melanizing effect of MRCH in *Pseudaletia* larvae a similar effect might have been expected in *I. io* pupae, which means a promotion of melanin instead of an inhibition. However, partially purified MRCH injected into prepupae of *I. io* from yellow background, which yield uniformously light pupae, did not activate the melanization of the pupal cuticle (data not shown).

Discussion

The site of PMRF formation

Hitherto PMRF was known to occur in the anterior part of nymphalid pupae (Koch and Bückmann, 1984). This was especially demonstrated in *I. io* and *Aglais urticae* by ligation experiments, nerve transections (Bückmann, 1969) and extractions from head-prothorax fragments (Koch *et al.*, 1990).

After ligation, the anterior part of the body is able to adapt its pigmentation to the background color, which shows that it must contain the source of melanization inhibiting factor, while posterior parts, becoming maximum melanized seemed to lack such a factor. Nerve transections between brain and SOG abolishes the reduction of melanin

in *A. urticae,* leading to uniformously black pupae. This demonstrates a control of color adaptation by the brain. Transections posterior to SOG diminished the reduction of pupal melanization. This effect was the weaker, the more ganglia remained in connection with the brain. Even transection between abdominal ganglia diminished the melanization somewhat (Bückmann, 1969). This means that several ganglia are involved in the formation of PMRF. Extracts of head-prothorax fragments from prepupae of *I. io, A. urticae, Pieris brassicae, Galleria mellonella (*Koch *et al.,* 1990) and *Precis coenia* (Bückmann *et al.,* 1990) contained a factor which reduced pupal melanization in *I. io.*

Similarily, in pierids and in papilionids with pupal color adaptation the pigmentation is controlled by factors from the anterior part of the body (Hidaka, 1956, 1961; Ohtaki, 1963; Bückmann, 1971).

The results from extraction of PMRF from whole anterior body fragments of *I. io* (Bückmann and Maisch, 1987) are now supplemented by those from isolated parts of the nervous system. They show that PMRF is, indeed, located in the anterior ganglia and, furthermore, that PMRF is distributed equally throughout all anterior ganglia including the brain (Table I), but not in the CC-CA complex (Table III). Unexpectedly, however, abdominal ganglia contain even more PMRF activity than the anterior ganglia. Again, the subdivision of this part of the abdominal nerve cord indicates, that the melanization reducing factor is present in every ganglion. Thus, our results demonstrate an even distribution of PMRF throughout the entire central nervous system. This is in agreement with the nerve severing experiments in *A. urticae* (Bückmann, 1969), where dissection even at the level of the third AG diminished the melanization reducing effect.

A hormone distributed through all the nervous system is not necessarily produced by all ganglia, as a transport of neuropeptides through parts of the ganglionic chain is known *e.g.* from diuretic hormone of the SOG of *Locusta migratoria* (Proux and Rougan-Rapuzzi, 1980; Remy and Girardie, 1980; Morgan and Mordue, 1984) and the eclosion hormone of the brain of *Manduca sexta* (Truman and Copenhaver, 1989).

However, in *I. io* the production of the hormone in all ganglia is more likely than a transport *via* connectives, because no PMRF activity is measurable in extracts of connectives between AG (Table III). This means, that the site of release is probably near the site of production.

The site of PMRF release

As the same PMRF content is found in AG, whether segmental branching nerves are cut or not (Table II) and extracts from these segmental nerves show no PMRF effect (Table III), probably no neurohemal areas in this peripheral region are sites of PMRF release. More likely neurohemal organs (NHO) in close proximity of the ventral nerve cord are involved, which could not be separated from AG in our preparation.

Metameric NHOs in close proximity of the ventral cord connected to the median nerve have been described in different insect species, especially in lepidopteran families by Raabe *et al.* (1971) and Provansal (1972). Actual release of neuropeptides close to the ganglia has been reported for diuretic hormone in *Rhodnius* and *Glossina* (Maddrell and Gee, 1974) and for melanization controlling hormone in first instar larvae of *Schistocerca gregaria* (Padgham, 1976).

The control of PMRF release

As the anterior segments of ligated animals exhibit normal color adaptation they must contain both, NHOs responsible for PMRF release and a releasing stimulus. This stimulus is affected by the brain through nervous transmission, as indicated by the nerve transection experiments (Bückmann, 1969, 1971). Similarily, in *Papilio xuthus* pupal color adaptation depends on intact connection between brain, SOG and prothoracic ganglion (Hidaka, 1956).

However, the large content of PMRF activity in abdominal ganglia of *I. io* is in contrast to the results of ligation experiments, where isolated abdomina were always maximally melanized. This, however, can be understood by the assumption that release of PMRF into the abdomen is controlled by the anterior nervous system and this control is excluded by isolating the abdomina, so that the factor remains unreleased.

Interspecific effects and the relation of PMRF to PBAN

MRCH of the larval heads of *P. separata* which controls larval pigmentation has sequence homol-

ogies with the pheromone biosynthesis activating neuropeptide (PBAN) of the adult *B. mori* (Matsumoto *et al.*, 1990, 1992a, b). Both peptides can replace each other in their mutual effects. In *I. io*, we also found an PBAN-like factor in the anterior nervous system (Br+SOG+TG) as well as in the posterior part (AG_{1-7}). Furthermore, it has been demonstrated that some insect myotropins also have MRCH activity as well as PBAN activity (Fonagy *et al.*, 1992b; Matsumoto *et al.*, 1993). These functional cross-reactivities reside in the C-terminal pentapeptide, Phe–X–Pro–Arg–Leu–NH_2. Since myotropins and pyrokinins also contain this C-terminal pentapeptide and these peptides are widely distributed in central nervous systems of insects (Schoofs *et al.*, 1993), the PBAN-like factor from *I. io* may belong to this FXPRLamide peptide family.

On the other hand, synthetic *Pseudaletia* pheromonotropins failed to reduce pupal melanization in *I. io*. Furthermore, PBAN and PMRF activity can be separated by two different HPLC systems into different fractions (Starnecker *et al.*, 1994). Therefore, PMRF and the PBAN-like factor of *I. io* are different molecules. As the *Pseudaletia* pheromonotropin is identical with MRCH, PMRF and MRCH are also different.

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