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Neuroscience Letters 368 (2004) 186–191
Neuroscience Letters
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Constitutive activity of *N*-β-alanyl-catecholamine ligase in insect brain

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Received 4 May 2004; received in revised form 1 July 2004; accepted 5 July 2004

Abstract

N-β-Alanyldopamine (NBAD) is the primary catechol tanning agent precursor in typical brown or yellow insect cuticle. The insect integument enzyme responsible for the synthesis of NBAD was reported to be expressed solely in the epidermis, and only at the time of cuticle sclerotization. However, in this study we demonstrate directly that the enzyme also is expressed in a constitutive manner in the neural system of insects. The requirements and kinetic parameters of the brain-associated enzyme appear similar to those of the epidermis-associated enzyme in *Ceratitis capitata*. The brain-associated enzyme also was able to catalyze the in vitro synthesis of *N*-β-alanylnorepinephrine (NBANE) and β-alanyl derivatives of other biogenic amines. A melanic mutant of *C. capitata*, *niger*, was unable to conjugate β-alanine with dopamine or other amines in either the epidermis or the brain. This result strongly supports the idea that these enzymes actually are expressed from a single gene and that differences in regulation must exist that account for the constitutive expression in the neural system. Similar results were obtained in *Drosophila melanogaster* and other insects. From these data, a number of questions arise about the role of β-alanyl derivatives of biogenic amines and other compounds in insect brain and similarly, in the mammalian CNS.
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Keywords: β-Alanine; *N*-β-Alanyldopamine; *N*-β-Alanylnorepinephrine; *Ceratitis capitata*; Medfly; Biogenic amines

N-β-Alanyldopamine (NBAD) is required for tanning (sclerotization and pigmentation) of the arthropod cuticle, and is the main precursor of the typical brown or yellow insect cuticle [1,8]. We previously characterized the enzyme required for its synthesis [15,16,23], using cell-free extracts of the Medfly (*Ceratitis capitata*) pre-pupae, collected at the onset of metamorphosis. The activity of this *N*-β-alanyl-catecholamine ligase is induced in the epidermis just at the time of sclerotization [23]. A similar enzyme might be involved in the pigmentation of swallowtail butterfly wings [13]. We determined that the enzyme is an ATP-dependent ligase with broad substrate specificity that also is capable of synthesizing *N*-β-alanylnorepinephrine (NBANE) [16]. Due to the rapid loss of activity in vitro [16] the enzyme has been difficult to purify. In addition, we demonstrated that a Medfly melanic mutant, *niger*^{1} (*nig*^{1}), is unable to synthesize NBAD or NBANE [15,16,23]. The recessive point mutation in the single copy gene *niger* was obtained using ethyl methanesulfonate (EMS) as a mutagenic agent. The ligation group of *niger* was assigned to the Medfly chromosome two, which is poorly characterized. The mutation triggered pleiotropic melanic effects on the puparium, the posterior larval spiracles and the adult body. An early, striking observation from our laboratory was that small but significant amounts (in terms of enzyme specific activity) of NBAD were synthesized by extracts of non-sclerotizing Medfly adult heads. This was in contrast to results supporting the accepted idea that only the epidermis of molting insects was capable of synthesizing NBAD. Moreover, Krueger et al. [14] detected the presence of NBAD in neural tissue of the tobacco hornworm, *Manduca sexta*. Therefore, we wanted to investigate the synthesis of NBAD and other β-alanine derivatives in insect brain and study the characteristics of the enzyme involved in its synthesis. To our knowledge, this is the first in vitro study showing that NBAD synthase is expressed in a constitutive manner in the central nervous system (CNS) of *C. capitata* and of all other insects tested.

Insects: Wild type *C. capitata* ("INTA Arg 17") strain and the mutant strain *niger* (*niger*^{1}) were reared in carrot-based

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doi:10.1016/j.neulet.2004.07.015

medium as previously described [17]. All of the results below refer to time-dependent events occurring during the standard life cycle under controlled culture conditions [19]. The insect cultures were synchronized, and the exact age of each one of the insects used in the experiments was assessed using a binocular microscope [20]. The fly age within the puparium is expressed in hours after puparium formation (apf), starting from the definitive immobilization of the third-instar larva defined as "Zero time" [19]. Dissections were performed on a refrigerated plate; ganglia (brains) were freed from other tissues and the pigment layer (adults) was eliminated. Wild type *D. melanogaster Oregon R* and the mutant strain *ebony*[4] were reared in commercial fly medium and maintained at 22 °C. Other insects were grown using standard procedures. *Chemicals*: All the available biogenic amines and/or standards were from Sigma Chem. Co. [^{14}C]β-alanine was from New England Nuclear (54.5 μCi/μmol). *N*-β-Alanyldopamine (NBAD) and other standards were generously synthesized by Dr. J.M. Aguirre (Luján University, Argentina) as described in Yamasaki et al. [24] with slight modifications. *N*-β-Alanylnorepinephrine (NBANE) was a generous gift from Dr. K. Kramer (USDA-ARS, Manhattan, KS, USA), which also provided the initial standards of NBAD. Sarcophagine (*N*-β-alanyl-tyrosine) was generously synthesized by Dr. J. Drijfhout from the Medical Center of Leyden University, Netherlands. *Cell extracts*: Slightly purified enzymatic extracts from *C. capitata* were prepared exactly as described [16]. The extracts contained an *N*-β-alanyldopamine hydrolase that was not active under our standard conditions for synthesis. Other insect extracts were prepared in the same way. *Synthase assay and product characterization*: Cell-free synthase activity was measured as previously described [16]. Catecholamine derivatives were isolated using alumina columns and analyzed by reversed phase HPLC (Econosphere-C-18, Altech) as previously described [16]. Other biogenic amine derivatives were analyzed directly using the same HPLC column. Radioactivity in the samples was measured in a Rackbeta 1214 Pharmacia liquid scintillation counter using Ultimagold (Packard) as scintillant. Protein concentrations were determined using the Folin reagent and BSA as the standard.

Previously, we established [16] that *C. capitata* extracts from wild type pre-pupae show high levels of *N*-β-alanyldopamine synthase activity, since at this stage the larval cuticle is being tanned to give rise to a golden-brown puparium. Using [^{14}C]β-alanine as tracer substrate and dopamine (DA) or norepinephrine (NE) as alternative second substrates, [^{14}C]NBAD and [^{14}C]NBANE, respectively, were synthesized by the epidermis enzyme from 3 h apf pre-pupae (Table 1). Similar levels of enzymatic activity were detected in the integument of exarate adults, having just emerged from the puparium (Table 1 and Fig. 3). However, negligible enzymatic activity could be detected in the integument during intermolt periods (i.e., larva III, Table 1 and Fig. 3) or after final sclerotization of adults (Fig. 3).

We tested the ability of head homogenates from sclerotized 12-day-old wild type *C. capitata* adults to synthesize radiolabeled NBAD. Since no sclerotization occurs in adult flies after several hours after ecdysis, the synthesis of catecholamine derivatives containing [^{14}C]β-alanine by late adult insects must be unrelated to sclerotization. Table 1 shows that the cell-free extracts of heads from non-sclerotizing *C. capitata* 12-day-old adults were able to synthesize 21.15 ± 1.16 pmol min^{-1} mg^{-1} of a substance that was the only labeled material retained in alumina, thus behaving as a catecholamine. The labeled conjugate behaved similarly to NBAD in C-18 reverse-phase HPLC (Fig. 1A) and in silica-gel TLC (not shown). The apparent K_M for DA was 20.2 μM. These parameters are in the range of the epidermis-associated enzyme synthesized from 3 h apf sclerotizing pre-pupae, which showed a specific activity of 23.11 ± 1.67 pmol mg^{-1} min^{-1} (Table 1) and an apparent K_M (DA) of 29.5 ± 3.5 μM. When the brain product was boiled in 1.2N

Table 1
Biosynthesis of β-alanyl derivatives by insect brain extracts

Insect/tissue	NBAD[a] (pmol min^{-1} mg^{-1})	NBANE[a] (pmol min^{-1} mg^{-1})
Ceratitis capitate		
wt pre-pupal integument[b]	23.11 ± 1.67	11.66 ± 1.20
wt just emerged exarate adults	36.44 ± 1.97	5.63 ± 0.72
wt larva III integument	0.00	0.00
wt 12 days old adult head	21.15 ± 1.10	6.71 ± 1.19
wt 12 days old beheaded adult body	0.70 ± 0.12	0.27 ± 0.25
wt larva III brain	4.34 ± 0.01	3.34 ± 0.08
wt 12 days old adult brain	35.00 ± 2.82	32.55 + 0.65
niger pre-pupae[b]	0.10 ± 0.01	0.06 ± 0.01
niger adult head	0.0	0.0
niger larva III brain	0.0	0.0
Drosophila melanogaster		
wt adult head	43.00 ± 7.00	73.10 ± 6.01
wt adult body	0.0	0.0
ebony adult head	0.0	0.0

wt: wild type.
[a] Apparent enzymatic activity.
[b] 3 h apf.

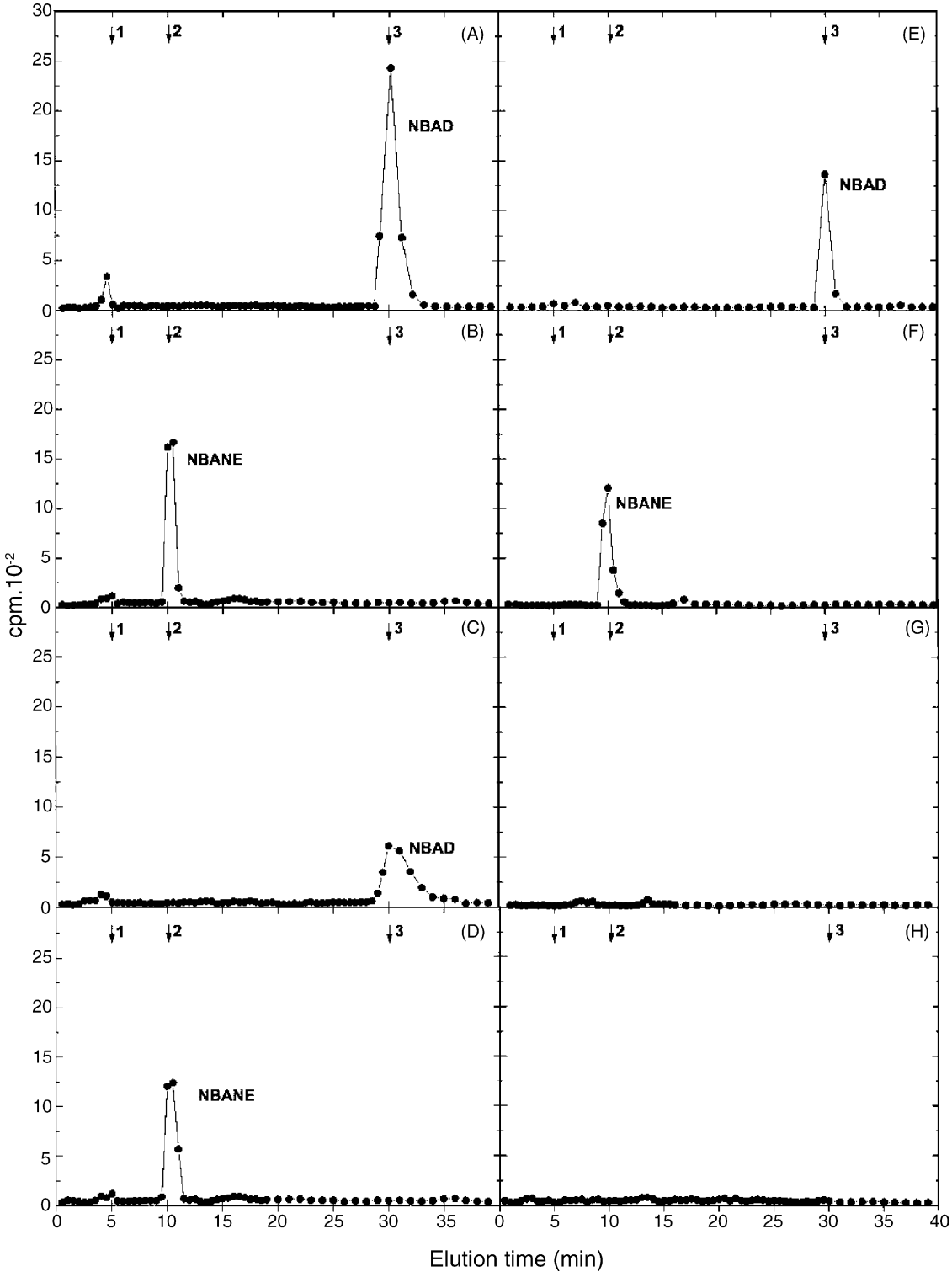

Fig. 1. HPLC analysis of NBAD and NBANE synthesized by neural tissue. (A–D) synthesis by extracts of wild type *C. capitata*: (A, B) adults heads, (C, D) larva III ganglia. (E, F) synthesis by *D. melanogaster* head extracts of NBAD (E) and NBANE (F). The mutant strains *C. capitata niger*[1] (G) and *D. melanogaster ebony*[4] (H) were unable to synthesize NBAD or NBANE. (1) β-Alanine, (2) NBANE and (3) NBAD.

HCl for 4 h (typical conditions for NBAD hydrolysis), most of the radioactivity was recovered as [^{14}C]β-alanine. Moreover, without exogenously added DA, traces of a radioactive substance behaving similarly to NBAD in HPLC were synthesized, thus reflecting endogenous levels of DA (not shown). Homogenates from the rest of the body showed only traces of enzymatic activity (0.70 ± 0.12 pmol mg^{-1} min^{-1}) (Table 1). The head extracts of non-sclerotizing *D. melanogaster* wild type adults also were able to synthesize NBAD (Table 1 and Fig. 1E). The apparent kinetic properties of the *D. melanogaster* brain-associated enzyme were similar to those of *C. capitata* (not shown). Significantly, the head extracts of both flies were able to use norepinephrine as a substrate (instead of DA) to synthesize [^{14}C]NBANE, as demonstrated by HPLC analysis (Table 1 and Fig. 1B–F). To confirm these results, we used homogenates of carefully isolated ganglia/brains of *C. capitata* larvae III and 12-day-old adults (Table 1). Due to the lability of the enzyme, the dissections (more than 50 brains per batch) must be performed rapidly (1 brain/min) at 3–4 °C. The results confirmed that all the synthesized [^{14}C]NBAD and [^{14}C]NBANE measured in the head extracts corresponded to brain-associated enzymatic activity (Table 1). As shown in Fig. 1C and D, the HPLC profiles of the corresponding ganglia products were identical to those obtained from whole heads as shown in Fig. 1A and B. The synthetic capacity exhibited by ganglia from larvae III occurred during intermolt, and therefore contrasts with the results in epidermis, where no synthesis of NBAD occurred (Table 1). The same reasoning could be applied to adult brains. As with the epidermis-associated enzyme [16], the *C. capitata* brain NBAD synthase was found to be extremely sensitive to temperatures higher than −70 °C (not shown), thus making purification and further kinetic analysis difficult. The above results were confirmed in insects other than dipterans since extracts from brain (adult heads) of several hemimetabolous insects like *Aeschna bonariensis*, the dragon fly, *Triatoma infestans*, the blood-sucking bug, and the cockroach *Blatella germanica* as well as holometabolous like *Apis mellifera*, the honey-bee, and *Tenebrio molitor*, the yellow mealworm, also synthesized NBAD and NBANE (not shown). To support the physiological relevance of our in vitro results, we performed a preliminary analysis of endogenous biogenic amines in batches of Medfly brains. In spite of the scarcity of the available material and the extreme complexity of the HPLC profiles (not shown), substances with retention times similar to NBAD and NBANE were detected at an estimated level of 40 pg/mg of brain tissue. *C. capitata* brain-associated enzymatic extracts also were able to accept other biogenic amines as alternative substrates (instead of dopamine), to synthesize the corresponding derivatives. Fig. 2 shows that, as judged from HPLC analysis, *N*-β-alanyl derivatives of octopamine (NBAOA), tyramine (NBATA) and histamine (carcinine, NBAHA) were formed (Fig. 2A–C). A serotonin (5HT) derivative, *N*-β-alanyl-serotonin (NBA5HT) also was synthesized (Fig. 2D). The biosynthesis of these substances has not been reported previously and will require

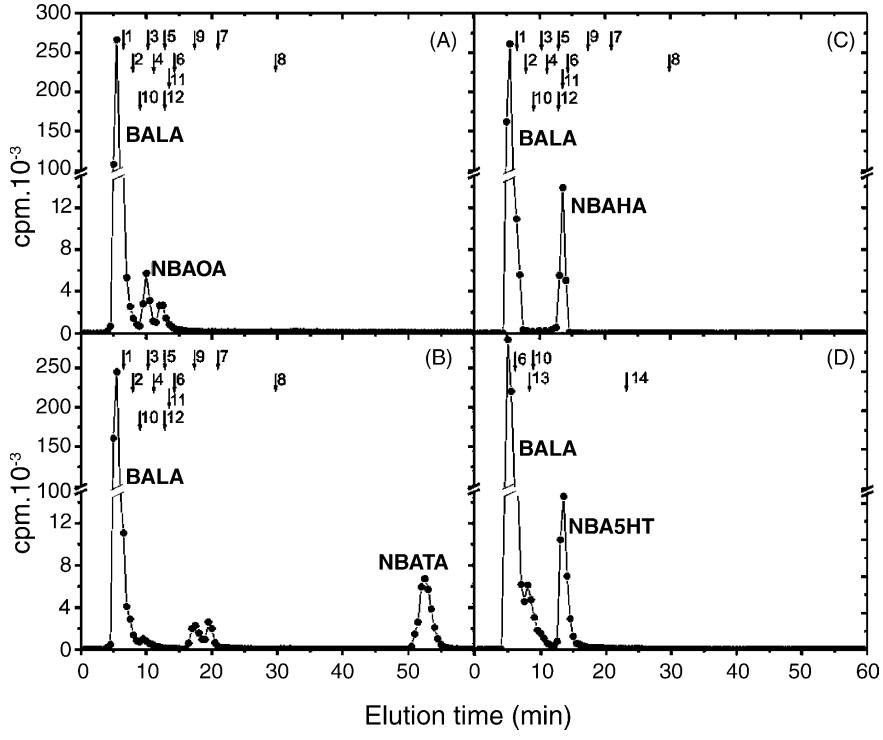

Fig. 2. HPLC analysis of β-alanyl derivatives synthesized by brain extracts of *C. capitata*. The incubation mixtures contained [^{14}C]β-alanine and (A) octopamine, (B) tyramine, (C) histamine and (D) serotonin (5-HT). (1) NE, (2) OA, (3) NBANE, (4) DOPA, (5) NADA, (6) DA, (7) TA, (8) NBAD, (9) NBATyr (sarcophagine), (10) tyrosine, (11) NBAHA (carcinine), (12) HA, (13) tryptamine, (14) 5-HT. Other overlapping standards are not shown.

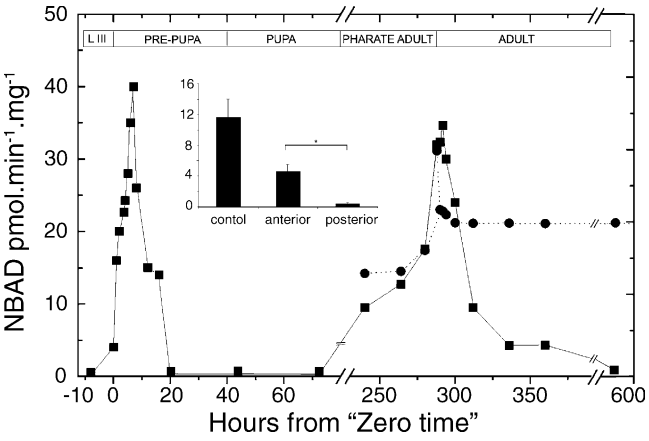

Fig. 3. Expression of *N*-β-alanylcatecholamine ligase during the life cycle of *C. capitata*. Epidermal expression (–■–) in larva, pre-pupa, pupa, pharate adult and adult. Neural expression (· · ·●· · ·) in pharate adult and adult. Ages are expressed in hours before and after the onset of pupariation ("Zero time"). The inset shows the enzymatic activity in anterior and posterior body regions 5 h after ligation of larvae. Control: whole body expression.

further studies under physiological conditions. Competition experiments in which 0.1 mM tyramine was present in the incubation mixture, in addition to 0.1 mM dopamine, generated 69% NBATA and 31% NBAD (not shown). Competition experiments using 0.1 mM histamine, instead of tyramine, only reduced the synthesis of NBAD by 25%.

Fig. 3 shows the expression profile of NBAD synthase. The brain-associated enzyme of the wild type adult *C. capitata* is expressed constitutively (Fig. 3, right), whereas the expression of the epidermis synthase is transiently induced at the end of the larval III stage (Fig. 3, left) and at the time of adult ecdysis (Fig. 3, right). To demonstrate that the epidermis enzyme is induced by brain ecdysteroid hormone(s) (probably the molt hormone, 20-OH-ecdysone), we ligated larvae III [18]. Five hours after ligation, we prepared extracts from both parts of the body. As expected, enzymatic activity was detected only in the anterior part (Fig. 3, inset), thus confirming that the enzyme was not induced in the posterior part due to the lack of the proper brain hormonal message.

Both the pre-pupal extracts (integument undergoing sclerotization) as well as the whole head extracts from the melanic mutant *C. capitata niger* (*niger*) adults were unable to synthesize β-alanine derivatives of dopamine and norepinephrine (Table 1). The same results were obtained when homogenates from ganglia of *C. capitata niger* and adult heads of the melanic *D. melanogaster ebony*^{4} (*e*^{4}) were tested (Table 1 and Fig. 1G and H). These mutants also were unable to synthesize any of the β-alanine derivatives described above (not shown). The data strongly support the hypothesis that the enzymatic protein in both flies is related to a corresponding single gene (*niger* and *e*, respectively) that can be expressed either in epidermis or ganglia.

Our results demonstrate directly, for the first time, that the insect *N*-β-alanyl-catecholamine ligase (also known as NBAD synthase), an enzyme of the catecholamine pathway previously known to be expressed only in epidermis [23] and only induced at the time of sclerotization, is constitutively expressed in the neural system. Therefore, regulatory factors driving differential expression must exist in the two tissues in order to allow the transcription of *niger* (or *e*) in a tissue-specific and time-dependent manner during the life cycle. We found that the promoter of the *D. melanogaster ebony* gene (GenBank/AF301896) contains a consensus sequence for the transcription factors expressed by the so-called early metamorphosis genes E74 and Br-C. This reinforces the idea that induction of the homologous *C. capitata niger* gene is triggered by a typical 20-OH-ecdysone-induced cascade, as indirectly demonstrated in the inset to Fig. 3. In spite of the functional similarity of the proteins ebony and niger, we concluded that little if any sequence homology exists between the two genes. After many Northern and Southern hybridization studies using ^{32}P-labeled *ebony* cDNA, no positive signal was obtained in Medfly nucleic acid samples. However, we can assume that ecdysone-dependent transcription factors binding to enhancers would trigger the expression of *niger* only in epidermis undergoing molt. In addition, the constitutive activation of *niger* could be maintained in brain/ganglia cells. According to Kim et al. [12], possible infection-induced gene expression in the epidermis must also be taken into account. It remains to be determined whether the epidermis and neural synthases, encoded by the *niger* gene, are identical proteins or different variants generated by alternative splicing and/or post-translational modifications. Our results are concordant with in vivo data from Krueger et al. [14] in *M. sexta*, and from Richardt et al. [22] who described in situ expression of the *ebony* gene in *Drosophila* brain.

The wide substrate specificity of the brain-associated *N*-β-alanyl-catecholamine ligase is a known characteristic of other enzymes of the catecholamine pathway, like dopa decarboxylase and acetyl dopamine synthase [5]. Novel β-alanyl conjugates of tyramine, octopamine and serotonin, NBATA, NBAOA and NBA5HT are described here (Fig. 2). To our knowledge, this is the first report of synthesis of these substances by a brain-associated enzyme from any biological system. Since we previously described the synthesis of sarcophagine (*N*-β-alanyl-tyrosine) by the epidermis-associated enzyme [16], we tested the synthesis of this and other known dipeptides containing β-alanine by the brain-associated enzyme [2,6,11]. The brain enzyme was able to synthesize carcinine (NBAHA; Fig. 2C) but not sarcophagine. The in vitro synthesis of carcinine by insect brain extracts reported here is in agreement with recent data on in vivo synthesis by Borycz et al. [3]. The presence of carcinine in mammalian tissues was reported previously [4,7]; therefore our results might represent a link with mammalian β-alanine derivative metabolism. Another enzyme with properties very similar to those of NBAD synthase and carcinine synthase (i.e., ligating β-alanine with histidine to generate carnosine (β-alanyl-histidine)) exists in mammalian tissues. Carnosine is present in muscles and neural tissue of many vertebrates, including humans. It apparently provides a neuroprotective

function [9], and may play a role in synaptic transmission. We have been unable to synthesize carnosine using insect extracts.

Taken together, the results agree well with a recent report describing the properties of a chimeric ebony protein from *Drosophila* expressed in transformed S2 cells [21]. A number of questions arise about the physiological role of NBAD, NBANE and eventually other β-alanine derivatives in the insect brain. Preliminary tests with the Medfly *niger* mutant showed that a strong correlation exists between the lack of β-alanine derivatives and abnormal behavior (i.e., delay in larvae jumping and less mobility of adults in T-maze and vertical cylinders). These results are in agreement with previous reports of behavioral deficiencies in the *D. melanogaster* mutant *ebony* [10]. This strongly supports the idea of NBAD, NBANE and related derivatives as important insect brain metabolites. These molecules might play a role as regulatory molecules, sequestering and eventually liberating the respective neurotransmitter (DA, NE or other). In this case, β-alanine derivatives must be regulated by the action of specific hydrolases or peptidases. We know from our preliminary results that an NBAD hydrolase exists in the adult brain of *C. capitata*. An alternative possibility is that insect NBAD and NBANE might be substances directly involved in neuroregulation, by analogy with mammalian carnosine and carcinine. This points to possible homology with mammalian systems and may have pharmacological implications.

Acknowledgements

We are grateful to M. Bravo for excellent technical assistance and to Drs. K. Kramer, J. Drijfhout, L. Centanin, P. Wappner and J. Aguirre for helpful discussions. M. Pérez was a FOMEC-University of Buenos Aires Fellow. L.A. Quesada-Allué is Professor at the same University and Principal Investigator of the CONICET (Argentina). This work was supported in part by grants from the University of Buenos Aires and from the ANPCyT-SECyT (Argentina).

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