Comparative Biochemistry and Physiology, Part B 168 (2014) 70–75

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Brain norepinephrine identified by mass spectrometry is associated with reproductive status of females of the linden bug *Pyrrhocoris apterus*

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ARTICLE INFO

Article history:
Received 13 September 2013
Received in revised form 1 November 2013
Accepted 6 November 2013
Available online 13 November 2013

Keywords:
Adult diapause
Brain complex
Dopamine
Norepinephrine
Insects
Mass spectrometry

ABSTRACT

Several biogenic amines, including controversial presence of norepinephrine (NE), were identified by the high performance liquid chromatography equipped with electrospray ionisation mass spectrometry in brain complexes of adult females of *Pyrrhocoris apterus*. Quantitative analysis was performed by the high performance liquid chromatography coupled to electrochemical detector. Levels of NE, dopamine (DA), octopamine (OA) and 5-hydroxytryptamine (5-HT) in brain complexes were measured in reproductive vs. diapause females. In field collected samples, levels of NE and DA were significantly higher in reproductive (May) than in non-reproductive (Sep, Oct, Feb) females. In laboratory females, NE is higher in long day photoperiod (reproduction) than in short day photoperiod (diapause) already from the first week of the adult age, while DA shows differences between the two contrasting photoperiods only from the second week of the adult age. No association between reproductive status and levels of OA and 5-HT was found.

1. Introduction

Major biogenic amines in the insect nervous system include dopamine (DA), tyramine (TA), octopamine (OA), serotonin (5-hydroxytryptamine, 5-HT) and histamine (Evans, 1980; Roeder, 1994). According to recent reviews, vertebrate adrenergic transmitters, norepinephrine (NE) and epinephrine (E), have no physiological relevance in insects; their role is fulfilled by their invertebrate counterparts OA and TA (Blenau and Baumann, 2001; Roeder, 2005; Hauser et al., 2006). On the other hand, both NE and E were detected by the high performance liquid chromatography with electrochemical detection (HPLC-ECD) in several insect species (e.g. Sparks and Geng, 1992; Natsukawa et al., 1996; Awad et al., 1997; Sasaki and Nagao, 2001; Matsumoto and Takeda, 2002).

The first goal of this study has been to characterize biogenic amine profile in the central nervous system (CNS) of our model species, the linden bug *Pyrrhocoris apterus*. Several biogenic amines, including NE were identified using high performance liquid chromatography equipped with electrospray ionisation mass spectrometry (HPLC-ESI-MS). For their quantitative determination the high performance liquid chromatography coupled to electrochemical detector was used (HPLC-ECD).

P. apterus exhibits reproductive diapause induced by short-day photoperiod. Long-day photoperiod prevents or terminates diapause (Hodek, 1971). Under field conditions, diapause is induced in late summer/early autumn and diapause completion is associated with the loss of photoperiodic response. Thus, post-diapause adults are stimulated to reproduce in spring by increase in temperature although photoperiod may be still short (Hodek, 1971).

Increasing evidence suggests that biogenic amines play a key role in the transmission of photoperiodic signals to the endocrine system. In seasonal mammals, the photoperiodic regulation of reproductive dormancy is mediated by daily rhythm in the synthesis pineal hormone melatonin. Synthesis of melatonin is stimulated at night by the secretion of NE that is controlled by circadian clocks in the suprachiasmatic nucleus of hypothalamus (Reiter, 1993; Ganguly et al., 2002). Although melatonin has been detected in several insect species (Vivien-Roels et al., 1984; Tilden et al., 1994; Linn et al., 1995), its role in the photoperiodic regulation of diapause is not known. Published data on the relation of biogenic amines to diapause are focused on larval and pupal diapause. Induction of larval diapause in a drosophilid fly, *Chymomyza costata*, is associated with the maintenance of a high level of total body DA, while continual development is associated with decrease of DA level in both wild-type and mutant non-diapause strain (Kostal et al., 1998, 1999). Moreover, DA level increases at night, which indicates the role of DA in measurement of day/night length (Kostal et al., 2000). It is suggested that DA supports induction and maintenance of pupal diapause of large white *Pieris brassicae* (Puiroux et al., 1990; Isabel et al., 2001) and the cabbage moth *Mamestra brassicae* (Noguchi and Hayakawa, 1997). A dual regulatory system consisting of the ecdysiotropin inhibitory pathway by DA, NE and E metabolism and ecdysiotropin stimulatory pathway by 5-HT metabolism is proposed for the regulation of pupal diapause in the Chinese oak silk moth *Antheraea pernyi* (Matsumoto and Takeda, 2002). Relation of biogenic amines to adult diapause has not been studied.

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http://dx.doi.org/10.1016/j.cbpb.2013.11.003

The second goal of this study has been to explore a potential relationship between the level of several catecholamines in CNS, NE, DA and OA, and adult diapause. We measured biogenic amine levels (1) in adults collected in the field in autumn (diapause), late winter (diapause terminated, but reproduction still prevented by low temperature), and spring (reproduction); (2) in wild type laboratory adults kept from the egg stage under two contrasting photoperiods, long days (reproduction) or short days (diapause), and (3) in a non-diapause laboratory strain kept from the egg stage under short days (reproduction).

2. Material and methods

2.1. Insects

Colonies of *Pyrrhocoris apterus* (L.) (Heteroptera) were reared at 25 ± 2 °C under either a reproduction-promoting long day (LD) photoperiod of 18 h light/6 h darkness or diapause-promoting short day (SD) photoperiod of 12 h light/12 h darkness and supplied *ad libitum* with linden seeds and water. Two types of females were used: wild type (WT) females and non-diapause (ND) females. Non-diapause laboratory strain was selected from several females which oviposited under SD (Socha and Hodkova, 1994; Hodkova and Socha, 1995). In addition to laboratory colonies, samples collected in field near to Ceske Budejovice (49 °N) were used. Brain-suboesophageal ganglion-corpora cardiaca-corpus allatum (BR-SG-CC-CA) or BR-SG were dissected under Ringer insect saline, immediately placed on dry ice, and stored at −85 °C until analysis. Brain complexes from field-collected insects were dissected every two hours, 1–11 h after light on. Because there was no statistical difference between individual time intervals (1–3 h, 5–7 h, 9–11 h after light on) brain complexes from laboratory insects were dissected 5–7 h after light on.

Brain complex from one individual and complexes from five individuals were used as one sample for HPLC-ECD and HPLC-ESI-MS analysis, respectively.

2.2. Analysis of biogenic amines

NE, DA, OA and 5HT standards, and other chemicals used for biogenic amine extraction, derivatization and HPLC-MS and HPLC-ECD analyses were purchased from Sigma–Aldrich (Praha, Czech Republic). All solvents used were of HPLC grade.

2.2.1. HPLC-ESI-MS

Tissue samples were homogenized in 200 μl of 0.2 M perchloric acid on ice. After centrifugation (14,000 g for 20 min at 4 °C) the pH of the supernatant was adjusted to pH = 10 with 100 μl of carbonate buffer and the analytes were treated with 10 μl of ethyl chloroformate for 5 min. Corresponding N (O) –ethyloxycarbonyl derivatives (EOC) were extracted twice with dichloromethane. The pooled organic layer was evaporated to dryness, the residue was dissolved in 50 μl of a mobile phase and an aliquot of 5 μl was injected into the HPLC-ESI-MS system.

A quadrupole ion trap LCQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with an ESI source, a Rheos 2000 quaternary HPLC system (Flux, Basel, Switzerland), a FAMOS autosampler and a Thermos thermostat (both LC Packings-Dionex, Amsterdam, The Netherlands). Chromatographic separation was performed on a Symmetry C8 (150 × 1 mm, 3.5 μm) column (Waters, Milford, MA, USA). The mobile phase consisted of 10 mM ammonium formate in methanol and water and the isocratic elution with 57% MeOH was used. The column flow rate was 50 μl/min at 35 °C.

The mass spectrometer was operated in positive electrospray (ESI) ionization mode at 4.5 kV, with the capillary temperature at 195 °C, and nitrogen was used as a shielding and an auxiliary gas. The full scan mass spectra were acquired in the mass range 150–500 amu, which was scanned every 0.3 s. To confirm the presence of biogenic amines the tandem mass spectra (MS2) were recorded with isolation width, 3; normalized collision energy, 25%; 1 microscan; and maximum ion time, 100 ms. The molecular ions $[M + H]^+$ of the derivatives were set as precursor ions, except for OA and NE, where $[M + H\text{-}18]^+$ ions corresponding to the loss of H_2O were used. The data were acquired and processed by an Xcalibur 1.2 software (Thermo Fisher Scientific). Target compounds were identified by associating their retention times and positive electrospray ionization MS2 spectra with corresponding standards.

2.2.2. HPLC-ECD

Tissue samples were homogenized in 50 μl of 0.2 M perchloric acid as described above. The supernatant was diluted 5 times with a mobile phase and a 50 μl aliquot was injected into the HPLC-ECD system.

An HPLC system (LC-10 AD Shimatzu, Japan) was coupled to an ESA Coulochem II detector (Chelmsford, MA, USA). Biogenic amines were

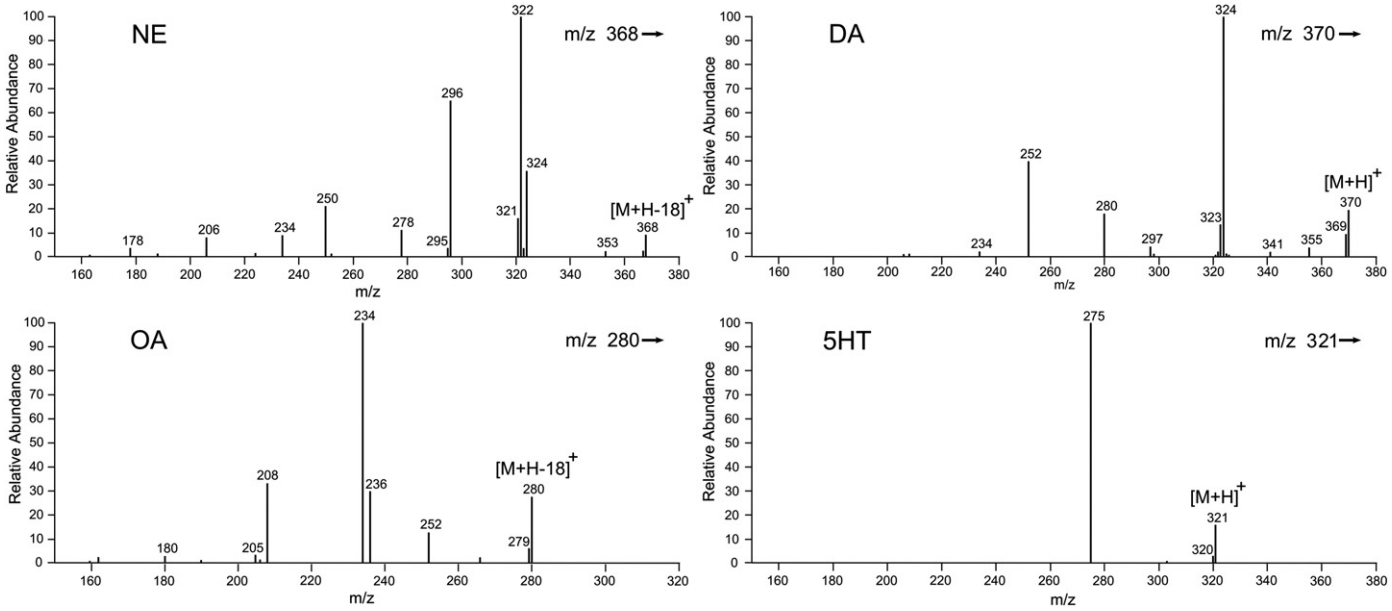

Fig. 1. Positive ESI MS2 spectra of the EOC derivatives of NE, DA, OA and 5HT detected in *P. apterus* brain complexes.