Journal of Insect Physiology 57 (2011) 94–107

Contents lists available at ScienceDirect

Journal of Insect Physiology

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

Nitric oxide/cGMP signaling in the corpora allata of female grasshoppers

Andrea Wirmer, Ralf Heinrich *

Institute for Zoology, University of Göttingen, 37073 Göttingen, Germany

ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form 27 September 2010
Accepted 29 September 2010

Keywords:
Corpora allata
Nitric oxide
Citrulline
Neuroanatomy
Grasshopper
Chorthippus biguttulus

ABSTRACT

The corpora allata (CA) of various insects express enzymes with fixation resistant NADPHdiaphorase activity. In female grasshoppers, juvenile hormone (JH) released from the CA is necessary to establish reproductive readiness, including sound production. Previous studies demonstrated that female sound production is also promoted by systemic inhibition of nitric oxide (NO) formation. In addition, allatotropin and allatostatin expressing central brain neurons were located in close vicinity of NO generating cells. It was therefore speculated that NO signaling may contribute to the control of juvenile hormone release from the CA.

This study demonstrates the presence of NO/cGMP signaling in the CA of female *Chorthippus biguttulus*. CA parenchymal cells exhibit NADPHdiaphorase activity, express anti NOS immunoreactivity and accumulate citrulline, which is generated as a byproduct of NO generation. Varicose terminals from brain neurons in the dorsal pars intercerebralis and pars lateralis that accumulate cGMP upon stimulation with NO donors serve as intrinsic targets of NO in the CA. Both accumulation of citrulline and cyclic GMP were inhibited by the NOS inhibitor aminoguanidine, suggesting that NO in CA is produced by NOS. These results suggest that NO is a retrograde transmitter that provides feedback to projection neurons controlling JH production. Combined immunostainings and backfill experiments detected CA cells with processes extending into the CC and the protocerebrum that expressed immunoreactivity against the pan-neural marker anti-HRP. Allatostatin and allatotropin immunopositive brain neurons do not express NOS but subpopulations accumulate cGMP upon NO-formation. Direct innervation of CA by these peptidergic neurons was not observed.

1. Introduction

Juvenile hormone (JH), produced in the corpora allata (CA), is a master regulator for various aspects of female reproductive behavior (Hartfelder, 2000). JH modulates the phonotactic behavior in cricket females (Stout et al., 1991), regulates the release of pheromones in cockroaches (Schal et al., 1997) and both stimulates vitellogenin synthesis and determines caste status of social insects like *Bombus terrestris* (Larrere and Couillaud, 1993) and *Apis mellifera* (Robinson et al., 1991). Female grasshoppers (*Gomphocerus rufus*) that lack JH after removal of the corpora allata never reach a state of reproductive readiness, do not answer male calling songs and lifelong refuse mating with a male (Loher, 1962).

The gaseous transmitter nitric oxide stimulates the production of cyclic guanosine monophosphate (cGMP) by activation of the soluble guanylate cyclase in its target cells in central nervous systems of both vertebrates and invertebrates including insects (Garthwaite and Boulton, 1995; Heinrich and Ganter, 2007). Systemic application of the NOS inhibitor aminoguanidine enhanced sound production of *Ch. biguttulus* females and extended it to periods when females normally remain mute, e.g. shortly before and after oviposition (Weinrich et al., 2008). Female sound production which is regarded as a sign of high reproductive readiness (von Helversen, 1972; Wirmer et al., 2010) is also suppressed by NO/cGMP signaling in the central complex. In particular, injection of NO donors and cGMP analogs into the central complex inhibit muscarine-stimulated sound production (Wenzel et al., 2005) while inhibitors of nitric oxide synthase (NOS) release species specific singing (Weinrich et al., 2008). Since both JH and NO/cGMP signaling modulate the same components of female grasshopper reproductive behavior, both systems may either be regulated by common upstream signals or directly influence each other. Brain neuropils, the corpora allata and their neural connections may serve as substrates for the convergence of regulatory pathways to mediate a coherent influence on reproduction.

Mating activity of the cockroach *Diploptera punctata* affected the intensity of NADPHdiaphorase activity in the corpora allata which suggested a regulatory function of NO for JH release (Chiang et al., 2000). However, since immunocytochemistry against NOS

* Corresponding author at: Department of Neurobiology, Institute for Zoology, Berliner Strasse 28, 37073 Göttingen, Germany. Tel.: +49 551 3991183; fax: +49 551 395438.
E-mail address: rheinri1@gwdg.de (R. Heinrich).

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doi:10.1016/j.jinsphys.2010.09.010

produced no or weak staining in the corpora allata, NADPHdiaphorase activity could have resulted from other enzymes involved in JH production or some other metabolic pathway (Skinner et al., 2000).

The brain controls the corpora allata via the nervi corporis allati I and II (NCA I and NCA II) (Vullings et al., 1999). Most of the direct connections with the corpora allata originate from somata in the neurosecretory pars intercerebralis and pars lateralis that project axons through the nervus corporis cardiaci II, the corpora cardiaca (CC) and the NCA I.

Peptidergic brain factors that affect JH synthesis are generally classified as stimulatory allatotropins and inhibitory allatostatins (Gilbert et al., 2000). Only one allatotropin has been identified in *Manduca sexta* (Mas-AT). However, its stimulatory effect on JH production is restricted to lepidopteran species and the honey bee *Apis mellifera* and not detected in the cockroach *Periplaneta americana* and the grasshopper *Schistocerca nitens* (Kataoka et al., 1989). Other substances with stimulatory effects in vivo failed to increase JH generation upon direct application to isolated corpora allata (Gilbert et al., 2000).

Allatostatins (ASTs) have been assigned to three different families, the A-ASTs, B-ASTs, and C-ASTs, that share some portions of their amino acid sequences (Stay and Tobe, 2007). Though they have been detected in several insect taxa their functions related to JH synthesis are still under debate. Some insect species express peptides of the AST families that apparently have no inhibitory effect on JH synthesis and those peptides that really suppress JH production may do this indirectly by modulation of central nervous neuronal activities that affect corpora allata activity (Stay and Tobe, 2007).

Authors of a few studies already speculated about the possibility that NO could influence JH production by acting on allatostatin or–tropin releasing neurons. Kurylas et al. (2005) assumed colocalizations of allatostatin or allatotropin with NOS and NADPHd in protocerebral neurons, and Skinner et al. (2000) found allatostatin positive fibers in very close proximity to NOS containing fibers in the central complex.

In this study we aimed to substantiate speculations about NO/cGMP signaling in the corpora allata by labeling both sources and targets of NO in the retrocerebral complex of female *Chorthippus biguttulus* grasshoppers with NADPH diaphorase histochemistry and anti NOS, anti citrulline and anti cGMP immunocytochemistry. In order to reveal the potential convergence of neural pathways that regulate corpora allata function, we performed backfills of axons that connect the brain with the corpora allata and combined these with immunocytochemical detections of *Diploptera punctata* allatostatin 7 (Dip-Ast-7), *Manduca sexta* allatotropin (Mas-AT), HRP-like antigens and components of the NO/cGMP system in the brain.

2. Materials and methods

2.1. Animals

Studies were performed with female nightingale grasshoppers of the species *Chorthippus biguttulus* and female pacific beetle cockroaches (*Diploptera punctata*).

Most of the grasshopper females were reared from eggs that had been collected during the previous summer and kept at 4 °C for at least four months. After approximately one week at 26 °C, the nymphs hatched and were fed with grass (*Poa spec.* and *Dactylis spec.*) and supplemental food for crickets (Nekton, Pforzheim, Germany) ad libitum.

Cockroaches were purchased from J. Bernhardt (www.schaben-spinnen.de). Until usage, they were kept in a 55 cm × 35 cm × 27 cm box at 24 °C and fed with potatoes and fruits.

2.2. General protocol for immunocytochemical stainings

Animals were anesthetized by cooling to 4 °C. Subsequently, they were fixed to a Plasticine (Pelikan) bed and the head was opened dorsally. After removal of tracheae, muscles and fatty tissue, the brain was dissected. For fixation, brains were incubated in a solution containing 4% paraformaldehyde (PFA) in 0.1 M PO_4 buffer for two hours at 10 °C. Embedding in gelatine albumine and postfixation in the same fixative were followed by cutting the brains into 50 μm thick sections with a vibratome (Leica, VT100S). After overnight permeabilization of the tissue with Triton X-100 (Sigma) dissolved in PBS, the sections were incubated in blocking buffer consisting of PBS containing either 5% normal donkey serum (Jackson ImmunoResearch) or 5% normal goat serum (Amersham Biosiences), 0.25% bovine serum albumine (MP Biomedicals, Inc.) and Triton X-100. Primary antibodies were applied in blocking buffer for at least 24 h. Afterwards, excess primary antibodies were removed by several rinses in PBST. Secondary antibodies were applied in blocking buffer for three hours at a concentration of 1:300. The sections were rinsed with PBST two times for 10 min and three times for 10 min with PBS. Thereafter, the sections were mounted on microscope slides with PBS/glycerol (Sigma). Immunofluorescence was analyzed with a confocal microscope (Leica TCS SP2) using the Leica confocal software. Brightness and contrast of images were adjusted with Image J (NIH) or Photoshop (Adobe).

The following antibodies were used in this study:

uNOS (Affinty BioReagents): The antibody detects a conserved region of nitric oxide synthases. It labels proteins of similar expected molecular weights in hawkmoths (Gibson and Nighorn, 2000) and locusts (Elphick et al., 1995; Stern et al., 2010). It has been used to label brains of *Ch. biguttulus* (Wenzel et al., 2005).
Citrulline (donated by G. Martinelli, New York, USA): The antibody is directed against a citrulline-glutaraldehyde conjugate. Its specificity has been determined by Martinelli et al. (2002). The antibody has been used to label brains of locusts (Siegl et al., 2009, 2010) and *Ch. biguttulus* (Weinrich et al., 2008).
Cyclic GMP (donated by J. deVente, Maastricht, The Netherlands): Specificity has been described by Tanaka et al. (1997). The antibody has previously been used on locusts (Siegl et al., 2009; Stern et al., 2010) and *Ch. biguttulus* (Weinrich et al., 2008).
Dip-Ast-7 (Developmental Studies Hybridoma Bank): The antibody developed by Stay and coworkers has been demonstrated to be most sensitive to allatostatin 7 from *Diploptera punctata* (Stay et al., 1992).
Mas-AT (donatet by D. Nässel, Stockhom, Sweden): The antibody developed by Veenstra and Hagedorn (1993) labels an allatotropin molecule from *Manduca sexta* that was initially described by Kataoka et al. (1989). It has been used for studies on various insects including locusts (Homberg et al., 2004).
Horse radish peroxidase (HRP, Sigma): The rabbit α-HRP serum labels the pan-neuronally expressed surface protein Nervana (Jan and Jan, 1982; Sun and Salvaterra, 1995a,b). It has been used as general marker for neurons in various insects including *Drosophila melanogaster* (Sun and Salvaterra, 1995a,b), honeybees (Kreissl and Bicker, 1992), cockroaches (Loesel et al., 2006), crickets (Cayre et al., 1998) and grasshoppers (Jan and Jan, 1982; Gocht et al., 2009).

Antibodies were either used after neurobiotin (Vector) tracing or in combination with each other. Specific modifications of the general staining procedure are mentioned below.