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CHANGES IN *LYMANTRIA DISPAR* PROTOCEREBRAL NEUROSECRETORY NEURONS AFTER EXPOSURE TO CADMIUM

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Abstract - Gypsy moth 4th instar caterpillars were fed for 3 days with an artificial diet supplemented with increasing cadmium (Cd) concentrations (0, 10, 30, 100 and 250 μg/g of dry food weight). Changes in the morphometric characteristics of A1' dorso-medial and L2 dorso-lateral neurosecretory neurons (nsn) were analyzed. In the A1' nsn, Cd supplements led to an enhanced nuclear size, except in the group treated with 250 μg Cd/g in the form of dry food. The size of L2 type nsn was increased in the groups provided with 30 and 100 μg Cd/g, while no differences in the size of nuclei was detected in L2 neurons among the experimental groups.

Key words: Cadmium, *Lymantria dispar,* protocerebral neurosecretory neurons

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INTRODUCTION

Lymantria dispar is a polyphagous herbivorous insect pest with a host range estimated at more than 500 plant species (Lance, 1983). Cadmium accumulation in plants causes changes in the dynamics of animal populations, including *L. dispar* and other phytophagous insects (Williams and Liebhold, 1995).

The amount of Cd in nature has been increasing as a consequence of anthropogenic activities. Numerous data indicate the influence of this heavy metal on insect development, growth, reproduction and/or hatching (Gintenreiter et al., 1993; Rayms-Keller et al., 1998; Sildanchandra and Crane, 2000), respiration (Ortel and Vogel, 1989) and metabolic processes (Bischoff, 1995; Niu et al., 2002).

The neuroendocrine reorganization in the insect's protocerebral region (dorsomedial part) of the brain during nutritive stress changes protein and lipid metabolism as well as digestive enzyme amounts and activities (Ivanović and Janković-Hladni, 1991; Mrdaković et al., 2008).

Our previous investigations revealed that acute exposure of gypsy moth caterpillars to a Cd supplemented diet induced changes in the activity of the neurosecretory neurons (nsn) from the *pars intercerebralis*. We found alterations in bombyxin (a small form of PTTH) immunopositive A2 nsn (Ilijin et al., 2010; Ilijin et al., 2011) and L2' nsn, suggesting they are the sites of synthesis of the large form of PTTH in *L. dispar* caterpillars (Ilijin, 2009). To further elucidate the hormonal reorganization in response to heavy metal stress, we analyzed the morphometric changes in two other types of nsn: A1' and L2. The aim of this work was to overview specific and nonspecific responses to the acute effects of Cd in gypsy moth caterpillars by analyzing two different types of neurosecretory neurons.

MATERIALS AND METHODS

Insect rearing

Egg masses were collected in a poplar forest at Opovo 30 km from Belgrade and kept in a refrigerator at 4°C until they were set to hatch. Larvae were reared on a synthetic HWG (high wheat germ) diet (O'Dell et al., 1985) in transparent plastic containers (V=200ml) at 23˚C and a 12h light/dark photoperiod. On the first day of the 4th instar, larvae were fed on diets with 10, 30, 100, 250 μg of added Cd *per* g of dry food (10 μg, 30 μg, 100 μg and 250 μg groups) or without Cd (control group) for 3 days and then sacrificed by decapitation. The caterpillars were randomly assigned to five groups for histochemistry (n=15).

Histological techniques

After decapitation, the head capsules were fixed in Bouin's solution for 24 h (Merck). The brain complexes were dissected, dehydrated in a graded series of ethanol (from 80% to 100%) (Hemos, Belgrade), and then embedded in paraffin wax (59°C, Merck). Serial sections (3.5 μm) of the brain complexes were cut for histochemistry (microtome "820"Spencer). After drying, the sections were deparaffinized in xylene (Hemos), and stained by the modified (Panov, 1980) Ewen paraldehyde fuchsin technique (Ewen 1962). The neurosecretory neurons were stained dark purple - paraldehyde fuchsin positive. The size of the A1'and L2 nsn and their nuclei (expressed as means of the shortest and longest diameters in μm) were analyzed. Measurements were made using the image processing and analysis system (QWin image analysis tool kit) linked to a Leica DMLB light microscope (Leica). Data were evaluated by one-way ANOVA and a *post-hoc* multiple range test (Fisher's least significant difference (LSD)).

RESULTS

A1'nsn are located in the dorsomedial part of the *L. dispar* protocerebrum, and their average size was 25.96 μm (Fig. 1). After exposure of the caterpillars to different Cd concentrations we observed a tendency in the A1'nsn to increase in size (Fig. 2A). The average nuclear size was significantly greater in the groups of caterpillars fed with diets containing 10, 30 and 100 μg of added Cd *per* g dry weight (one-way ANOVA, P<0.001) (Fig. 2B). The majority of A1'nsn in all groups had a large amount of neurosecretory material in the cytoplasm (Fig. 2C). In the A1' nsn from the caterpillars receiving the diets with 10 and 100 μg of added Cd, a fine-grained neurosecretory material was present, while in the other groups the neurosecretory material was large-grained (Fig. 2D). All these results point to elevated synthetic and secretory activity in A1'nsn in response to Cd.

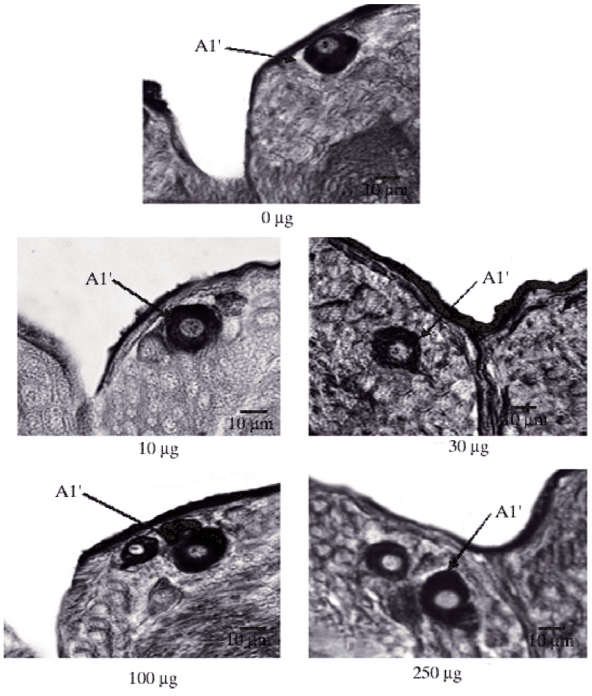

Fig. 1. – Brain transverse cross-sections of *Lymantria dispar* 4th instar caterpillars after feeding for 3 days with an artificial diet supplemented with various cadmium concentrations (0 μg; 10 μg; 30 μg; 100 μg; 250 μg Cd/g of dry food weight). Arrows indicate the protocerebral A1' neurosecretory neurons. The bar represents 10 μm.

The L2 nsn are located in the dorsolateral part of the caterpillar protocerebrum, and their average size is 16.15 μm (Fig. 3). Statistically significant increases in nsn size were detected in the groups re-

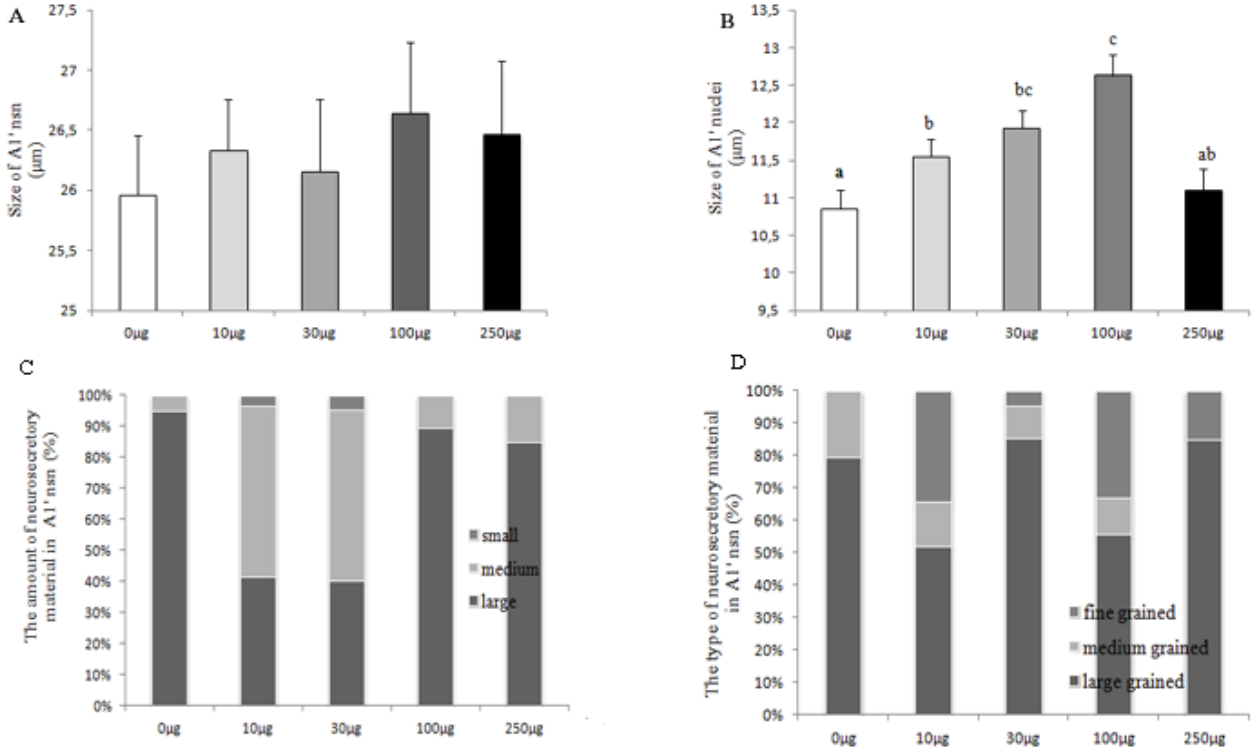

Fig. 2. - The size of A1' neurosecretory neurons (nsn) (**A**) and their nuclei (**B**), the amount (**C**) and the type of neurosecretory material (**D**) in protocerebrum of *L. dispar* 4th instar caterpillars after receiving artificial diets supplemented with various cadmium concentrations (0 µg; 10 µg; 30 µg; 100 µg; 250 µg Cd/g of dry food weight) for 3 days. Error bars indicate the standard error of the mean (SEM) for (n = 15). Different letters (a,b,c) indicate significant differences between groups (LSD test, P<0.01).

ceiving 30 and 100 µg of added Cd *per* g of dry food weight in comparison to the control group (Fig. 4A). There were no significant changes in nuclei size in any of the experimental groups (Fig. 4B). The cytoplasm of most of the L2 nsn was filled with a large amount of large-grained neurosecretory material (Figs. 4C and 4D). These cytological characteristics indicate a low level of secretion and the retention of neurosecretory material in the cytoplasm of L2 neurons.

DISCUSSION

Neurosecretory cells from the *pars intercerebralis* in the brain of insects release various hormonal factors that control the essential physiological and developmental functions and these cells are therefore of considerable biological significance. These neurohormones are included in all phases of the stress response mechanism, providing the energy necessary for the mechanisms of stress tolerance (Gäde and Goldsworthy, 2003).

The insect's gut is an accumulative organ for Cd. The gut epithelium and peritrophic matrix of insects is an important target organ for heavy metals (Lauverijat et al., 1989). Clearly, cadmium in food destroys the midgut structure and this impacts fundamental processes (digestion and absorption of food) and could cause starvation and have a dramatic impact on an insect's life processes and survival (Rayms-Keller et al., 1998, 2000). High dose Cd toxicity causes effects similar to starvation. Intoxication with a starvation effect obviously influences both the quality and quantity of midgut proteins, including digestive enzymes and brain

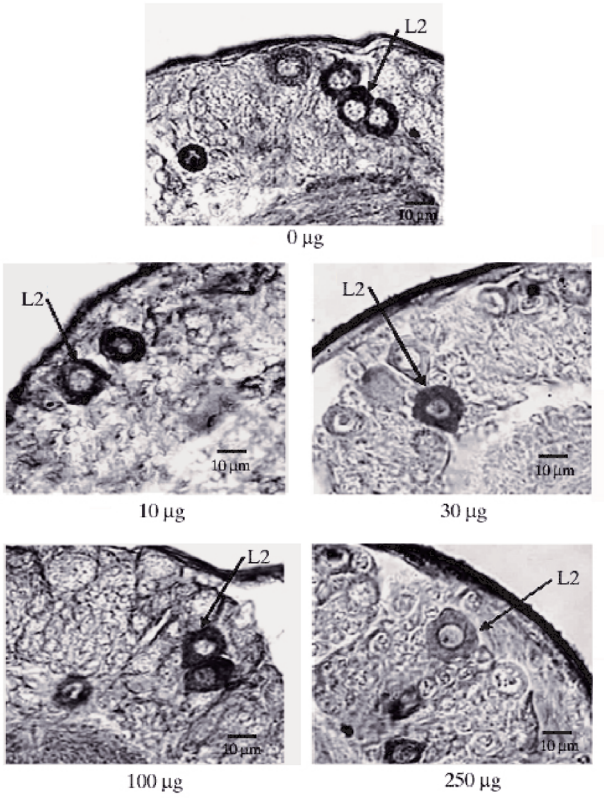

Fig. 3. – Brain transverse cross-sections of *Lymantria dispar* 4th instar caterpillars after receiving artificial diets supplemented with various cadmium concentrations (0 µg; 10 µg; 30 µg; 100 µg; 250 µg Cd/g of dry food weight) for 3 days. Arrows indicate the protocerebral L2 neurosecretory neurons. The bar represents 10 µm.

proteins. These changes may indicate the role of the neuroendocrine system in environmental changes (Ivanović et al., 1991). Neurosecretory neurons, like A1'nsn, located in the medial part of the *pars intercerebralis,* synthesize neuropeptides that have a regulatory role in the synthesis and release of the adipokinetic neurohormone, secretion of midgut digestive enzymes (proteases and amylases), diuresis, water balance, etc. (Gäde and Goldsworthy, 2003), (Raabe, 1989). Activation of these neurons could be necessary for a digestive and metabolic reorganization which compensates for the disturbed homeostasis under stressful conditions. Prolonged larval development (Ilijin et al., 2010), reduction of larval mass (Ilijin et al., 2010) and inhibition of alkaline phosphatase, trypsin, leucine aminopeptidase, α and β glucosidase and esterase (Vlahović et al., 2009, 2011), changes in protein, lipid and carbohydrate levels in the hemolymph and whole body (Ortel 1995 a, 1995 b, 1996) were detected under the influence of cadmium.

In this work we have revealed an elevation of the synthetic and secretory activity of A1' medial nsn. The results presented here led us to assume that the defense strategy of *L. dispar* caterpillars in the presence of cadmium includes digestive reorganization allocation of resources towards the activation of energy metabolism, regulated by neurohormones, synthesized in *pars intercerebralis* nsn. All defense mechanisms, such as increasing excretion, detoxification or repair of the damage are energetically expensive.

In the L2 nsn from the lateral part of the protocerebrum the retention of neurosecretory material in their cytoplasm was obvious and a low level of secretion was detected. One of the responses of insect larvae to Cd intoxication is the changing of cell Ca^{2+} concentration (Craig et al., 1998). These two ions compete for passage into the cell through the same site of entry. Bickmeyer et al., (1994) showed a dose-dependent block of Ca^{2+} channel currents in *Locusta migratoria* neurons treated with Cd. Calcium is a universal intracellular messenger that controls secretion, metabolism, or gene expression. Also, Cd can effect transmitter release (Soliakov and Wonnacott, 1996). It is plausible that the retention of neurosecretory material in L2 nsn is the consequence of an inhibited Ca^{2+} influx caused by cadmium. The results also show the different sensitivity to certain types of nsn to Cd intoxication. Research into the neuroendocrine response in stress could provide useful information about the regulation of the endocrine system in *Lymantria dispar* caterpillars, and could contribute to the development of pest-controlling substances.

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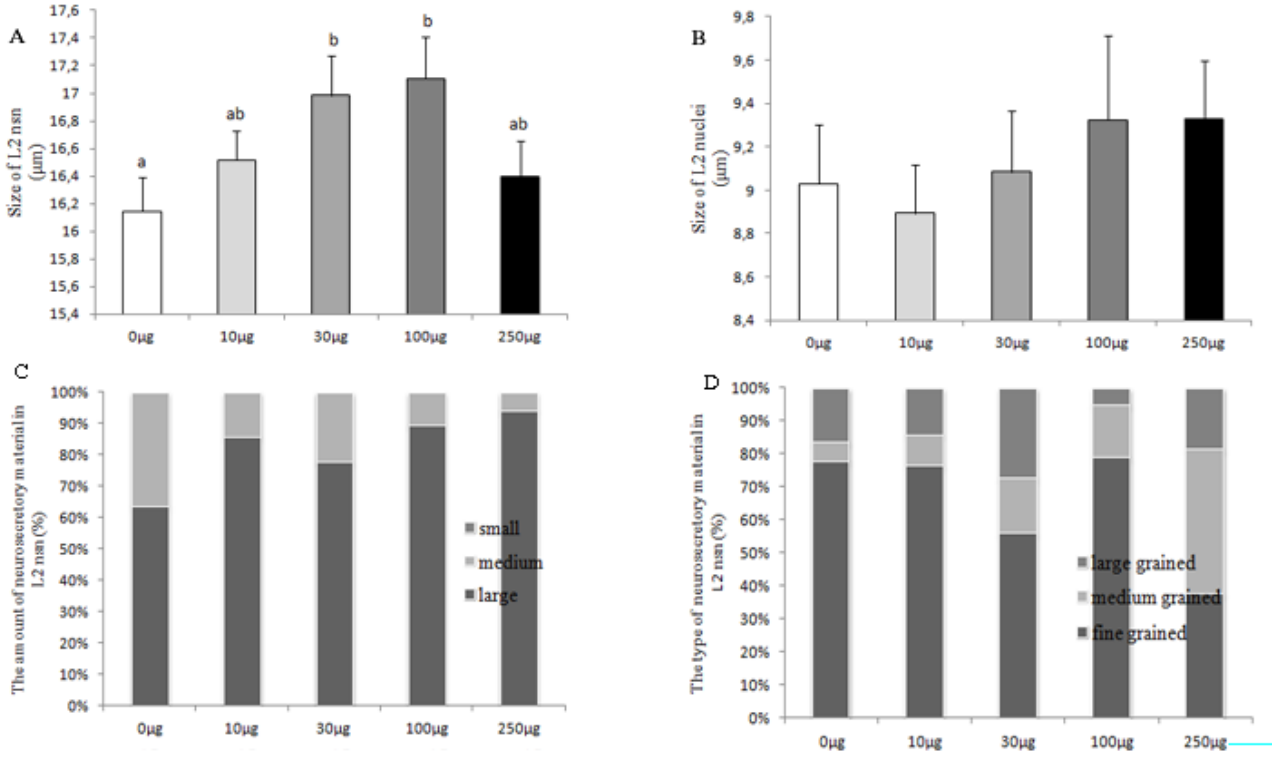

Figs. 4. - The size of L2 neurosecretory neurons (nsn) (**A**) and their nuclei (**B**), the amount (**C**) and the type of neurosecretory material (**D**) in protocerebrum of *L. dispar* 4th instar caterpillars after receiving artificial diets supplemented with various cadmium concentrations (0 µg; 10 µg; 30 µg; 100 µg; 250 µg Cd/g of dry food weight) for 3 days. Error bars indicate the standard error of the mean (SEM) for (n = 15). Different letters (a,b) indicate significant differences between groups (LSD test, P<0.01).

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