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Short Communication

Ventral nerve cord remodeling in a stingless bee (*Melipona quadrifasciata anthidioides,* Hymenoptera, Apidae) depends on ecdysteroid fluctuation and programmed cell death

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ABSTRACT The reorganization of the ventral nerve cord (VNC) during metamorphosis of *M. quadrifasciata* was observed to be characterized by shortening of connectives and subsequent fusion of the 2nd and 3rd thoracic and the 1st abdominal ganglia. Also, the 5th to 7th abdominal ganglia came into very close contact. These changes were accompanied by increasing levels of endogenous ecdysteroids, as determined by a radioimmunoassay. Incubation of VNC in the presence of 5 µg 20-hydroxyecdysone, caused significant shortening of connectives in the thoracic region, but not in the abdomen, evidencing a segment-specific response to this hormone. Cell death in the ventral ganglia was revealed by transmission electron microscopy and TUNEL-reaction. Detection of labeled cells in the region where contiguous ganglia come into close contact suggests that programmed cell death is involved in ganglionic fusion.

KEY WORDS: *stingless bee, Hymenoptera, apoptosis, ecdysteroid, ventral nerve cord*

During metamorphosis of holometabolous insects, the larval nervous system is remodeled in accordance with the adult body pattern and the different functions required in adult life. The most evident changes consist in the anatomical reorganization of the VNC, which occurs by shortening of connectives and consequent fusion of some ventral ganglia (Pipa, 1967; Amos and Mesce, 1994; Cantera *et al.,* 1995). At the cellular level, most of the larval neurons are remodeled or inevitably die, whilst new imaginal neurons are generated (Truman and Reiss, 1976; Levine and Truman, 1982; Booker and Truman, 1987; Levine *et al.,* 1995). These neurodevelopmental events, observed in Lepidoptera and Diptera, have been associated with changes in the circulating levels of ecdysteroid hormones (Pipa, 1969; Robertson and Pipa, 1973; Truman, 1988; Levine, 1989; Truman *et al.,* 1993; Amos *et al.,* 1996).

Although VNC reorganization is a striking characteristic of the larval-pupal transition, this process in eusocial insects has remained undescribed. In the present study, we examined in detail the gradual changes in VNC occurring during metamorphosis in a stingless bee, *M. quadrifasciata*. The observed changes were correlated with the variation in hemolymph ecdysteroid level, determined by a radioimmuoassay. The role of this hormone was further assayed in an *in vitro* incubation system established to monitor the response of isolated VNC to 20E. For a cellular level understanding of ganglia fusion, programmed cell death was investigated by electron microscopy and TUNEL labeling.

In defecating *M. quadrifasciata* larvae (5th instar larvae in preparation to metamorphosis), the VNC consists of 3 thoracic (T1–T3) and 7 abdominal (Ab1–Ab7) ganglia linked by paired connective nerves. During larval-pupal transition, the VNC shrinks mainly due to the shortening of the connectives linking the ganglia T2, T3 and Ab1, forming a single ganglion, and of those linking ganglia Ab4, Ab5, Ab6 and Ab7 that, in consequence, come into close proximity (Fig. 1A). This VNC remodeling correlates with a gradual increase in the hemolymph ecdysteroid titer (Fig. 1B). We could evidence a significant shortening in the connectives linking all the thoracic and Ab1 ganglia, and also in the connectives linking the ganglia Ab4 to Ab6, 48h as soon as the ecdysteroid titer started to increase (Fig. 2A). This developmental period (48h from the beginning of defecation process, identified as "time 0") was chosen to set up a series of VNC *in vitro* incubations in the presence (or absence) of 20E. These experiments showed a significant ($p<0.05$) shortening of the connectives linking the thoracic and Ab1 ganglia, in presence of 5 µg 20E (Fig. 2B), indicating that 20E alone is sufficient to cause their shortening. This response was dose-dependent since shrinking was not

Abbreviations used in this paper: 20-E, 20-hydroxyecdysone; VNC, ventral nerve cord.

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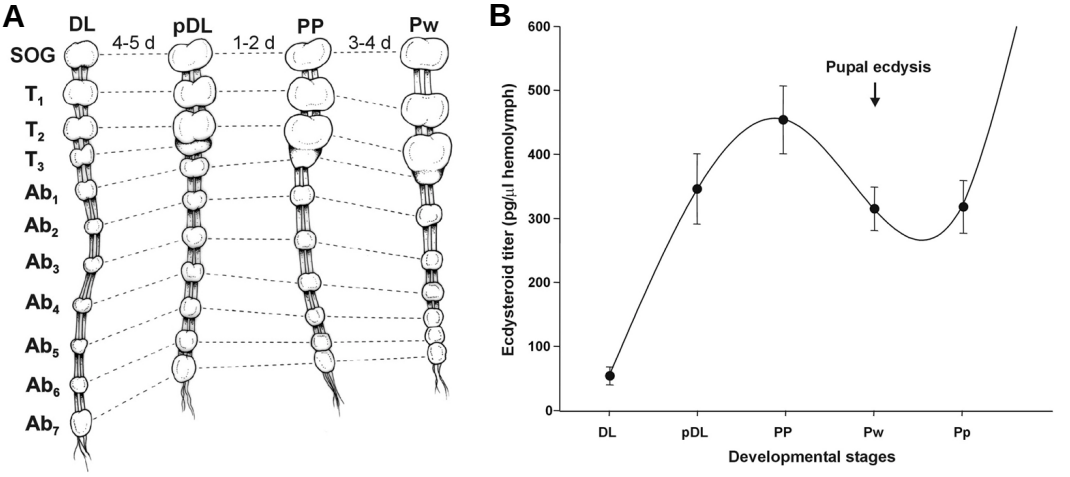

Fig. 1. Relationship between VNC remodeling and ecdysteroid titer fluctuation during *M. quadrifasciata* larval-pupal transition. (A) *(DL) Defecating and (pDL) post-defecating larvae, (PP) prepupae, (Pw) newly ecdysed white-eyed pupae, and the subsequent pupal stage, (Pp) pink-eyed pupae. (SOG) suboesophageal, (T) thoracic and (Ab) abdominal ganglia.* **(B)** *The graph shows the fluctuation in hemolymph ecdysteroids. Values represent means and standard errors of 15 samples for each stage.*

observed when a lower 20E dose (1 μg) was used (data not shown). In contrast, the connectives linking ganglia Ab4 to Ab6, did not significantly shorten *in vitro*, in the presence of 20E (1 or 5 μg) as they did *in vivo* (Figs. 2 A,B).

Post-embryonic changes in the CNS of *M. quadrifasciata* were studied by Cruz-Landim and Höfling (1972) that also referred to the modifications in VNC. In the present work, the metamorphic modifications in VNC were positively correlated with the endogenous ecdysteroid titer, and the direct effect of 20E on VNC remodeling was demonstrated *in vitro*. To our knowledge, Pipa (1969) was the first to show the function of ecdysteroids on interganglionic connective shortening by injecting α-ecdysone in *Galleria mellonella* pupae. Later, Robertson and Pipa (1973), demonstrated shortening of connectives incubated *in vitro*, in the presence of 20E or analogues. Different from our incubations, where entire larval VNCs were used, they used only the T2-T3 and the Ab4-Ab5 pupal segments. As observed here in *M. quadrifasciata*, the thoracic and abdominal segments of *G. mellonella* also responded differentially to the ecdysteroid doses used.

It is not known how some connectives shorten while others maintain their size, or even enlarge, when exposed to the same hormonal signal. Obviously, steroid hormones typically have access to all cells bathed in insect hemolymph, yet the responses of individual cells can be remarkably different. Neurons and other ecdysone target tissues show qualitative and quantitative changes in ecdysone receptor (EcR) expression, which in turn are correlated with distinct patterns of ecdysteroid response (Talbot *et al.,* 1993; Truman *et al.,* 1994). The differential expression of EcR isoforms in the cells could account for regulating different genes when the hormone-receptor complex binds to upstream recognition sites. Furthermore, the pattern of ecdysteroid binding sites in the nervous system of *M. sexta* was shown to be dependent on developmental stage (Fahrbach, 1992). This could make the response spatially and developmentally restricted, and could explain why, in an anterior-posterior series of ganglia, as those forming the VNC, some are responsive to 20E whereas others are not.

Signs of cell death, such as condensed chromatin in the periphery of the nuclear envelope, presence of autophagic vacuoles, nucleolar fragmentation and cytoplasm vacuolization were frequently observed in ganglionic cells of metamorphosing *M. quadrifasciata* (Fig. 3). TUNEL labeled cells (apoptotic cells) were characteristically detected in the regions of ganglionic fusion (Fig. 4A). In non-fusing ganglia, apoptotic cells were observed only in the periphery of the ganglionic mass (Fig. 4B), showing that although apoptosis is a general phenomenon in all ganglia examined, its spatial distribution differs between fusing and non-fusing ganglia. Recently, Usui-Aoki *et al.,* (2002) observed failure in shortening of the abdominal nervous system in *Drosophila* pupae where apoptosis had been inhibited by mutations, suggesting strongly that programmed cell death is part of the nervous system remodeling program.

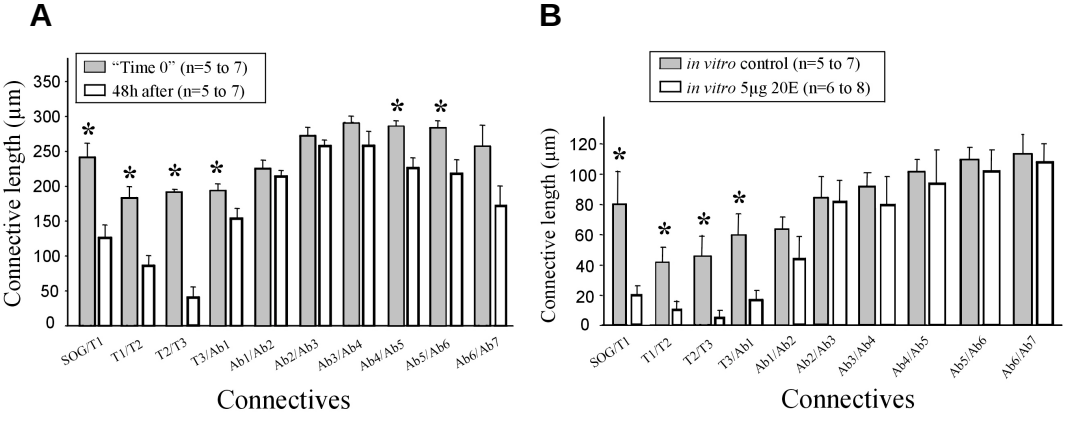

Fig. 2. Connective length in VNCs from *M. quadrifasciata* larvae. (A) *Larvae starting defecation ("time 0") compared to 48 h older larvae.* **(B)** *VNCs incubated* in vitro *for 48 h in the presence of 20E (5 μg/ml) or without hormone (* in vitro *control). The connectives in (A) are longer than those in (B), because the latter were measured 48 h after being released from their attachment to the body wall. We assumed that the shortening effect observed* in vitro *occurred equally for all connectives. * p<0.05.*

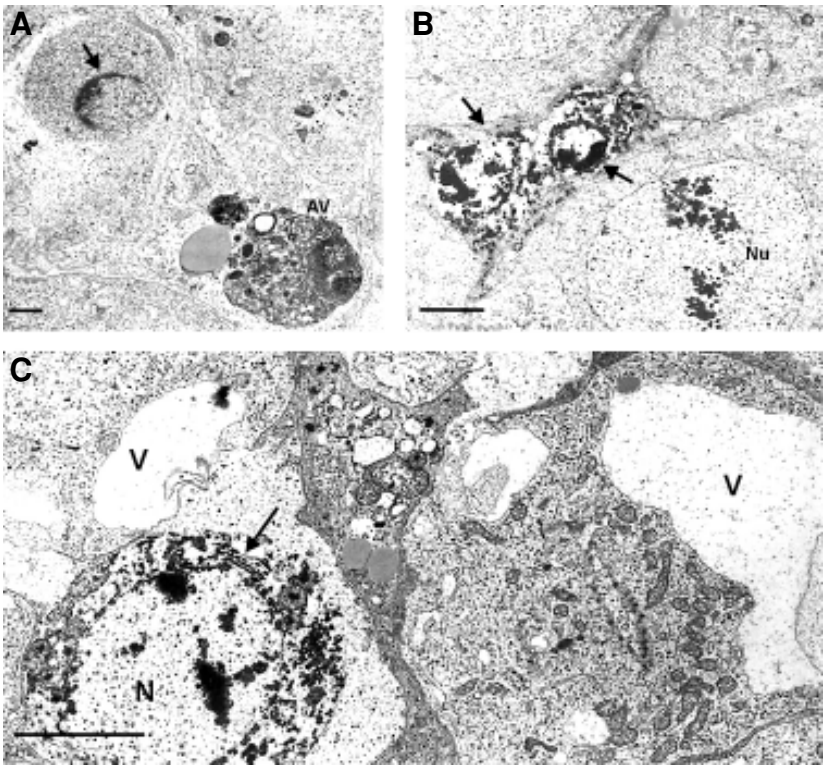

Fig. 3. Ultrathin sections of degenerating VNC cells from metamorphosing *M. quadrifasciata*. (A) *Condensed chromatin (arrow) in the periphery of the nuclear envelope, and autophagic vacuole (AV) in defecating larvae.* **(B)** *Advanced cell death (arrows), and fragmented nucleolus (Nu) in prepupae.* **(C)** *Condensed chromatin (arrow) in the nuclear (N) periphery and large cytoplasm vesicles (V) in white-eyed pupae. Bars, A, 2 μM; B,C, 4 μm.*

It is well known that programmed cell death in a variety of metamorphosing tissues is under ecdysteroid control (Truman and Schwartz, 1984; Weeks *et al.,* 1992; Jiang *et al.,* 1997; Kinch *et al.,* 2003). Specifically in the honey bee brain, the onset of apoptosis in neuronal precursors of mushroom bodies coincides with elevated levels of pupal ecdysteroids (Ganeshina *et al.,* 2000). Apparently, apoptosis involved in connective shortening and ganglionic fusion constitutes a cell response to increasing ecdysteroid levels that ultimately results in *M. quadrifasciata* VNC remodeling.

The fact that in our *in vitro* assays of *M. quadrifasciata* VNC, abdominal connectives did not responded to 20E suggests that additional factors are necessary for their shortening. A signal transmitted from the pterothoracic ganglion was showed to be necessary to regulate post-eclosion death of abdominal motoneurons in adult *M. sexta* (Choi and Fahrbach, 1995), showing for the first time that in addition to the ecdysteroidal signal, a neural peptide is implicated in remodeling the insect nervous system. This finding reinforces that certain aspects of the VNC reorganization could also depend on other factor(s) besides the endocrine signal.

In conclusion, the connective shortening and ganglionic fusion in metamorphosing *M. quadrifasciata*, was correlated with endogenous ecdysteroid titer modulation. Specifically, the shortening of thoracic connectives turned out to be caused by 20E. Also, the occurrence of apoptotic cells in the fusing region of adjacent ganglia strongly suggests that programmed cell death may drive ganglionic fusion.

Experimental Procedures

VNCs from metamorphosing *M. quadrifasciata* were prefixed *in situ* in 2% paraformaldehyde, 2% glutaraldehyde and 4% sucrose in sodium cacodylate buffer 0.05M, pH 7.2 before being dissected, fixed in fresh fixative, and washed in the same buffer for stereomicroscope analysis of the time course of VNC remodeling.

To establish the effect of 20E on connective length, VNCs from 5th instar larvae that just started to defecate were dissected and incubated in culture medium (Rachinsky and Hartfelder, 1998) during 48 h at 28°C, under shaking, in the presence or absence of 20E (1 or 5 μg/ml medium). After incubation, the VNCs were fixed and washed as described above, for connective measurements under stereomicroscope using an ocular micrometer. To establish the shortening *status* of the connectives before *in vitro* incubations, measurements were taken from larvae that just started to defecate. To compare the *in vitro* results with connective shortening occurring *in vivo*, measurements were also taken from a batch of larvae of corresponding age.

VNCs ultrathin sections were obtained as previously described for fat body cells (Pinto *et al.,* 2000). The sections were analyzed using a Philips EM208 transmission electron microscope. To visualize cells undergoing apoptosis, VNC whole mounts were TUNEL-labeled using the TdT-FragEL™ kit (Calbiochem), and analyzed using a Zeiss Axioskop II.

Ecdysteroid titers in hemolymph were quantified by radioimmunoassay as previously described (Feldlaufer and Hartfelder, 1997; Pinto *et al.,* 2002).

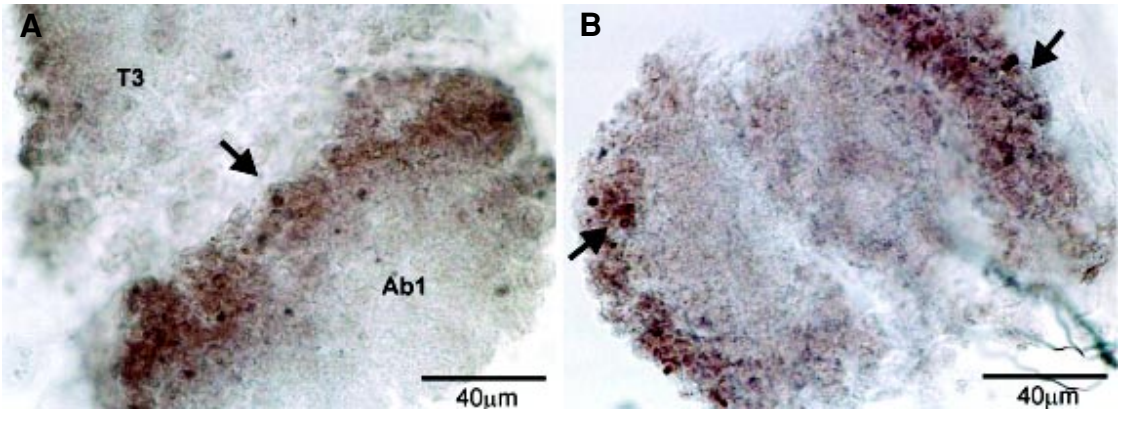

Fig. 4. TUNEL-labeled cells in VNC ganglia of *M. quadrifasciata*. (A) *Apoptotic cells in the fusion region (arrow) of ganglia T3 and Ab1 in prepupae.* **(B)** *Apoptotic cells labeled in the periphery of non-fusing ganglion (arrows) from defecating larvae.*

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