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Silencing Allatostatin Expression Using Double-Stranded RNA Targeted to Preproallatostatin mRNA in the German Cockroach

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YXFGL-NH_2 family allatostatins (ASTs) were isolated from cockroach brain extracts based on their capacity to inhibit juvenile hormone (JH) biosynthesis in corpora allata (CA) incubated in vitro. Subsequently, the inhibitory activity of synthetic ASTs was demonstrated experimentally, although these peptides were shown to be active as JH inhibitors only in cockroaches, crickets, and termites. Here, we sought to examine whether ASTs are true physiological regulators of JH synthesis. To this end, we used RNA interference methodologies and the cockroach *Blattella germanica* as a model. Treatments with double-stranded RNA targeting the allatostatin gene in females of *B. germanica* produced a rapid and long-lasting reduction in mRNA and peptide levels in both brain and midgut during the reproductive cycle. Nevertheless, while brain AST levels were reduced approximately 70–80%, JH synthesis did not increase in any of the age groups tested. Arch. Insect Biochem. Physiol. 62:73–79, 2006.

KEYWORDS: allatostatin; juvenile hormone; *Blattella germanica*; RNAi; dsRNA

INTRODUCTION

Insect neuropeptides are involved in most physiological and developmental processes, including growth, moulting, metamorphosis, reproduction, diapause, feeding, and metabolism. Among the insect neuropeptide families, one of the most thoroughly studied is that of the YXFGL-NH_2 allatostatins (ASTs). The first members of this group were identified in the cockroach *Diploptera punctata* based on their activity as inhibitors of juvenile hormone (JH) synthesis by the corpora allata (CA) (Woodhead et al., 1989; Pratt et al., 1989). Since then, orthologous peptides have been reported in other species of Dictyoptera, as well as in Orthoptera (locusts and crickets), Phasmida, Lepidoptera, Diptera, and Crustacea Decapoda (see Bendena et al., 1999; Dircksen et al., 1999; Lorenz et al., 2000; Duve et al., 2002; Bellés and Maestro, 2005).

Studies using synthetic versions of the peptides and biological tests ad hoc, revealed that ASTs can affect a number of biological processes. These include inhibition of muscle contraction in the digestive tract, oviducts, and heart, inhibition of vitellogenin release by the fat body, neuromodulatory action on neuromuscular transmission, stimulation of digestive enzyme release, and inhibition of food intake (see Martín et al., 1996; Fusé et al., 1999; Stay 2000; Aguilar et al., 2003). The most characteristic effect of AST, the inhibition of JH produc-

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tion, was demonstrated in cockroaches, crickets, and termites, but not in locusts, stick insects, moths, and flies (see Bendena et al., 1999; Yagi et al., 2005). Immunocytochemical studies also showed that ASTs are ubiquitously spread throughout the nervous system, peripheral nerves to the visceral muscle, in midgut cells, and in haemocytes (see Stay, 2000). Indeed, such a wide distribution suggests that they play a multiplicity of roles.

In the cockroach *B. germanica*, four ASTs (BLAST-1 to -4) have been identified from brain extracts (Bellés et al., 1994), while nine more have been deduced from the gene encoding the AST prepro-hormone (Bellés et al., 1999). Synthetic peptide activity has been tested using different bioassays, including radiochemical assays to measure effects on JH production (Bellés et al., 1994). BLAST-1 to -4 inhibited JH production in highly active CA, although the maximal inhibition achieved was only around 60% at a concentration of 10^{-5}M. Indeed, only BLAST-3 exhibited a modest 40% inhibitory activity at the dose of 10^{-8}M (Bellés et al., 1994).

All of the data suggest that the true physiological function(s) of allatostatins awaits further clarification, and even that the physiological sense of the allatostatic effects in sensitive insects remains unclear. With these premises in mind, we approached the study of AST physiological functions in *B. germanica* by reducing their levels using an RNA interference (RNAi) strategy (Zamore, 2001; Hannon, 2002). Treatments employing a double-stranded RNA (dsRNA) that targets the preproAST gene would reduce the corresponding mRNA levels, and, therefore, those of the AST peptides. Thus, if ASTs are indeed key inhibitory modulators of the cockroach CA, we would then expect JH synthesis to increase in AST knock-down specimens.

MATERIALS AND METHODS

Insects

Adult females of *B. germanica* (L.) (Dictyoptera, Blattellidae) were obtained from a colony fed dog chow and water, and reared in the dark at 30 ± 1°C and 60–70% relative humidity. Freshly molted last instar nymphs or adult females were isolated and used at the appropriate ages. Virgin females were used in the experiments carried out during the first 7 days of the gonadotrophic cycle. To study the period of ootheca transport, mated females were used instead, since they retain the ootheca throughout the entire embryogenesis.

Tissue Extraction and Allatostatin Quantification

Tissue dissection and ELISA assays were performed as previously described (Vilaplana et al., 1999). Briefly, brains and midguts were homogenized in phosphate buffered saline, boiled for 5 min, centrifuged, and the supernatant collected and lyophilized. AST measurements using competitive ELISA were performed utilizing BLAST-3-ovoalbumin as conjugated, with the same peptide in its unconjugated form used as a standard. Secondary antiserum consisted of goat anti-rabbit conjugated to peroxidase (Sigma), while the substrate solution was prepared in citrate buffer (pH 5), with 3,3′,5,5′-tetramethylbenzidine and H_2O_2. Results are expressed as BLAST-3 equivalents (Vilaplana et al., 1999).

Quantification of Juvenile Hormone Synthesis

JH III synthesis in CA incubated in vitro was quantified according to the method published by Piulachs and Couillaud (1992). Essentially, individual pairs of CA were incubated in 100 µl of TC 199 medium (Flow) containing L-methionine (0.1 nM), Hank's salts, Hepes buffer (20 mM) plus Ficoll (20 mg/ml), to which L-[^{3}H-methyl] methionine (Amersham Biosciences) had been added, to achieve a final specific activity of 7.4 GBq/mmol. CA were incubated for 2 h, after which JH III was quantified in the medium plus homogenized glands.

RNA Interference

To generate the dsRNA targeting the *B. germanica* preproAST transcript (dsBgAST), a 928-bp fragment spanning positions 258 to 1,185 of the preproAST

gene reported by Bellés et al. (1999), which included all 13 ASTs, was sub-cloned into the pSTBlue-1 vector (Novagen). As a control, a non-coding sequence of 92 bp from the pSTBlue-1 vector was used (dsControl). RNAs were generated by in vitro transcription using either SP6 or T7 RNA polymerases (Promega) from the respective plasmids, and then resuspended in water. To obtain dsRNAs, equimolar amounts of sense and antisense RNAs were mixed, heated for 10 min at 95°C, cooled down slowly to room temperature, treated with RQ1 DNase (Promega) and RNase A (Sigma), and stored at –20°C until use. Formation of dsRNA was confirmed by running 1 µl of the reaction products in a 1% agarose gel. dsRNAs were suspended in diethyl pyrocarbonate-treated water and diluted in Ringer saline to a final concentration of 1 µg/µl. Two microliters of the dsRNA solution, or the corresponding solvent, were injected into the abdomens of females that had just molted into sixth instar nymphs or into adults.

RNA Isolation, cDNA Preparation, Polymerase Chain Reaction (PCR), and Southern Blot Analysis

Tissues for RNA extraction were frozen in liquid nitrogen immediately after dissection, and stored at –80°C until use. Total RNA was isolated using the GenElute Mammalian Total RNA kit (Sigma). All RNA samples were treated with RQ1 DNase. cDNAs were prepared using SuperScript II reverse transcriptase (Invitrogen), as described by Aguilar et al. (2003). Negative controls without the reverse transcriptase step were used to check for genomic contamination.

PCR and Southern-blot methodologies were followed as previously described (Aguilar et al., 2003). Primers used for amplifying preproAST transcript were: forward, 5′-CAATTCGGAACTGGACTTAGTAA-3′, reverse, 5′-CCAAGACCAAAGGAAAACCTGTG-3′ (positions 234 to 863). As a reference, the same cDNAs were amplified with a primer pair specific for Actin-5C, as described by Maestro et al. (2005). To estimate mRNA levels semi-quantitatively, a non-saturating number of cycles in the PCR system were used. Southern blot analysis was carried out as described by Aguilar et al. (2003).

RESULTS

To determine the time required to obtain a significant reduction in preproAST mRNA and AST levels, a time-course study following dsBgAST treatment was conducted. For this purpose, freshly molted adult females were treated with 2 µg dsBgAST, and brains and midguts were dissected out on days 1, 4, and 7 after treatment. Tissues were then processed for RT-PCR and ELISA analysis.

Reductions in brain mRNA for preproAST were not detected 1 day after treatment. However, a readily apparent reduction in mRNA levels was observed on day 4, whereas on day 7 mRNAs were almost undetectable (Fig. 1). Similarly, AST levels (in terms of BLAST-3 equivalents) as measured by ELISA, were similar to those of controls on day 1, but decreased significantly on days 4 and 7 (35 and 42% reduction, respectively) (Fig. 1). In midgut tissues, the silencing effects were somewhat more rapid. PreproAST mRNA levels were practically undetectable 1 day after treatment, and this effect was maintained on days 4 and 7 (Fig. 2). In terms of BLAST-3 equivalents, significant reductions were observed on days 4 and 7 (53 and 37% reduction, respectively) (Fig. 2).

To study the effects on JH synthesis, freshly molted adult females were treated with 2 µg dsBgAST, and CA activity was examined on days 3, 6, 7, and 14 (that is, 6 days following formation of the ootheca, and during the transport period). For each treatment, brain AST contents (BLAST-3 equivalents) were similarly quantified by ELISA. Results (Fig. 3) demonstrated that JH synthesis in dsBgAST-treated specimens was similar to that of controls for all tested age groups, despite the fact that brain AST contents were significantly reduced to approximately 40–50% on days 3, 6, or 7 and as much as approximately 80% on day 14. Given that the highest reduction in brain AST was observed with the longest treatment (Fig. 3), we treated freshly molted last (sixth) instar nymphs with dsBgAST. In this instance, controls included

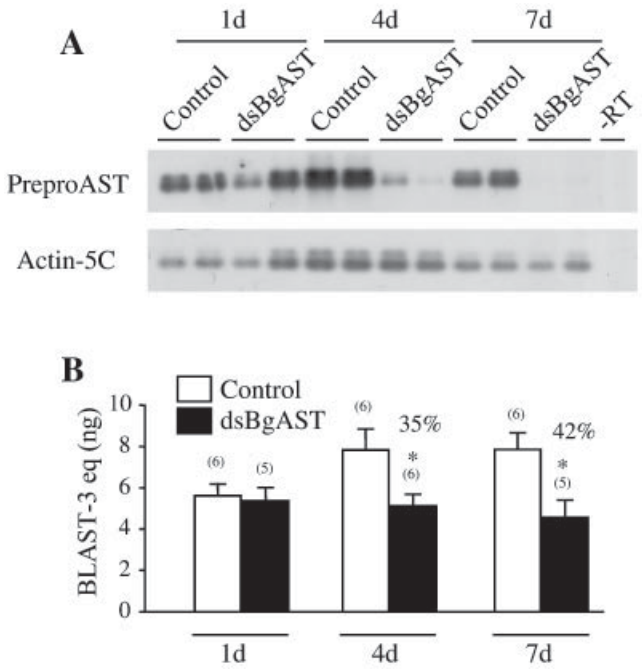

Fig. 1. Effects of dsBgAST treatment on preproAST mRNA and AST levels in brain tissues of *B. germanica* females. **A:** PreproAST mRNAs from control and dsBgAST-treated animals were analyzed using RT-PCR, followed by Southern blot. Actin-5C levels were used as a reference. –RT: Negative controls without the reverse transcriptase step. **B:** Brain AST content, in terms of BLAST-3 equivalents, was measured by ELISA. Results are expressed as the mean ± SEM. The number of replicates is indicated at the top of each column. Percentages of inhibition are also indicated. Asterisks represent significant differences (Student's *t*-test) (*$P < 0.05$).

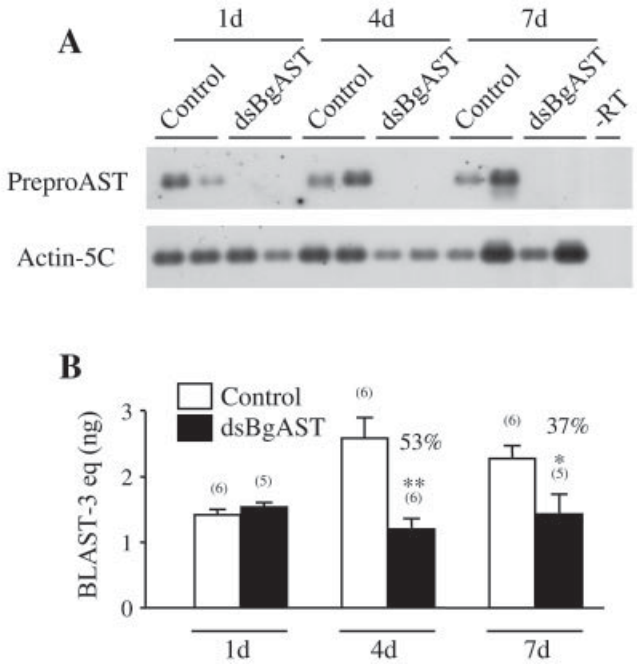

Fig. 2. Effects of dsBgAST treatment on preproAST mRNA and AST levels in midgut tissues of *B. germanica* females. **A:** PreproAST mRNAs from control and dsBgAST-treated animals were analyzed using RT-PCR, followed by Southern blot. Actin-5C levels were used as a reference. –RT: Negative controls without the reverse transcriptase step. **B:** Midgut AST content, in terms of BLAST-3 equivalents, was measured by ELISA. Results are expressed as the mean ± SEM. The number of replicates is indicated at the top of each column. Percentages of inhibition are also indicated. Asterisks represent significant differences (Student's *t*-test) (*$P < 0.05$; **$P < 0.01$).

specimens treated with the dsControl. Results, in terms of brain BLAST-3 content and JH synthesis, were checked on days 3 and 7 of adult life. They reveal (Fig. 4) that while a significant AST reduction in brain tissues was obtained in all dsBgAST-treated animals (around 70%), JH synthesis remained practically unaffected (Fig. 4).

DISCUSSION

ASTs were first discovered (and named) for their inhibitory activity on JH synthesis in the cockroach CA (Woodhead et al., 1989; Pratt et al., 1989), and this has remained their most extensively studied activity in cockroaches (see, Bendena et al., 1999). The question we sought to answer was whether ASTs play the role of physiological regulator of JH synthesis during the reproductive cycle of the German cockroach.

The RNAi methodology used in the present study has proven to be an effective and reliable method to knock-down YXFGL-NH_2 allatostatins expression. Our results indicate that treatment with dsBgAST targeting the preproAST mRNA triggers a rapid reduction in preproAST mRNA levels in the brain and the midgut. In addition, a subsequent decline in AST peptide levels followed the drop in mRNA levels, although the effects on peptides were less pronounced than those on mRNA. Our results also indicate that these effects are maintained over

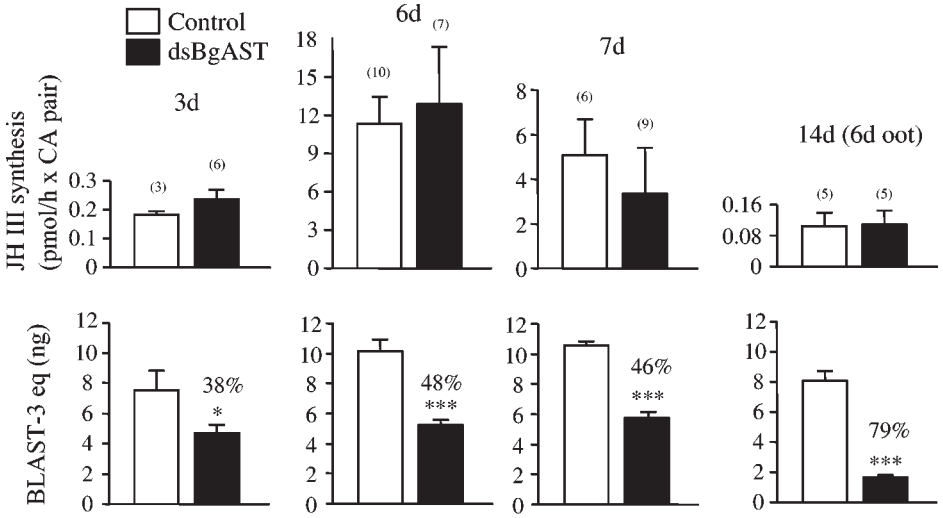

Fig. 3. Juvenile hormone III (JH III) synthesis and brain AST content (in terms of BLAST-3 equivalents) measured by ELISA in control and dsBgAST-treated adult females. Specimens were treated immediately following the imaginal molt and dissected 3, 6, 7, or 14 days later (6 days after ootheca formation). The same specimens were used for JH synthesis and ELISA assays. Results are expressed as the mean ± SEM. The number of replicates is indicated at the top of each column. Percentages of inhibition are also indicated. Asterisks represent significant differences (Student's *t*-test) (**P* < 0.05; ****P* < 0.001).

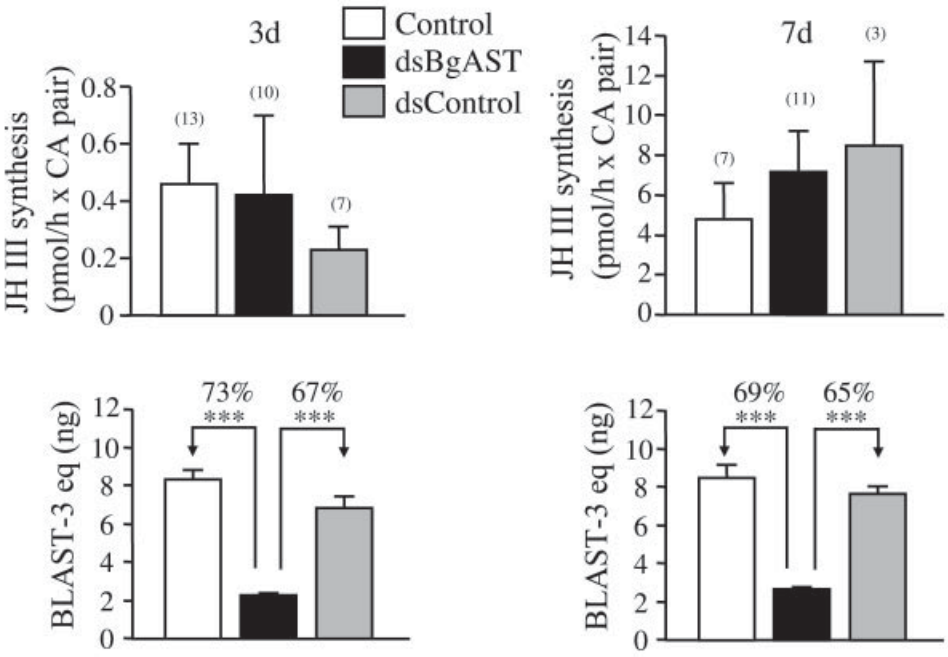

Fig. 4. Juvenile hormone III (JH III) synthesis and brain AST content (in terms of BLAST-3 equivalents) measured by ELISA in control females, and females treated with dsBgAST or dsControl. Specimens were treated as recently molted last instar nymphs and dissected 3 or 7 days after the imaginal molt. The same specimens were used for JH synthesis and ELISA assays. Results are expressed as the mean ± SEM. The number of replicates is indicated at the top of each column. Percentages of inhibition are also indicated. Asterisks represent significant differences (Student's *t*-test) (****P* < 0.001).

long periods, and that the largest reductions, in terms of peptides, were achieved during the longest treatment periods. However, while brain ASTs were significantly reduced (even to 70–80%) for all tested age groups, JH production failed to increase in any of the dsBgAST-treated animals.

Different hypotheses may explain these results. A possible explanation is that control of JH production may involve redundant regulatory factors. Therefore, the reduction of ASTs would be compensated by another factor. Nonetheless, there is no clear evidence in this regard in cockroaches. We must also consider the possibility that ASTs are not true physiological modulators of JH production, at least in the female reproductive cycle of *B. germanica*. If this is indeed the case, then the inhibitory activities observed in the in vitro incubated CA (Bellés et al., 1994) might be the result of pharmacological effects produced by relatively high peptide concentrations. This does not exclude, of course, that YXFGL-NH_2 allatostatins could indeed play the role of physiological regulators of JH production in other species and processes. Finally, we cannot rule out the possibility that the reduction in brain AST levels achieved with RNAi was insufficient to generate a detectable effect on JH production, and that the remaining 20–30% of brain AST in knock-down females were capable of sustaining the CA at the same inhibition level as those of controls. The testing of this hypothesis, however, would require a complete knockout of the allatostatin gene, a goal unaffordable, by the moment, given that *B. germanica* is not yet amenable to genetic transformation.

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