Maturation and Responsiveness to Extracts of Corpora Allata from Male *Locusta migratoria* containing Allatotropic Factors

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Corpora allata (CA) from male *Locusta migratoria* were tested for their ability to synthesize juvenile hormone (JH) and to respond to stimulating brain/corpora cardiaca (CC) extracts under *in vitro* conditions. We found that a preincubation of the CA of both sexes at 4 °C for 24 h lowers their basal rate of synthesis and retains their responsiveness to allatotropic factors. Male CA can be stimulated by brain/CC extracts as well as female CA. JH biosynthesis stimulating factors are also present in male brain/CC extracts. Thus such extracts from male locusts can be used for the isolation of locust allatotropin. Furthermore male locust CA are appropriate for bioassaying such allatotropic factors.

Introduction

JH biosynthesis in insect CA appears to be controlled by stimulating (allatotropins) and/or inhibiting (allatostatins) neuropeptides [1–3]. In female *Locusta migratoria* there is some evidence for one or more allatotropins [4–7] inducing cyclic activity of the CA which results in gonocycles [8]. In male *Locusta migratoria* mating behaviour, yellow coloration and development of the accessory reproductive glands are controlled by JH but no cyclic JH biosynthesis occurs. JH synthesis increases steadily with age and remains at a high level [9]. However, nothing is known about its neuroendocrine control nor have such factors been assayed in the retrocerebral complex of male *Locusta migratoria*.

We have investigated the response of CA from male and female locusts to methanolic brain and CC extracts from locusts of both sexes and the response of male CA at different ages towards increasing concentrations of brain/CC extracts. We found that male CA respond to stimulatory extracts like female CA, and that male *Locusta migratoria* are thus a good system for isolating and testing of allatotropic factors.

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Materials and Methods

Preparation of brain/CC extracts

Brains and CC were dissected from crowded unanesthetized female and/or male locusts, immediately shock-frozen in liquid nitrogen, and stored at −70 °C until use. The brains and CC were sonicated in 100% methanol, centrifuged at 5000 rpm and the supernatant dried in a vacuum concentrator (Speed Vac). For experiments the dried brain and CC extracts were redissolved in Minimal Essential Medium (MEM) (Kibbutz Beth Haemek, Israel) containing unlabelled methionine and [methyl-^{3}H] methionine (spec. activity 83.2 Ci/mmol; Amersham) (final methionine concentration: 0.01 µmol/100 µl medium) as described previously [10].

Radiochemical assay of juvenile hormone synthesis in vitro

Dissected CA were placed in MEM and cleaned of fatbody and trachea. Individual glands were then transferred into glass vials containing 100 µl MEM with 5 µCi [methyl-^{3}H]methionine and with or without brain/CC-extracts. The glands were incubated for 2 h at 32 °C and the JH was extracted with 300 µl hexane, which was added directly to the vial without removal of the glands. After centrifugation aliquots of the hexane phase were measured in a liquid scintillation counter (1209 Rackbeta, LKB).

Results

JH synthesis after long-term in vitro culture of CA

CA from mature male and female locusts were kept at different temperatures and for different time periods in MEM in order to observe their ability to synthesize JH under *in vitro* conditions. In preliminary experiments we noticed a dramatic decrease of JH biosynthesis when incubating CA up to 72 h at 4 or 32 °C. CA incubated at 32 °C lost their ability to respond to allatotropic factors within 24 h whereas those incubated at 4 °C had a reduced spontaneous activity but could be stimulated well by such compounds. Thus we explored the possibility to use such glands with reduced basal activity for assaying extracts containing allatotropic factors. Immediately after dissection (0 h) excised CA of male locusts spontaneously produced about 30 pmol JH hr^{-1} $gland^{-1}$ (Fig. 1 A). The addition of 0.25 brain/CC equivalents caused an increase in JH synthesis to about 50 pmol JH hr^{-1} $gland^{-1}$. After measurement of the spontaneous rate and of the stimulated JH synthesis at 0 h the glands were transferred individually to fresh MEM without radioactive tracer and without additions, divided into 2 groups and kept at 4 °C or 32 °C for 24 h. Thereafter they were incubated again for 2 h at 32 °C in MEM containing the radioactive tracer and with or without 0.25 brain/CC equivalents. After 24 h at 4 °C the basal rate of JH synthesis was about 50% lower than right after dissection. However, after the preincubation at 4 °C the glands responded very well to the addition of 0.25 brain/CC equivalents and produced about 45 pmol JH hr^{-1} $gland^{-1}$. In contrast the glands kept at 32 °C for 24 h had a very low basal rate of JH biosynthesis and did not respond to allatotropic stimulation (Fig. 1 A). CA of female locusts responded in essential the same manner (Fig. 1 B).

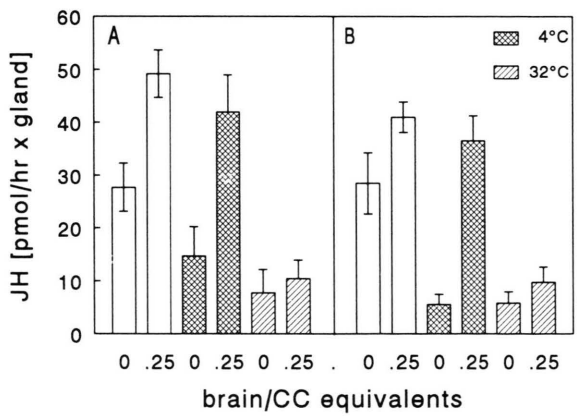

Fig. 1. JH *in vitro* biosynthesis by CA of 21 days old male (A) and female (B) locusts in presence or absence of brain/CC extracts. CA were tested immediately after dissection (0 h, white bars) and after a preincubation of 24 h at different temperatures. Vertical bars indicate S.E.M. (number of replicates for each experiment, $n = 11-13$).

Responsiveness of mature CA to stimulation by extracts containing allatotropic components

Brain/CC extracts were prepared from adult locusts of different ages: 2, 17 and 42 days. These extracts were used to stimulate *in vitro* fully competent CA of 3–4 weeks old adult male and female locusts after a preincubation period of 24 h at 4 °C (Tab. I). All CA could be well stimulated: maximum stimulation was achieved with extracts obtained from 2 and 17 days old locusts. The relatively low stimulation of female CA by extracts from 17 days old males is not significantly different from the other results (t-test). Extracts from locusts 42 days old did not stimulate JH synthesis; the results are not significantly different from the control incubations without brain/CC extracts. No difference was found in the response of male and female CA.

Dose-response of CA from male locusts to extracts containing allatotropic components

CA from male locusts of different ages were incubated in MEM containing different concentrations of brain/CC extracts. CA of 1 week old male locusts barely responded to increasing brain/CC extract concentrations (Fig. 2 A). Maximum response was attained with 0.625 brain/CC equivalents. With CA of 14 days old male locusts, an age which corresponds to female locusts of the first ovarian cycle, maximum allatal response was attained with only 0.156 brain/CC equivalent per gland (Fig. 2 B). Higher concentrations of brain/CC extracts caused a decline in JH synthesis. Also CA from male locusts aged 1 month, comparable in age to females in the second ovarian cycle, were fully stimulated with 0.156 brain/CC equivalents (Fig. 2 C). High levels of JH biosynthesis were maintained by concentrations up to 1.25 brain/CC-equivalents.

Table I. JH biosynthesis in 3–4 weeks old male and female locust CA in the presence of brain/CC extracts. The brain/CC extracts were prepared from locusts of both sexes, 2, 17 and 42 days resp. after emergence. After dissection the CA were kept in MEM without radioactive precursor at 4 °C for 24 h and then transferred into fresh MEM containing and 0.1 brain/CC equivalents and incubated for 2 h at 32 °C. Each value represents mean ± S.E.M.

0.1 brain/CC equivalent	Female CA JH [pmol h^{-1} $gland^{-1}$]	*n*	Male CA JH [pmol h^{-1} $gland^{-1}$]	*n*
male, 2 days	52.1 ± 8.1*	11	51.1 ± 7.7*	11
female, 2 days	59.3 ± 10.7*	12	58.2 ± 12.3*	11
male, 17 days	38.7 ± 5.6	12	52.1 ± 6.5*	12
female, 17 days	50.5 ± 5.7*	12	55.6 ± 6.4*	12
male, 42 days	41.9 ± 8.8	6	37.3 ± 6.2	5
female, 42 days	27.4 ± 7.9	5	33.9 ± 6.4	6
control	22.2 ± 6.4	11	21.9 ± 6.4	11

* Results significantly different from controls (t-test).

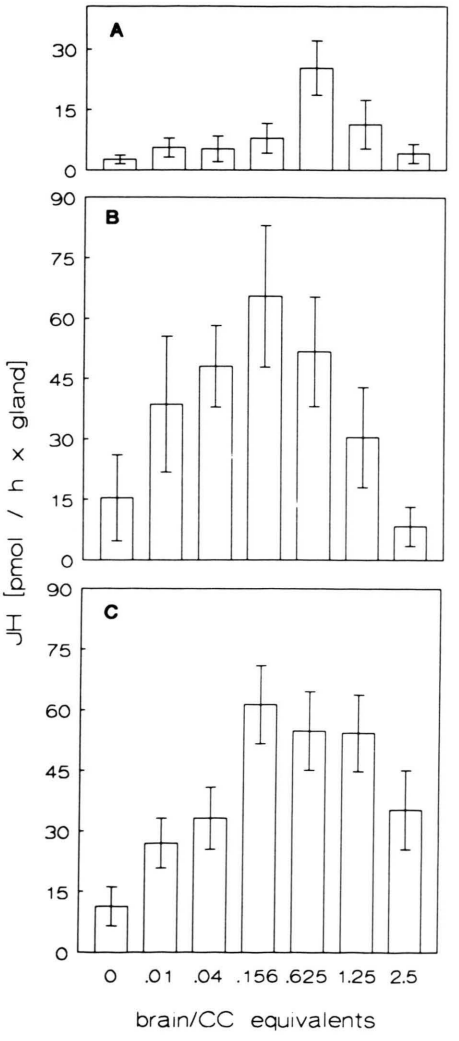

Fig. 2. Dose response of adult male locust CA to brain/CC extracts prepared from mature (21–28 days old) male and female locusts. Age of locusts incubated CA: A, 7 days (number of replicates for each experiment, $n = 6$); B, 14 days ($n = 7$) and C, 28 days ($n = 15–20$). Vertical bars indicate S.E.M.

Discussion

This paper addresses two problems of research on neuroendocrine regulation of CA activity. The first concerns the high variability of JH biosynthesis encountered with the radiochemical assay for extracts containing allatotropic factors. The second problem concerns the availability of starting material for such research. We examined the suitability of male locust CA for the bioassay of allatotropic factors, as well as the possibility to use male locust brain/CC extracts as a source for such factors. Different approaches have been proposed to overcome the problem of high variability of basal levels of JH biosynthesis of CA from *Locusta migratoria*. Ferenz and Diehl [4] used CA of adult females within 24 h after emergence. Such glands had a low basal JH biosynthetic activity with little variability; but these glands could not be stimulated to high activities. In addition the percentage of JH-diol produced is also much higher in glands with low spontaneous levels of JH synthesis [10]. Gadot and Applebaum [5] determined the basal JH release of each single gland prior to stimulation with putative allatotropic extracts and subtracted the spontaneous JH release from the total after stimulation. This experimental design is not appropriate if the initial basal rate is already close to the

maximum capacity of the glands. Couillaud and Girardie [7] proposed a surgical technique to depress the basal JH release of mature female CA by disconnecting the CA *in vivo* 3 days before using them for the bioassay. This method seems to be reliable but it is difficult to handle.

It is possible to lower the basal JH release *in vitro* by preincubating the CA for different periods and at different temperatures. If CA are kept in culture up to three days at 4 °C, their basal JH release then approaches zero (data not shown), which is in good agreement with the results of Couillaud and Girardie [7]. At 32 °C this effect occurred already after 24 h but these glands also lost their ability to react to allatotropic factors. There was also a quick and drastic reduction of JH biosynthesis in CA previously noted to have extremely high basal JH release rates. Within 24 h at 4 °C, such treated glands responded consistently and very well to allatotropic stimulation when incubated again at 32 °C. Thus a 24 h preincubation at 4 °C proved to be a helpful procedure to reduce variation and to increase responsiveness to extracts containing allatotropic components in locust CA.

The CA from male locusts responded to the various treatments like those from females did. There was also no difference between sexes in the dose response experiments. The amount of brain/CC equivalents we needed for maximum stimulation of maturing males (0.156) is similar to that needed for maturing females (0.1) [5]. For sexually mature locusts we obtained similar results. Beyond the optimum brain/CC extract concentration the JH synthesis declined. This phenomenon was also observed by Sreng *et al.* [11] and by Couillaud *et al.* [12] but not by Couillaud and Girardie [7] nor by Gadot and Applebaum [5]. A possible explanation for this decline could be the presence of an inhibiting factor in the methanolic extracts, which depresses the JH synthesis only in case of higher concentrated extracts. Since the brains and CC used for the extraction were dissected one by one and immediately shock-frozen in liquid nitrogen in order to protect the allatotropic factor, such an inhibitor might have been protected from degradation, too [13].

Brain/CC extracts both from male and female locusts contain JH synthesis stimulating factors, which were shown to be of peptidic nature [4, 5]. So far only the allatotropic factor from *Manduca sexta* has been purified [3]. For further attempts to isolate and characterize locust allatotropin it will be helpful to know that such factors are present in male brain/CC extracts and that male locust CA are appropriate for bioassaying such allatotropic factors.

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