Brain factor from *Galleria mellonella* (Lepidoptera) stimulating silk gland activity

J. Michalik, E. Szolajska and Z. Lassota

Department of Comparative Biochemistry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02-532 Warsaw (Poland)
Received 10 September 1991; accepted 3 March 1992

Abstract. Brain extracts from day 1–4 last instar larvae of *Galleria mellonella* (Lepidoptera) stimulate RNA synthesis in cultured silk glands from day 3 last instar larvae. When the fibroin-synthesizing posterior parts of silk glands were incubated for 3 h in vitro in the presence of brain extract (0.1 brain equivalent), [^{3}H]-uridine incorporation into RNA was stimulated more than twofold. The stimulating effect of brain extract showed a dose response relationship. It is suggested that the heat-resistant and protease-sensitive brain factor is a peptide.
Key words. Neuropeptide; silk gland; *Galleria mellonella*.

Crude extracts of neurohaemal tissues influence many processes in insects [1]. The silk glands of *Galleria mellonella* larvae, which, during the last instar, produce large amounts of silk proteins, have been shown to be under the control of juvenile hormone [2] and ecdysteroid [3]. The glands are also stimulated when brains are implanted into isolated abdomens [3]. This stimulation results in increased RNA and protein synthesis [3]. It is not clear, however, what part, if any, of the stimulatory effect of the brain tissue is due to the direct action of factors from the brain on the silk glands. To resolve this question we have tested in vitro the influence of dissected brains or crude brain extracts on silk gland tissues in a convenient incubation system described earlier [4].

Material and methods

Experimental insects. Larvae and pupae of *Galleria mellonella* (Lepidoptera, Pyralidae) were kept in constant darkness at 30 °C and fed on a semisynthetic diet [5]. Their age was measured in hours after the penultimate, last larval and pupal ecdysis.

Cultivation of silk glands in vitro. Silk glands and brains from water-anaesthetized larvae were dissected out in a laminar hood. The posterior part of the silk gland was incubated either alone or with brain extract in 100 µl Grace's medium (Gibco) supplemented with 2.0 µCi of [5,6^{3}H]-uridine (Amersham, 49 Ci/mmol). The activity of the gland tissue was expressed by [^{3}H]-uridine incorporation into total RNA during short time incubation in vitro. The activation was expressed as the ratio of radioactivity incorporated by a gland, in the presence of brain or brain extract, to the value for a parallel gland from the same animal incubated alone (control). The activation ratio (Ar) is the amount of [^{3}H]-uridine incorporated into RNA (dpm/gland) in the presence of brain or brain extract, divided by the amount of [^{3}H]-uridine incorporated into RNA by the control gland (dpm/gland)[7]. The presence of several other tissues dissected from the larval body, such as the suboesophageal ganglion, fragments of fat body or muscle, did not influence silk gland activity.

Brain extract preparation. All glass, tubes and pipette tips used in the assays were siliconized. Brains were homogenized briefly in cold distilled water, incubated at 65 °C for 5 min and centrifuged at 10,000 × g for 10 min in the cold. The supernatant contained 0.2 brain equivalents (BE)/µl, except if otherwise stated. In stimulation assays with brain extracts, controls were treated with suboesophageal ganglion extracts prepared according to the same procedure as for brain tissue and used in a comparable concentration (tissue equivalent per volume).

Brain factor characterization. The heat stability of brain factor was determined by heating at 100 °C for increasing periods of time. The brain factor sensitivity to proteases was determined by incubation of stock brain extract (0.2 BE/µl) with an equal volume of trypsin (Sigma) 1 mg/ml; 7,500-10,000 units/mg or pronase (Calbiochem) 2 mg/ml; 45,000 PUK/g, in 0.05 M Tris-HCl pH 8.1 at 37 °C for 1 h. The reaction was terminated by raising the temperature to 100 °C for 3 min. The denatured proteins were spun at 10,000 × g for 10 min and the supernatants were used in stimulation assays. The buffer used in this study did not affect the activity of the silk glands.

RNA measurements. Newly-synthesized nucleic acids from in vitro cultured silk glands were extracted with 120 µl of 0.5 N $HClO_4$ for 20 min at 70 °C [6]. Radioactivity of [^{3}H]-uridine incorporated into RNA was measured in 0.1-ml aliquots of these extracts in scintillator (dioxan, 50 g naphthalene, 7 g PPO, 0.05 g POPOP/l) in a liquid scintillation counter (1209 Rackbeta, LKB).

Results and discussion

We investigated the direct effect of explanted brain on the posterior part (the site of fibroin synthesis) of silk glands coincubated in vitro with [5,6^{3}H]-uridine. During short periods of incubation, the incorporation of the label into RNA in the posterior parts of parallel silk glands derived from the same larva was relatively uniform; the differences were never bigger than 20%. On the other hand, the discrepancies in RNA synthesis rate between glands derived from different larvae of the same age could

amount to 80%, despite the very careful selection of animals according to their stage of development. During 3 h incubation with isotope the incorporation into control posterior silk glands (PSG) ranged from 1650 to 2750 dpm/gland. The average incorporation for 20 independent experiments was 2112 ± 572 (dpm/gland ± SEM). Because of this variation, in each experiment treated and control glands were taken from the same animal. Activation of PSG by brain explant or crude brain extract was expressed as an activation ratio (Ar). Routinely we used last (VII) instar larvae of *G. mellonella*. The brains were explanted from day 4 (VII/4) larvae, because it had been observed previously that VII/4 brain implants can stimulate the growth of silk glands in isolated abdomens in situ [3]. The silk glands were derived from day 3 larval abdomens, depleted of endogenous hormones [8]. At this stage of development, at the 'accumulation phase', the synthesis of both nucleic acids and proteins reaches a high level in silk glands of *G. mellonella* [2]. The [^{3}H]-uridine incorporation into the RNA of brain-stimulated PSG, as compared with incorporation into control PSG coincubated with suboesophageal ganglion, was linear up to 6 h (fig. 1).

Double factor analysis of variance showed that the interactions between time and brain stimulation were at the significance level $\alpha = 0.005$. An independent one-way analysis of variance for 2-, 4- and 6-h incubations and two levels of stimulation was done. At each defined time of incubation the differences in mean values between controls and PSG in the presence of brain were at a significance level not higher than $\alpha = 0.002$. Analysis for control values showed no differences in mean values for 2, 4 and 6 h with probability $\alpha = 0.85$. Analysis for PSG + brain showed differences in mean values of groups for 2-, 4- and 6-h incubation at the significance level $\alpha = 0.004$. A model for linear regression is proposed; activation ratio (Ar) – time. The variance analysis allows us to present this model at the significance level $\alpha = 0.002$. The activation ratio (Ar ± SEM) after 2 h coincubation of PSG with two brains was 1.4 ± 0.1, and after 6 h it was 2.5 ± 0.28 of the control. These results showed that VII/4 brain tissue influences the activity of VII/3 PSG in vitro.

To confirm the specificity of this effect of brains on PSG we used crude brain extract in incubation assays in vitro. Figure 2 shows the dose-dependence of PSG stimulation by crude brain extract. Double factor analysis of variance demonstrated interaction: a brain equivalent-activation ratio at a significance level lower than $\alpha = 0.0001$. One-way analysis of variance was done for different BE values and two stimulation levels. Analysis of variance for determined BE values showed that for all BE values greater than 0.005, the differences in mean values between controls and activated PSGs were at a significance level not higher than $\alpha = 0.02$. One-way analysis of variance for controls demonstrated no differences in mean values (probability $\alpha = 1.0$). One-way analysis of variance for stimulated PSG showed differences in mean values for each BE value at a significance level lower than $\alpha = 0.0001$. A non-linear regression model, Ar – BE, was proposed.

Based on analysis of variance this model can be presented at a significance level lower than $\alpha = 0.005$. For a 3-h incubation, the upper limit of the activation ratio was found to be about 2.0 in the presence of 0.1 brain equivalent (BE). With 0.03 BE in the medium, the activation ratio was 50% of the maximal value, and with 0.005 BE the effect was not measurable.

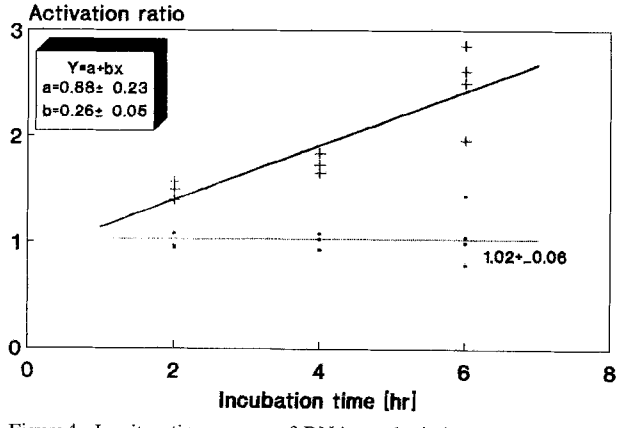

Figure 1. In vitro time-course of RNA synthesis in day 3 PSG in the presence of brain tissues dissected on day 4 of last instar larvae of *G. mellonella*. Glands were incubated in 100 µl Grace's medium either in the presence of 2 suboesophageal ganglions (■) or with isolated brains; 2 brains per gland (+). Control mean level (···). The linear model – (—). The incubation medium was changed every 2 h. The last portion contained 2.0 µCi [^{3}H]-uridine. The linear model $Y = a + bx$, where $a = 0.88 \pm 0.23$ and $b = 0.26 \pm 0.05$, was fitted to the experimental data.

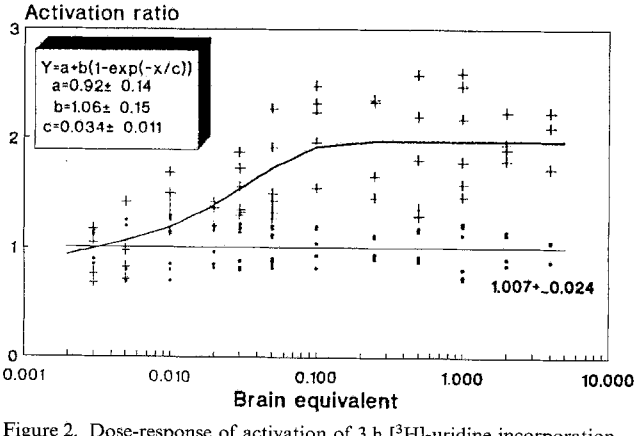

Figure 2. Dose-response of activation of 3 h [^{3}H]-uridine incorporation into RNA in day 3 PSG by crude extract of day 4 larval brain. VII/4 brains were homogenized shortly in cold distilled water, incubated at 65 °C for 5 min and centrifuged at 10,000 × g for 10 min in the cold. The supernatant (crude brain extract), containing 0.2 BE/µl, was used in activation assays (+). Control PSGs were incubated with an appropriate amount of crude suboesophageal ganglion extract (■) per silk gland. Control mean level – (·····); linear model – (—).
The non-linear model $Y = a + b(1 - \exp(-x/c))$, where $a = 0.92 \pm 0.14$, $b = 1.06 \pm 0.15$ and $c = 0.034 \pm 0.011$.

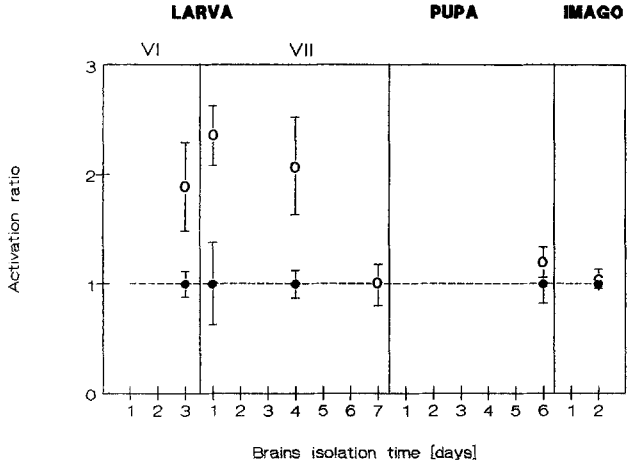

Figure 3. Stimulation of RNA synthesis in PSG day 3 last instar larvae by crude brain extracts. Brains were isolated in different developmental stages: penultimate, last larval, pupa and imago, homogenized in cold distilled water, incubated at 65 °C for 5 min and centrifuged in the cold. The supernatant (crude brain extract) containing 1 BE/µl, was used in activation assays. The incubation of PSG was carried out for 3 h in presence of 0.2 BE/PSG (o) or 0.2 suboesophageal ganglion equivalent extract in control assays (•). Each point represents the mean (±SEM) of 4–5 assays.

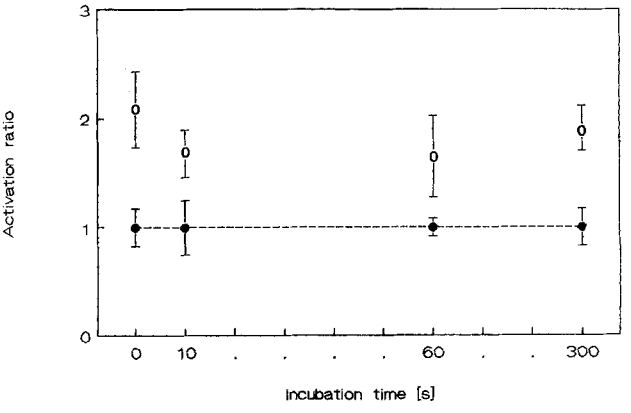

Figure 4. Thermostability of crude brain extract at 100 °C at a concentration of 0.2 BE/µl. Small volumes (25 µl) of crude brain extract were incubated at 100 °C for 10 s, 1 min or 5 min, cooled down and spun in the cold. The supernatant was used in PSG activation assays in vitro (o). Control PSGs were incubated with appropriate amounts of crude suboesophageal ganglion extract (•). Each point represents the mean (±SEM) of Ar values for 4–5 assays.

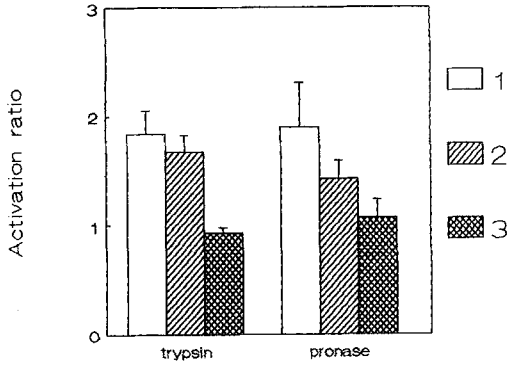

Figure 5. Sensitivity of brain extract to proteolytic hydrolysis by trypsin (A) and pronase (B). Brain extract (1.0 BE) was incubated at 37 °C for 1 h (1); preincubated at 37 °C for 1 h with denatured enzymes (2). The volume of 20 µl of crude brain extract (4 BE) was incubated either with 150–200 units of trypsin or 1800 PUK of pronase in the volume of 40 µl of 0.05 M Tris-HCl pH 8.1. Controls contained appropriate volumes of 0.05 M Tris-HCl pH 8.1 added to the incubation medium.

The factor from the brain tissue which directly stimulated RNA synthesis in PSG was also found in penultimate (VI) larval instar (fig. 3). The 0.2 (VI/3) brain equivalent activates PSG in vitro in a manner similar to VII/1 and VII/4 brain extract. However, 0.2 BE and even 2.0 BE of extract from larvae at very late last instar (VII/6) did not stimulate PSG in vitro, although the glands were sensitive to the factor(s) from the brains of younger individuals.

The effect of brain extracts of pupae and imagos in a dose 0.2 BE/PSG was also not measurable. Ten times more BE was required to obtain activation ratio (Ar ± SEM) of 1.9 ± 0.3 with pupal and of 1.6 ± 0.4 with imaginal brain extracts. The stimulatory activity of brain on PSG therefore depends on the stage of development and is correlated with the synthetic ability of this gland. The response of silk gland tissues to stimulators from brain is evident in vitro during 18–84 h of the last instar, i.e. during the preparatory and active phases of silk protein synthesis. Subsequently, during the regression phase, when silk protein synthesis ceases, no response even to high BE could be detected (data not shown).

We have compared the electrophoretic patterns in polyacrylamide gels of RNA synthesized in vitro in the posterior part of active silk glands stimulated by VII/4 larval brain factor with the pattern in controls. The stimulatory effect involves ribosomal 28 S and 18 S RNA, but mainly species higher than these two, which may be pre-ribosomal RNA, located in the area > 40 S.

The chemical nature of brain factor from VII/4 larvae was investigated by testing its sensitivity to temperature and proteases. After 5-min incubation of the crude aqueous extract of the brain at 100 °C, its activity towards silk glands remained practically unaffected (fig. 4).

This result shows that the factor has a molecular structure which allows it to retain its biological activity at high temperatures. One cycle of freezing at −20 °C and thawing resulted in partial loss of activity; three cycles resulted in complete inactivation (data not shown). Treatment of crude aqueous brain extract with pronase or trypsin resulted in considerably decreased activity of the brain factor in our in vitro test system (fig. 5).

These results indicate that the brain-tissue factor of *G. mellonella* which stimulates RNA synthesis in silk producing glands may be a peptide.

The insect neuropeptides described hitherto were shown to stimulate juvenile hormone synthesis, as in *Locusta migratoria*[7,10,11] or *Manduca sexta*[7,12]. Brains implanted into freshly ecdysed last instar larvae of *G. mellonella* also stimulate juvenile hormone production[13,14]. In *Bombyx mori* prothoracicotropic hormone (PTTH) stimulates in vitro RNA synthesis in the prothoracic gland, which synthesizes and releases ecdysone[15].

The action of the brain factor is independent of that of the hormones released by the endocrine glands. Ecdysterone present in the incubation medium at a concentration of 10^{-6} M does not affect the activation of PSG by the brain factor, nor does 10^{-6} M juvenile hormone II. It is noteworthy that in our system 10^{-6} M juvenile hormone II inhibits the incorporation of [^{3}H]-uridine into RNA of PSG by at least 30% during 3 h incubation in vitro, and this inhibition was abolished by the brain factor. It seems that at least a part of the observed effect of brain implantation reported earlier[3] should be ascribed to a direct, hormone-independent action of brain tissue factor(s). More work is needed on the isolation and structure of brain factor, which may show some similarities to other insect neuropeptides[16–18].

Acknowledgments. We are grateful to Piotr Pawlowski for his help in statistical evaluation of some of the experimental data.

1 Keeley, L. L., and Hayes, T. K., Insect Biochem. *17* (1987) 639.
2 Sehnal, F., Janda, V., and Nemec, V., J. Insect Physiol. *29* (1983) 237.
3 Sehnal, F., and Michalik, J., J. Insect Physiol. *30* (1984) 119.
4 Sehnal, F., Baronio, P., Michalik, J., and Kludkiewicz, B., Sericologia *27* (1987) 643.
5 Sehnal, F., Z. wiss. Zool. *174* (1966) 53.
6 Linzen, B., and Wyatt, G. R., Biochim. biophys. Acta *87* (1964) 188.
7 Granger, N. A., Mitchell, L. J., Janzen, W. P., and Bollenbacher, W. E., Molec. cell. Endocr. *37* (1984) 349.
8 Rohner, R. M., and Meyer, D. R., Experientia *36* (1980) 1327.
9 Rembold, H., Schlagintweit, B., and Ulrich, G. M., J. Insect Physiol. *32* (1986) 91.
10 Ferenz, H-J., and Diehl, I., Z. Naturforsch. *38c* (1983) 856.
11 Gadot, M., and Applebaum, S. W., Archs Biochem. Biophys. *2* (1985) 117.
12 Kataoka, H., Toschi, A., Li, J. P., Carney, R. L., Schooley, D. A., and Kramer, S. J., Science *243* (1989) 1481.
13 Rembold, H., and Sehnal, F., Insect Biochem. *17* (1987) 997.
14 Sehnal, F., and Rembold, H., Experientia *41* (1985) 684.
15 Aizono, Y., Matsuo, N., Yoshida, Y., Funatsu, G., Funatsu, M., and Kobayashi, M., J. Insect Physiol. *32* (1986) 711.
16 Girardi, J., Huet, J.-C., and Pernollet, J.-C., Insect Biochem. *20* (1990) 659.
17 Nachman, R. J., Holman, G. M., Haddon, W. F., and Ling, N., Science *234* (1986) 71.
18 Gazit, Y., Dunkenblum, E., Benichis, M., and Alstein, M., Insect Biochem. *20* (1990) 853.

0014-4754/92/080762-04$1.50 + 0.20/0

Development of melatonin rhythm in the pineal gland and eyes of chick embryo

M. Zeman, E. Gwinner* and E. Somogyiová

*Institute of Animal Biochemistry and Genetics, SASci, CS-900 28 Ivanka pro Dunaji (Czechoslovakia), and *Max-Planck-Institut für Verhaltensphysiologie, D-W-8138 Andechs (Germany)*
Received 1 July 1991; accepted 16 January 1992

Abstract. A melatonin rhythm was observed in the pineals of 18-day-old chick embryos incubated under a light-dark regime of 18 : 6 h. A low pineal melatonin content was found during the light phase of the day. Concentrations started to increase 2 h after dark onset and reached maximum levels after 4 h of darkness. The amplitude of the pineal melatonin rhythm increased considerably after 2 days and night-time concentrations in 20-day-old embryos were more than 5 times higher than in 18-day-old ones. Significant day/night differences in melatonin production were found both in pineals and eyes. Exposure of eggs to 1 h of light during the dark period decreased the high melatonin concentrations in the eyes but not in the pineals of the 20-day-old chick embryo. The results suggest that in this precocial bird at least part of the circadian system may already operate during embryonic life.
Key words. Melatonin; circadian rhythm; embryo; ontogeny.

In the mammalian fetus, circadian rhythms are entrained by the rhythmicity of the mother. Maternal melatonin transmits the information on day-length to the fetus[1,2], and this information can influence subsequent body development, especially in photoperiodic species[3,4]. In birds, a direct neuroendocrine signal between mother and developing embryo is naturally excluded. However, through its incubatory behavior the mother could influence the characteristics of endogenously developing rhythms in embryos. As melatonin may be directly involved in the circadian organization of birds[5–7], its possible rhythmic production during embryonic life could be important for the development and entrainment of other behavioral and physiological rhythms.

Melatonin has been found in the pineals of chick embryos[8–10]. Moreover, a circadian rhythm of N-acetyltransferase (NAT), which is responsible for rhythmic melatonin production, was observed in 18-day-old chick embryos[9]. Therefore the aim of the present study was to find out whether pineal melatonin exerts a daily rhythm in chick embryos incubated under a light-dark cycle, and whether melatonin synthesis in the embryonic pineal gland and eye can respond to lighting conditions.

Materials and methods

Broiler breeder hen eggs were incubated in a forced draught incubator (Bios, CSFR) at a temperature of 37.8 + 0.2 °C and a relative humidity of 55–65% under