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Comp. Biochem. Physiol. Vol. 103C, No. 1, pp. 91–95, 1992
Printed in Great Britain

0306-4492/92 $5.00 + 0.00

MODULATORY EFFECT OF LEARNING AND MEMORY ON HONEY BEE BRAIN ACETYLCHOLINESTERASE ACTIVITY

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(*Received* 14 *February* 1992; *accepted for publication* 18 *March* 1992)

Abstract—In the honey bee, the proboscis extension reflex elicited by an antennal sucrose stimulation was conditioned to a scent using a Pavlovian procedure. Brain acetylcholinesterase (AChE) activity was measured, immediately or one week after the learning session, in good and bad learners (respectively GL and BL) and in controls. There was a specific decrease of AChE activity in GL immediately after learning. A global decrease of enzyme activity was observed one week after learning in GL and BL. These results are discussed in relation to learning and memory processes and in terms of possible cholinergic regulation.

INTRODUCTION

Nearly all known neurotransmitters have been investigated from an anatomical point of view in the honey bee brain. Using immunological or histochemical methods, biogenic amines [dopamine (Mercer, 1987), serotonin (Bicker *et al.*, 1987; Mercer, 1987), octopamine (Mercer, 1987), noradrenaline (Mercer, 1987)], amino-acids [glutamate (Bicker *et al.*, 1988), GABA (Bicker *et al.*, 1987), taurine (Schafer *et al.*, 1988)] or peptides [prolactin (Schmid *et al.*, 1989), gastrin (Noble and Goodman, 1987), cholecystokinin (Noble and Goodman, 1987) or FRMF-amide (Schurmann and Erber, 1990)] were localized in the different parts of the brain. Some of these chemicals are involved in behavior or in the electrophysiological properties of the bee brain as was shown essentially by delivering drugs directly into the head haemolymph (Mercer and Menzel, 1982; Mercer, 1987; Macmillan and Mercer, 1987; Michelsen, 1988) or by using iontophoretic applications (Mercer and Erber, 1983).

It is well established that acetylcholine (ACh) is present in high concentrations in the insect brain (Breer, 1987). Some recent works show that acetylcholinesterase (AChE, E.C. 3.1.1.7) or acetylcholine receptors are largely distributed in the bee brain (Belzunces *et al.*, 1988; Kreissl and Bicker, 1989; Huang and Knowles, 1990; Scheidler *et al.*, 1990; Abdallah *et al.*, 1991) but nothing is known about the role of the neurotransmitter in brain functioning. The following experiment was undertaken in order to investigate the role of the cholinegeric system, and specially AChE, in learning and/or memory processes in the honey bee.

MATERIAL AND METHOD

Behavioral experiment

Worker bees (*Apis mellifera*) were used. They were caught at the hive entrance, fixed in small tubes, and food deprived for eight hours to enhance their motivational state. The learning procedure was olfactory conditioning of the proboscis extension reflex (Menzel, 1990). The reflex was elicited by a drop of sugar water (unconditioned stimulus, US, 0.3 M) delivered to one antenna. The conditioned stimulus (CS) was a scent delivered to the same antenna through a syringe containing a small piece of paper soaked with 10 μl of vanillin extract. The number of bees which did not respond to sugar water was low (4%) as was low the number of bees displaying proboscis extension to the scent before conditioning (4.3%). These animals were discarded.

Bees were divided into two groups. In the conditioned group, there was a paired presentation of CS and US stimulations. CS was delivered to one antenna during six seconds. Three seconds after CS onset, US (antennal sucrose stimulation) was ipsilateraly applied during three seconds. The reflex response was rewarded by sucrose solution presented to the proboscis during three seconds. As soon as the honey bee reacted by a conditioned response to the odor alone, it was reinforced by applying sucrose to the mouthparts during three seconds. The learning session was comprised of five trials with a five minute intertrial interval. At the end of this session, the conditioned group was divided into good learners (GL) or bad learners (BL) on the following criterion when the honey bee performed successfully at least the two last trials of the session, it was classified as GL; a bad learner honey bee did not reach this criterion. (In the GL group, one-third of honey bees were conditioned after one trial, 37.4% emitted a conditioned response after two trials and 27% performed successfully the last two trials). For the control group, olfactory and gustatory stimulations were unpaired. Each animal of this group received three olfactory stimulations (six seconds each) at ten minutes intervals and three interpolated gustatory stimulations of three seconds each at ten minutes interval. This latter stimulation was followed by a reward of sucrose to the mouthparts during three seconds. Following the end of the learning experiment, in order to dissociate the effects of learning from those of memory on brain AChE activity, bees were either immediately sacrificed (groups T_0; GL: $n = 102$; BL: $n = 107$; C: $n = 101$). or kept in small cages for a week with food and water *ad libitum* and sacrificed thereafter (groups T_1; GL: $n = 102$, BL: $n = 90$; C: $n = 88$). Then the whole brains were removed, placed on

carbonic ice, and stored to −80°C until neurochemical experiments were conducted.

Neurochemical experiments

Lubrol PX, antipain, aprotinin, leupeptin, pepstatin A, benzamidin, soybean trypsin inhibitor and acetylcholine iodide were purchased from SIGMA (St Louis, MO, U.S.A.).

Solubilization of AChE

Bee brain AChE was solubilized by a quantitative extraction procedure (Belzunces *et al.*, 1988) with a 98% extraction rate. A known mass of bee brains (corresponding to 15–25 brains) was homogenized at 0°C using a glass Potter homogenizer in high-salt (HS) Lubrol buffer (1% (w/v) Lubrol PX, 1 M NaCl, 10 mM Tris–HCl pH 7.3) to make a 10% (w/v) extract. The extraction medium contained a mixture of 2 mg of antipain/ml, 1 mM benzamidin, 2 mg of leupeptin/ml, 25 units of aprotinin/ml, 10 mg of soybean trypsin inhibitor/ml and 2 mg of pepstatin A/ml. After homogenization, two half extraction volumes of HS-Lubrol buffer were used to wash the homogenizer and pooled to the brain exact. The homogenate was centrifuged at 100,000 g_{av}, for 1 hr at 4°C and the supernatant was used for volume, AChE and protein determinations.

AChE assay

AChE was assayed in 1 ml medium containing 1.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.3 mM acetylthiocholine iodide and 80 mM Tris–HCl pH 7.0 (Ellman *et al.*, 1961). The reaction rate was monitored by the increase in absorbance at 412 nm as a function of time.

Protein assay

Protein concentrations were determined by the method of Markwell *et al.* with bovine serum albumin as the standard (Markwell *et al.*, 1978).

For all determinations, each of the six groups was divided into four extracts, corresponding to 15–25 brains. Each extract was measured three times and 12 determinations were made for each group. One exception was made for the group BL at time T_1, with only nine measurements being taken.

RESULTS

The AChE activity is expressed in relation to the weight of the brain tissue (tissue activity; Fig. 1) or protein content (specific activity; Fig. 2). Each column represents the mean value of 12 determinations (except for BL T_1).

Tissue activity

A two-factor trend analysis shows a time-treatment interaction on the AChE activity of the bee brains ($F(2, 63) = 3.623$; $p < 0.03$). (Treatment refers to the three groups: conditioned bees (GL), unconditioned bees (BL), and controls (C)].

Conditioning has a strong effect upon brain AChE activity ($F(1, 2) = 26.187$; $p < 0.0001$), which appears globally as a reduction of the enzyme activity. The reduction is particularly important in GL groups at T_0 ($t(22) = -3.08$; $p < 0.005$) and T_1 ($t(22) = -7.557$; $p < 0.0001$), when compared with respective control groups.

For BL, there is a significant reduction of brain AChE activity only at T_1 [$t(19) = -3.355$; $p < 0.003$). It is noteworthy that time has no effect upon brain AChE activity of control bees ($t(22) = 1.103$; n.s.). (Fig. 3).

Specific activity

Results obtained for AChE expressed as specific activity are quite similar to those of AChE activity per gram of tissue. A two-factor trend analysis shows a stronger time-treatment interaction ($F(2, 63) = 5.588$; $p < 0.005$). Conversely, time effect is less pronounced ($F(1, 2) = 5.994$; $p < 0.01$ in spite of $F(1, 2) = 35.168$; $p < 0.0001$), probably due to the fact that, when expressed as specific activity, brain AChE modification with time in control animals is opposite to those observed for GL and BL groups (Fig. 4). However, control groups at T_0 and T_1 are not statistically different ($t(22) = -0.995$; n.s.)

As for the results of tissue activity, the global effect of conditioning is a reduction of brain AChE activity, which is especially pronounced in GL compared with

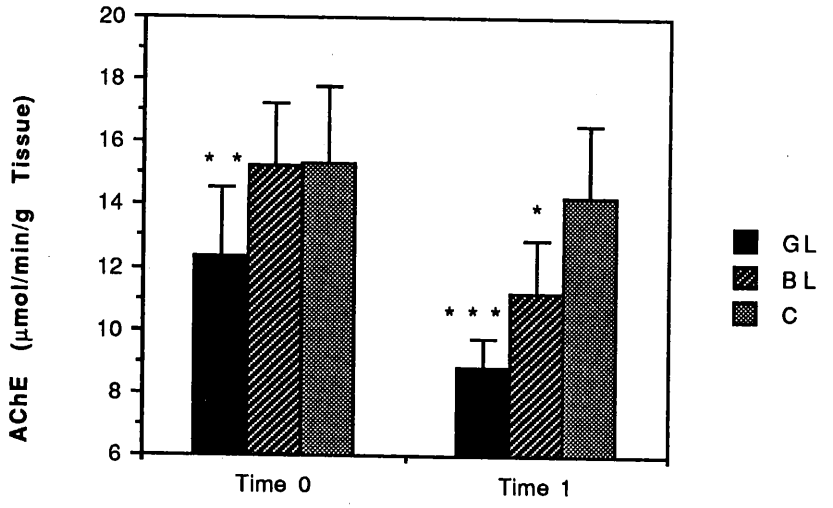

Fig. 1. Brain tissue AChE activity measured immediately (Time 0) or one week (Time 1) after learning in successfully (GL) or unsuccessfully (BL) conditioned bees and in controls (C). *$p < 0.003$; **$p < 0.005$; ***$p < 0.0001$.

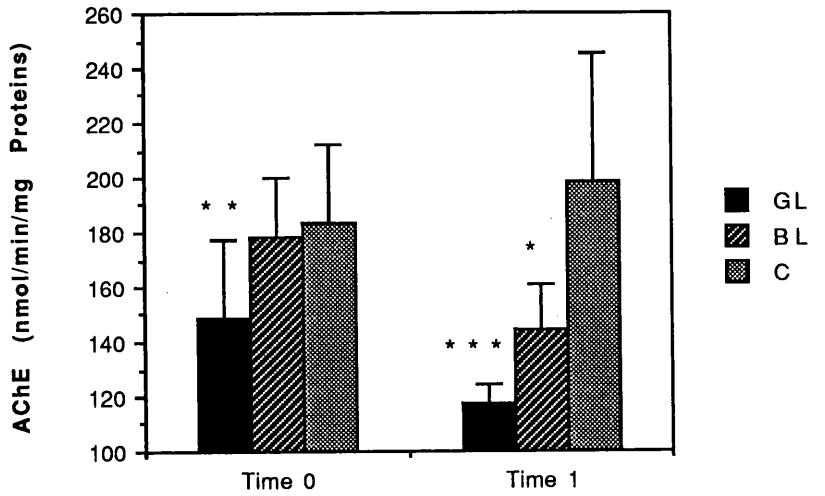

Fig. 2. Specific AChE activity measured immediately (Time 0) or one week (Time 1) after learning in successfully (GL) or unsuccessfully (BL) conditioned bees and in controls (C). $*p < 0.003$; $**p < 0.0006$, $***p < 0.0001$.

controls, at T_0 ($t(22) = -2.990$; $p < 0.006$) and T_1 ($t(22) = -5.959$; $p < 0.0001$), and in BL at T_1 ($t(19) = -3.293$; $p < 0.003$).

DISCUSSION

General commentary

These experiments show some specific modifications of cholinergic system activity in relation to learning and memory in the honey bee. Until now, the role of the cholinergic system in these functions was not investigated in insects (Menzel, 1990). Some previous works concerned biogenic amines (Mercer and Menzel, 1982; Mercer and Erber, 1983; Macmillan and Mercer, 1987; Michelsen, 1988) using direct or iontophoretic applications to the honey bee brain and looking for induced behavioral or electrophysiological modifications. The present experiment shows, for the first time in the honey bee, that neurochemical modifications are related to brain functioning.

Previous data in vertebrates showed an enhancement of AChE activity in rats submitted to an enriched environment during development (Rosenweig *et al.*, 1962) or a decrease of choline-acetyl-transferase (CAT) activity following a partial learning session in mice (Jaffard *et al.*, 1977). It was also shown that AChE inhibitors induce a time-dependent improvement of retention in rats (Deutsch, 1973). It is noteworthy that, in spite of a quite different nervous system organization, the cholinergic system seems also to be involved in learning and memory processes in the honey bee.

The main result from this experiment is a decay of AChE activity in relation to the learning experiment. There is, first, a rapid decrease of enzyme activity specific to GL compared with controls, followed one week later by a general decrease in the two experimental groups, being more pronounced in GL. The

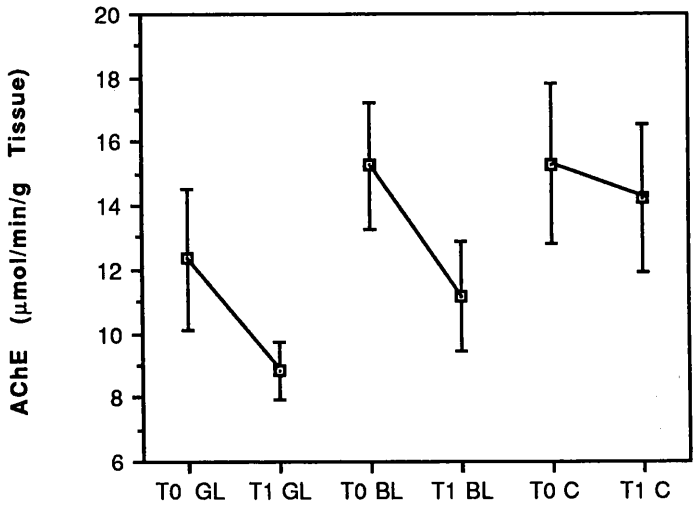

Fig. 3. Representation of the time-treatment interaction effect on brain tissue AChE activity. (GL, BL, C, T_0, T_1 as in Figs 1 and 2.)

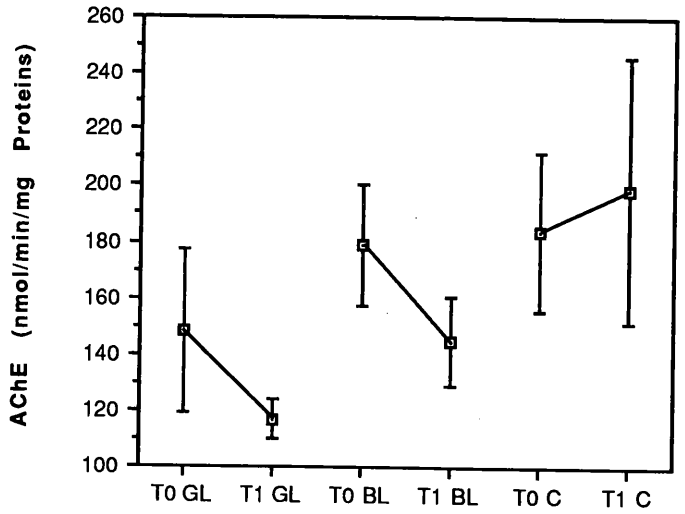

Fig. 4. Representation of the time-treatment interaction effect on specific enzyme activity. (GL, BL, C, T_0, T_1 as in Figs 1 and 2).

immediate reduction of enzyme activity in GL seems to be specifically related to the creation of a conditioned link between two events. It is not observed in BL which were submitted to the same experimental conditions including, particularly, the temporal contiguity of CS and US.

As the late decrease of AChE activity is observed in bad as well as in good learners, one might think that this modification is not related to the learning experiment. However, the decrease is not observed in control animals, which were subjected to the same restraint and food-deprivation conditions, and thus presumably in the same states of arousal and motivation. Therefore, this decay may be related to a late-acting memory process, perhaps a consolidation phase of previous information. For practical reasons, it was not possible to submit honey bees to a retrieval test before sacrificing them. Therefore, we cannot assume that, one week after learning, GL would have had a high retention level. However, on the basis of previous results (Menzel, 1990), we can hypothesize that the five-learning-trial session we used is remembered for the honey bees' entire life.

Bad learners apparently did not learn in the experimental situation. The criterion we chose (a conditioned motor response), only gives access to a performance level which might not be a good indicator of a learning process. We cannot, however, rule out the hypothesis that BL experienced latent learning, which was consolidated with time, leading to the observed decrease of brain AChE activity one week after training.

Possible mechanism of action at the cellular level

As we show a modulatory effect of nervous activity on AChE activity, it seems more logical to think that ACh acts on muscarinic receptors rather than on nicotinic ones. Effectively, the action of the muscarinic receptors is mediated by a G protein (Cohen-Armon *et al.*, 1988) while the nicotinic receptors act directly on ionic chanels. This hypothesis is likely as the pharmacological existence of muscarinic receptors is described in the honey bee (Huang and Knowles, 1990; Abdallah *et al.*, 1991) even though there anatomical location is not yet known.

On one hand, we know that *Apis mellifera* AChE is anchored in the membrane by a glycosyl-phosphatidyl-inositol (GPI) anchor, sensitive to the action of a phosphatidyl-inositol-specific phospholipase C (PI-PLC) which converts the membrane enzyme into a hydrophilic form (Belzunces *et al.*, 1990). One hypothesis is that this type of anchor would represent a way to regulate AChE activity by an endogenous PI-PLC (Lisanti *et al.*, 1990). This PI-PLC might be activated by G-proteins like phosphodiesterases involved in inositol phosphate metabolism (Birnbaumer *et al.*, 1990). On the other hand, AChE activity can be modulated by different chemicals, such as pyrethroids (Reddy *et al.*, 1991) which are able to bind to a G protein (Rossignol, 1991).

Taking into account these different data, it could be possible that the decay of AChE activity during and after learning could be linked to the action of ACh on muscarinic receptors. The activation of these receptors would activate a G protein, which would in turn activate a PI-PLC, converting anchored AChE into its hydrophylic form. The hypothesis of the modulation of AChE activity during learning is under investigation at this time.

Acknowledgements—This work was supported by the Région Midi-Pyrénées (Grant 9007795) and by the MRT (Grant 900134).

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