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Amino Acid Levels During Learning and Memory Consolidation of an Aversive Conditioning Task in Crickets

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JAFFE, K., S. BLAU AND N. A. ZABALA. *Amino acid levels during learning and memory consolidation of an aversive conditioning task in crickets.* PHARMACOL BIOCHEM BEHAV **43**(1) 205–214, 1992.—We followed the titer of free amino acids in nervous ganglia and hemolimph of the cricket *Pteronemobius sp.* at different times during and after a shock avoidance training that included one experimental group and three controls. The results showed that Tau, urea, Thr, His, GABA, and an unidentified compound (Q) increased their titer in ganglia and hemolimph during training, whereas Ala, Arg, Val, Glu, Ser, and one or all of the group formed by Cys, Phe, Ile, Leu, and Trp decreased theirs concomitantly to memory consolidation. The difference in the rate of experimental insects and their yoked slaves to consolidate the learned task was reflected in the changes of the titers of the amino acids mentioned above. The data add to the evidence for a direct involvement of these amino acids in modulating the memory consolidation process.

Amino acids Insects Memory consolidation Long-term memory Memory Crickets
Neuromodulation Aversive conditioning Learning Amino acid changes

AMINO acids are increasingly recognized as important neuromodulators. A few examples may illustrate this point: L-cysteine destroys neurons in vitro or in vivo (27), glutamate modulates NMDA receptors (29) and is involved in long-range glial signaling (7), and L-aspartate, *l*-glutamate, and NMDA activate the receptor channel complex of neurons (31). Long-term potentiation, thought to be related to mnemonic processes, is also affected by NMDA (16). In addition, amino acids have shown to be directly implicated in mnemonic processes: Free arginine increases its titer in the brain of the praying mantis after training (8,21), whereas in the cricket the urea cycle seems to be activated in nervous ganglia during training in an operand conditioned reward task (19). Injections of arginine enhance memory formation in the praying mantis (8), and injections of arginine, alanine, and glutamine enhance, whereas those of proline and ornitine block, memory formation in the cricket (22). Pools of amino acids enhance memory formation if injected into vertebrates (28). Intracranial injections of single nonessential amino acids and that of arginine, phenylalanine, and tryptophan block memory formation in the chick (15). L-Arginine has shown to be a precursor for nitric oxide (NO), which activates NMDA receptors (14), which in turn relates this chain with long-term potentiation phenomena in neurons (13).

Alternative hypotheses were put forward in an attempt to explain the action of amino acids on memory consolidation (8,19,22) and on opiate receptors (34,35,37) in the context of a two- or more stage model of memory formation. Although no strong conclusions can be drawn for the moment, much evidence suggests that some amino acids modulate biochemical reactions leading to memory consolidation (i.e., formation of long-term memory). Amino acids may play a role in triggering the processes from labile to consolidated memory (18,25), possibly by regulating intracellular *l*-arginine concentrations, which in turn modulate NO release. To clarify the role of some free amino acids on memory consolidation, it is important to know if changes in their titer during learning are specific for a particular training or, rather, if these changes are a general feature of learning situations among individuals of the same species. Thus, a novel system was used for training crickets, allowing for the monitoring of the concentration of 18 free amino acids in nervous tissue during and after learning two different aversive conditioning tasks.

METHOD

Training

Six hundred adult, female *Pteronemobius sp.* crickets were reared in the laboratory at 28°C and 70–85% RH following the method described before (20). Animals were trained individually in an aversive conditioning paradigm based upon a paradigm originally devised by Horridge (17) in which insects

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learned to raise their legs to avoid electric shocks. We adapted the system to crickets using a specially devised training apparatus (34). A pair of iron wire electrodes (0.9 mm diameter) were fixed with wax dorsally to the thorax of the cricket. The electrodes with the cricket were placed over a torsion balance that served as an electrical switch. When the animal pressed the balance, a relay system was activated, registering the cricket's leg position and controlling the administration of an electric shock through the electrodes (Fig. 1). Ten of these systems, protected by a wooden box, constituted the apparatus.

To fix insects to the electrodes, they were put to rest by applying anoxia, submitting them to an N_2 atmosphere for 3 min. Two hours after anoxia, they were mounted in the apparatus and trained for 5, 10, or 15 min in one of the following ways.

Experimental crickets (E) were trained to lift their legs to avoid moving the torsion balance. When the cricket pressed the balance, the relay system was activated and it received 10-V AC current until it lifted its metathoracic legs and the balance interrupted the punishment.

Yoked slave crickets (JS) were submitted to the same procedure as E but received the electric shock only when an E insect, connected to the JS shock administration system, received punishment. JS animals, although receiving the same amount of shock as E, could not associate their leg position with the punishment.

Passive control crickets (PC) were submitted to the same manipulation as E or JS but never received electric shocks.

Naive control crickets (NC) were taken from the rearing cage and killed without any previous manipulation.

Animals used for the retention test were trained for 5, 15, and 30 min. As the most significant results were observed with 15 min of training (see the Results section), animals used for biochemical analysis were trained for only 15 min.

For biochemical studies, animals were killed by plunging them into a mixture of dry ice and acetone immediately, 1 h, or 24 h after training, which produced the following groups as determined from the moment training started: 0, 5, 10, 15, 75, and 1,455 min. As the training lasted for a maximum of 15 min, insects in the groups labeled 5, 10, and 15 min were killed immediately after training. Animals were kept between training and killing, or training and the retention test, in a closed glass container at 90% RH and 28°C hanging from their electrodes. Group PC was not trained but animals were placed directly in the humid container for 2 h (PC 0 min), simulating the 2 h of rest experimental crickets received before training, or 26 h (PC 1,455 min) before they were killed. No anoxia was given to animals used for biochemical analysis after training, whereas half the animals used for the retention test were given anoxia immediately after training.

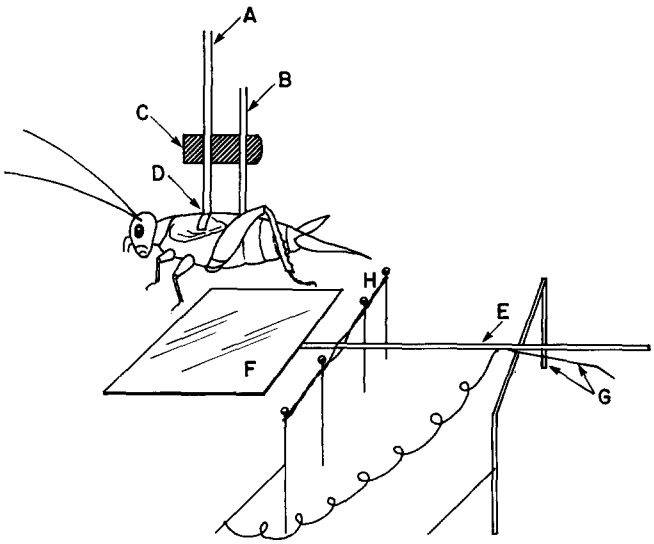

FIG. 1. Training device showing supporting electrodes (A and B), electrode supporter (C), wax fixing electrodes to insect (D), and balance (E) with surface (F) on which crickets rested their legs. The balance was supported by nylon cable whose tension was regulated by pins (H). Signals indicating the position of the balance went through cables (G).

Twenty-eight or more animals from each group (E, JS, PC, NC) were not killed but were tested 24 h after training [retention test (RT)]. RT consisted of placing animals into the training apparatus, each individual with its torsion balance connected to its own shock administration system. Animals were thus trained (PC and NC) or retrained (E and JS) for 10 min and the time they spent pressing the torsion balance and therefore receiving electric shocks was measured at 1-min intervals.

Amino Acid Analysis

The technique described by Jaffe et al. (22) was used. The nervous ganglia [brain (B), prothoracic ganglion (P), mesothoracic ganglion (M), and metathoracic ganglion (T)] were dissected from frozen crickets and each individual ganglion was then homogenized in 1.5 ml 80% ethanol. Hemolimph was collected from a separate batch of live crickets; after training, their cerci were cut and with the help of a capillary tube 50 μl of the hemolimph was collected from the abdomen through the cerci and homogenized, the same as the dissected ganglia, in 2.5 ml 80% ethanol at 4°C during 15 min. The homogenizer (Ika-Werk) was washed with 2.5 ml 80% ethanol and both solutions were pooled and centrifuged at 5,000× *g* during 10 min at 4°C. The supernatant was collected whereas the precipitate was homogenized again for 1 min in 5 ml 80% ethanol and centrifuged as mentioned above. This procedure was repeated three times, and all three supernatants were pooled. The final 15-ml solution was evaporated to dryness at reduced pressure and 60°C. The remaining precipitate was dissolved in 200 μl bidistilled water. A 50-μl sample was taken and dansilated by adding 50 μl 0.5 M sodium bicarbonate at pH 8.5 and 100 μl 6 mg/ml dansil cloride (Sigma Chemical Co., St. Louis, MO) in acetone. The mixture was incubated at 70°C during 20 min. To avoid formation of multiple derivates of His, Tyr, and Lys, 100 μl 10% formic acid was added. The final mixture was centrifuged at 1,000× g for 10 min and a sample of 50 μl was then injected into a high-performance liquid chromatographer (HPLC) (Waters Associates, Milford, MA) consisting of two pumps, a solvent programer, a fluorescence detector (excitation 360 nm, detection 495 nm), and a u-Bondapak C18 column.

Chromatographic separation of the amino acids in the sample was achieved using the following elution program: 5 min at isocratic conditions with 90% solution A (90% acetic acid 0.02 N and 10% acetonitrile) and 10% solution B (10% acetic acid 0.02 N and 90% acetonitrile). Then, the relation solution A:B changed from 90:10 to 30:70 linearly during 60 min. At the end, isocratic conditions at a 30:70 relation were maintained for 10 min.

RESULTS

Behavioral Assay

Figure 2 shows that both E and JS crickets learned with the training system used. E crickets avoided significantly more electric shocks the longer training they received during the retention test 24 h after training, whereas longer training in JS made them receive more shocks during the retention test ($p < 0.01$, Mann-Whitney *U*-test). When anoxia was given immediately after training, E crickets consolidated their memory (whatever they learned) faster compared to JS as evidenced during the retention test 24 h after training, that is, anoxia on JS crickets caused a significant reduction in the amount of shock received if given immediately after 5- or 15-min training but not after 30-min training. Anoxia on E crickets caused an increase in the amount of shock received during the retention test if given immediately after 5 min of training but not if given after 15 or 30 min of training, that is, after 5 min labile memory could not be disrupted any more with N_2 anoxia in E crickets, whereas only after 15 min did the memory of JS crickets become resistant to N_2 anoxia.

Biochemical Analysis

Chromatographic separation allowed the identification of Tau, Gln, His, Ser, Glu, Thr, Ala, Gaba, Arg, Pro, Val, Orn, Lys, Tyr, Urea, Gly, a peak composed by Phe, Leu, Ile, Cys, and Trp, and an unidentified amine called Q (19).

Figures 3-7 show the changes in amino acid levels for each ganglion and for the hemolimph. Data were analyzed statistically using analysis of variance (ANOVA), and for all the figures presented the test showed significant intergroup differences ($p < 0.05$). Due to the small sample size in some of the cases, single intergroup comparisons were performed using the more generally applicable Mann-Whitney *U*-test. The significant differences are indicated in the respective figures and can be summarized as follows.

Mechanical manipulation. Manipulation of the insect affected the titer of some amino acids as can be observed by comparing results for NC with PC (Figs. 3-7). His increased its titer in all ganglia and decreased in hemolimph in PC. For Pro, the changes are inverted: It decreased in all ganglia and increased in hemolimph. After 24 h, these amino acids do not restore their NC level. Lys showed a significant decrease in the prothoracic ganglion and hemolimph while the titer of Gln showed a significant increase in the brain and decreased in hemolimph.

Effect of electric shock. A Pearson correlation between the amount of electric shocks received by each individual during the first 5 min of training for both E and JC crickets (PC 2 h were taken as 0 shock group for both comparisons) and the titer of all amino acids in all ganglia for each animal immediately after learning showed a significant positive correlation between the amount of shocks received (total time the insect received punishment during training) and the titers of Orn ($r = 0.2238$, $p = 0.009$) and a negative correlation with that of Pro ($r = -0.2144$, $p = 0.012$).

Training. The effect of training can be assessed comparing PC with E and JS. Amino acids can be divided into three groups according to the tendency of the titer change observed. Tau, urea, Thr, and peak Q increased their titer during training (Fig. 3), whereas Ala, Arg, Val, and the peak containing

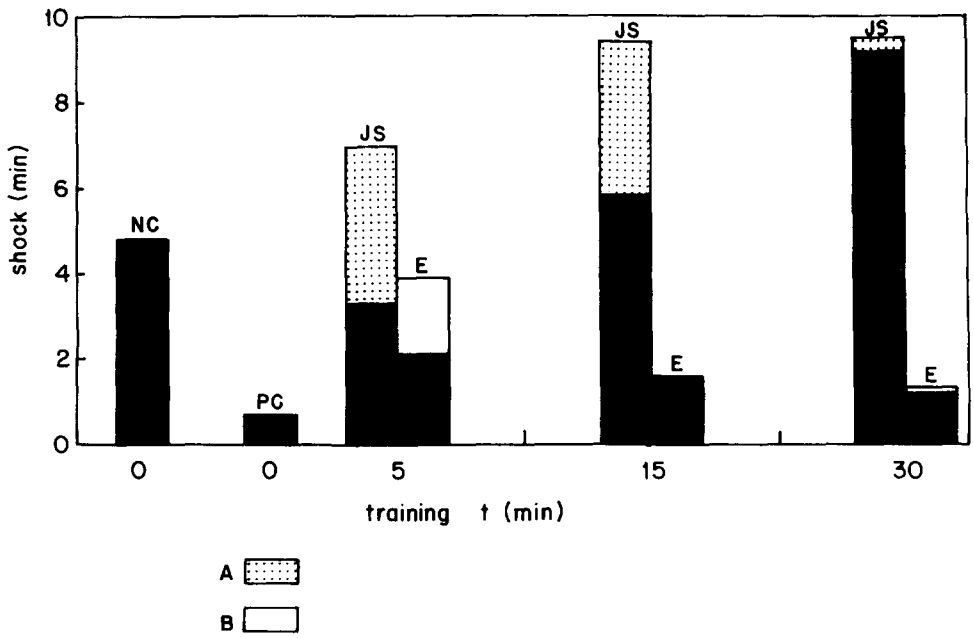

FIG. 2. Amount of time crickets received electric shock (i.e., did not avoid shocks) at the retention test (maximum of 10 min) is given for different groups of insects (NC, PC, JS, and E; the minimum number of replicates per group was 28). A and B indicate the reduction or increase of the amount of time crickets received shocks induced by the anoxia given immediately after training.

Phe, Leu, Ile, Cys, and Trp decreased (Fig. 4). These changes were more or less generalized, affecting at least three different ganglia and sometimes also the hemolimph. His showed a significant increase in its titer in the brains of E and JS insects and in metathorax and mesothorax of JS (Fig. 5). Glu and Ser decreased their titer in the brain, whereas GABA increased it immediately after training (Fig. 6).

The dynamics of the changes for compound Q (Fig. 3) and Tyr (Fig. 7) was oscillatory. The titers increased during the first 5 min of training, decreased during the next 5 min, and increased again during the last 5 min of training.

Some differences between titers in E and JS crickets were related to the time when the maximum change was observed. Tau, urea, peak Q, Thr, and Glu reached the maximum value always earlier or simultaneously in E crickets compared to JS. The time in which the minimum levels of amino acids that decreased their titer during training was reached was similar for E and JS crickets.

Twenty-four hours after training, few changes remained. Thr (Fig. 3) maintained high levels while Arg (Fig. 4) showed lower levels in nervous ganglia of E and JS when compared with PC. The levels in hemolimph were lower for Tau, Val, Tyr, and Lys, while Thr and His increased their titer 24 h after training when compared with PC.

Involvement of the diverse ganglia. The ganglion directly involved in controlling the metathoracic legs is the metathoracic ganglion, but all compartments of the insects nervous tissue showed changes in the titer of free amino acids. The direction of the change in the titers in all compartments during learning (comparing PC at 2 h with JS or E: data on 0 vs. 5, 10, and 15 min in Figs. 3-7) was the same for all amino acids. Surprisingly, even the changes in hemolimph followed that of the ganglia, excluding the possibility that the changes observed were due to fluxes between different compartments. This was not the case for more permanent titer changes due to manipulation of insects. As discussed above, the titer changes in hemolimph were opposite to that in the ganglia except for Lys, suggesting a possible passive or active transport of amino acids from the ganglia to hemolimph or vice versa.

DISCUSSION

Behavior

The results of the behavioral assays in this work may be summarized as follows:

1. Crickets submitted to electric shock learn to raise their legs to avoid the shock—experimental insects—or habituate to the shock—yoked slaves—confirming earlier findings (34), that is, both E and JS crickets learn but this learning, rather than an associative learning, seems to be more properly defined as a habituation to electric shock. Thus, the "controls" for learning experiments—originally designed by Horridge (17) to receive the same stimuli and performing the same activity as the experimental insects but without learning, that is, yoked slaves—showed a failure to avoid electric shocks after training that resembles that described for "learned helplessness" (24). Thus, these types of controls, generally used as controls for instrumental conditioning, have to be revised biochemically and behaviorally as they clearly are no controls.
2. Anoxia, in both types of training, causes the disruption of memory formation, inducing a partial retrograde amnesia. The times at which anoxia is effective as an amnesic agent differ between both types of training, suggesting that E insects consolidate memory faster than JS insects. Thus, biochemical reactions responsible for the passage from short-term memory or labile memory to long-term memory or consolidated memory (18,25) should occur earlier in E than in JS insects. This was confirmed in the present study as discussed below.

Biochemistry

Regarding the pattern of change of the concentration of free amino acids in nervous tissue during and after learning, no clear picture emerges, partly because little is known about the amino acid metabolism in nervous tissue in insects (5,12). Thus, the biochemical results reported here cannot be fully interpreted. However, the following may be concluded from the results.

1. *The titer of free amino acids in nervous tissue is affected by learning and memory consolidation.*
   - The changes in amino acid levels in both experimental and yoked control groups immediately after training relative to untrained controls are similar. Only some of these changes can be explained by manipulation or electric shock. For the latter, for example, only the concentrations of Pro and Orn proved to be directly correlated with the amount of shock received. This is congruent with the behavioral observation that on the retention test untrained passive controls performed better than either the experimental or yoked control group, which had received previous training, suggesting the existence of a strong nonassociative effect of electric shock irrespective of its relationship to the animals' behavior that could be more properly defined as a habituation to the electric shock.
   - The urea cycle is activated by learning, showing an increase in the titer of urea and ornitine at the expense of arginine. This finding is in agreement with earlier reports showing similar results using a different learning paradigm for the cricket (19,22) or praying mantis (8,21).
   - The unidentified basic amino acid (compound Q) that appeared associated with mnemonic processes in crickets during maze learning (19) was the same as that found here, increasing its titer during learning and shortly before memory formation.

In addition, other facts hint to the same direction. For example:

- The role of taurine may be important; its increase during training might be due to synthesis from cysteine (5), which decreases significantly. Taurine is not incorporated into proteins; therefore, its levels do not serve as indicators for protein synthesis activity. Bodnaryk (2,3) showed that taurine has an important role in brain mor-

FIGS. 3-7 (pp. 209-213) Titer (mean and SE) of free amino acids in nervous tissue relative to that in naive controls (NC = 1 in the abscissa). The ordinate indicates the moment the titer was measured, starting from the initiation of training (0 min). Training (for E and JC) occurred between 0 and 15 min. Each point represents the mean of a minimum of five individuals. (■), titer of passive controls (PC); (○), that of experimental crickets (E); (●), that of yoked slaves (JS). $*p < 0.05$, $**p < 0.01$, using Mann-Whitney *U*-test to compare NC vs. PC at 2 h (if indicated at the left of 0); PC at 2 h vs. PC at 24 h (if indicated on the discontinuous line); E vs. JS (if indicated at the exact time intervals); and PC 24 h with JS or E (if indicated at the right of 1,455 min).

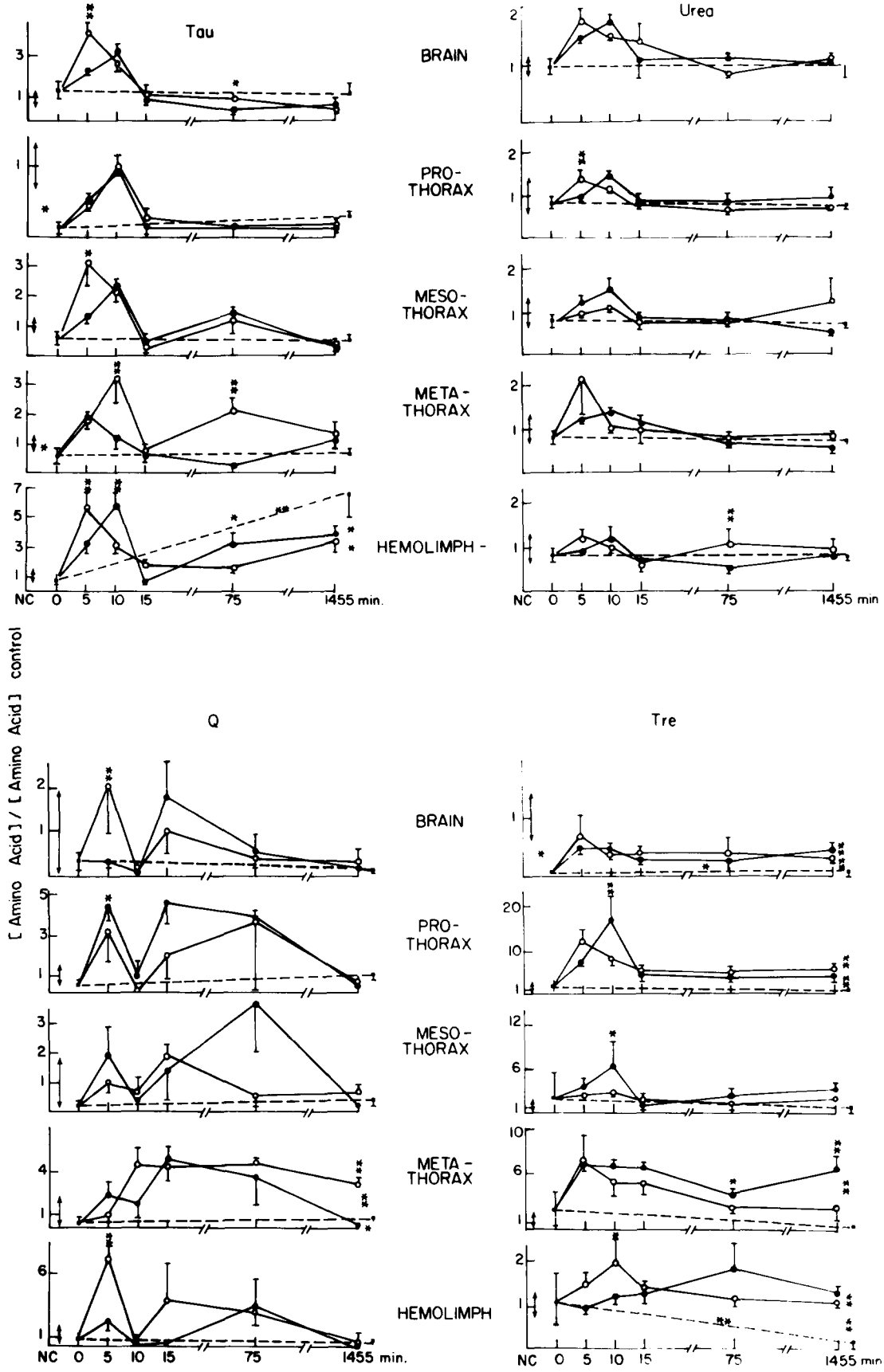

FIG. 3. (See p. 208 for legend.)

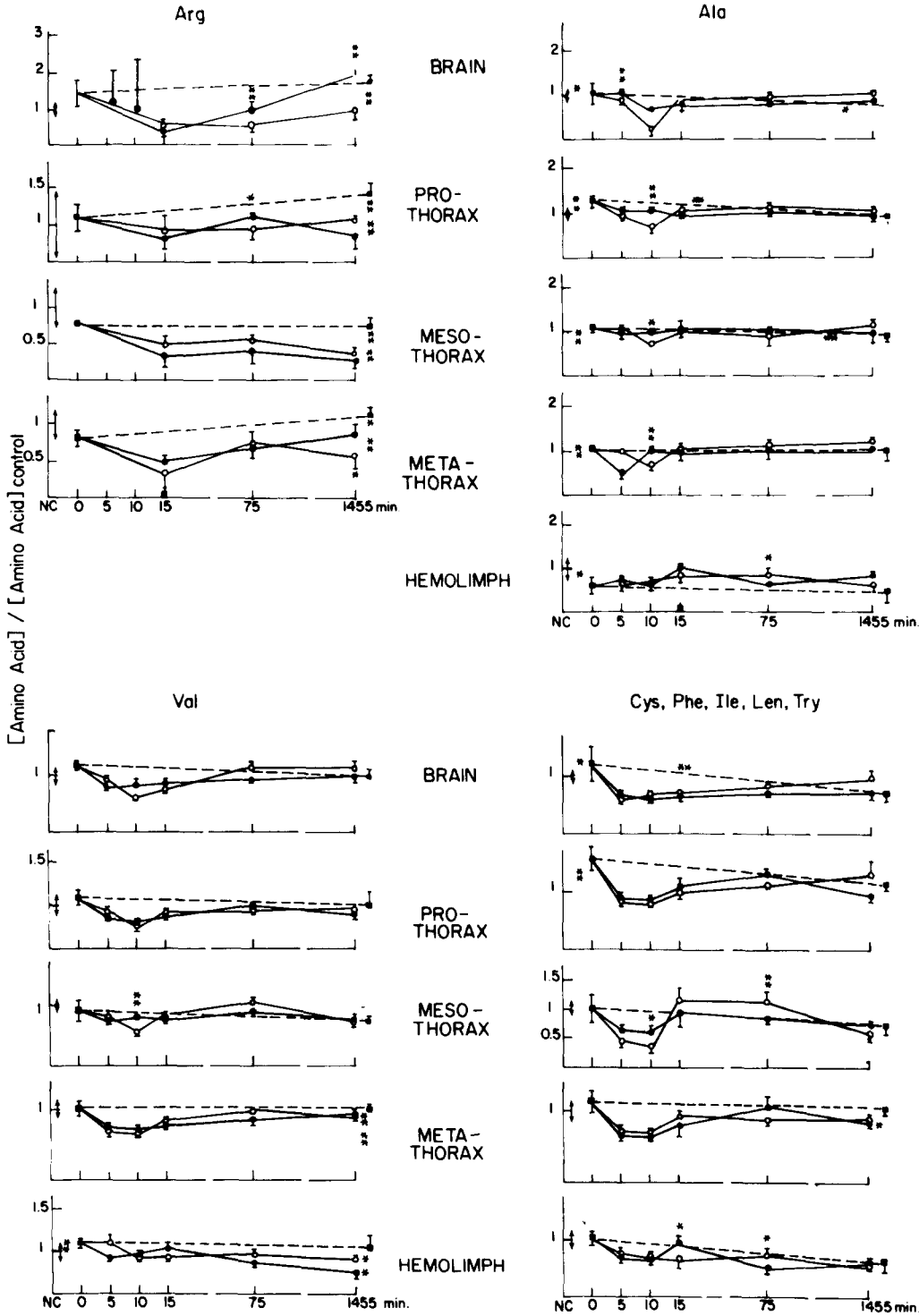

FIG. 4. (See p. 208 for legend.)

phogenesis of the Bertha armyworm *Mamestra configurata*. Thus, our results may indicate that morphological changes in the cricket's nervous tissue are taking place during learning and memory consolidation, in which Taurine could have a role.

• Alanine, which may be produced by insects from pyruvate by transamination (5), is known to produce proline via malate and glutamate (32). Alanine decrease its levels during training and its titer was very negatively correlated in all ganglia to that of proline in our results

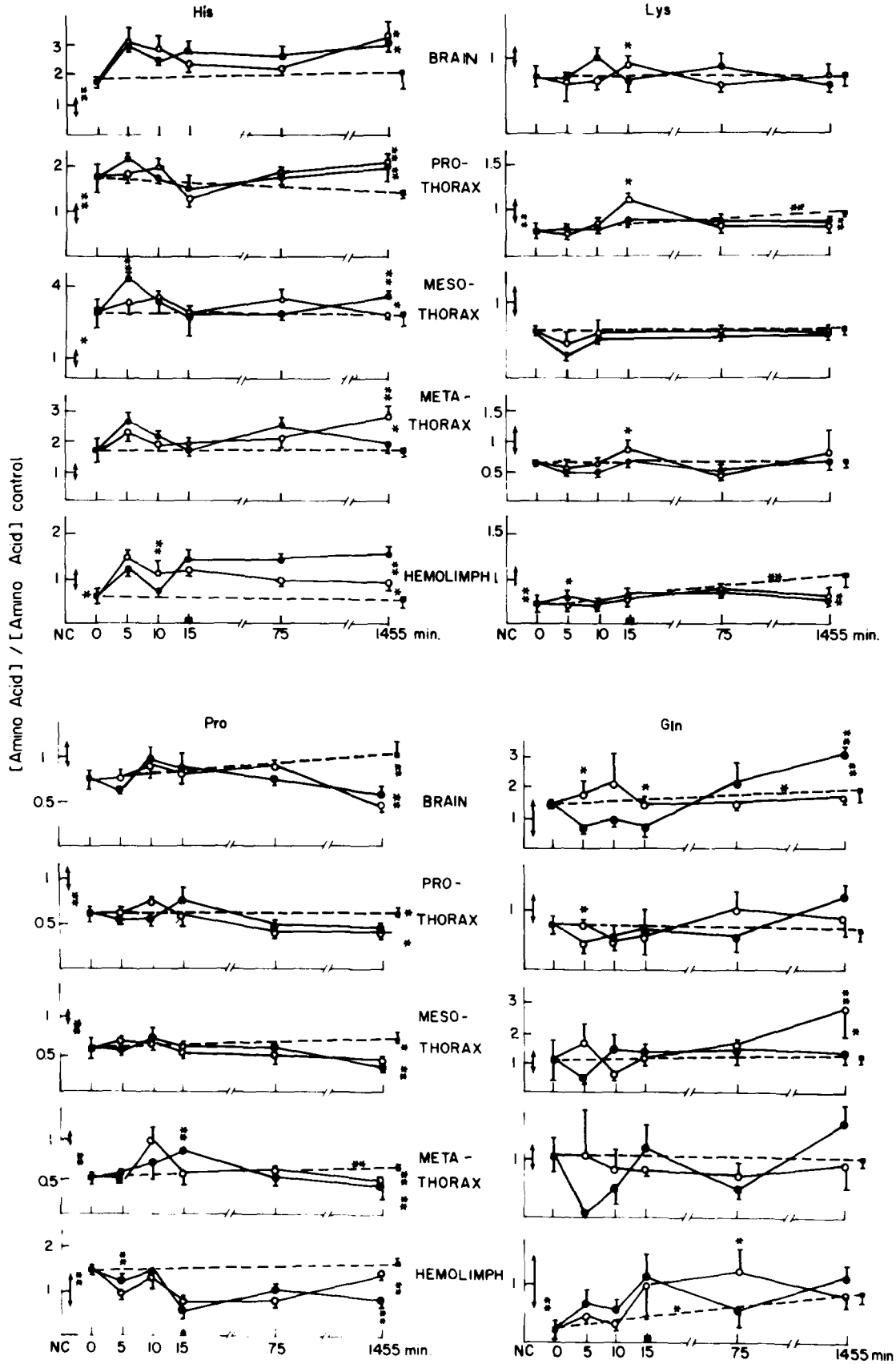

FIG. 5. (See p. 208 for legend.)

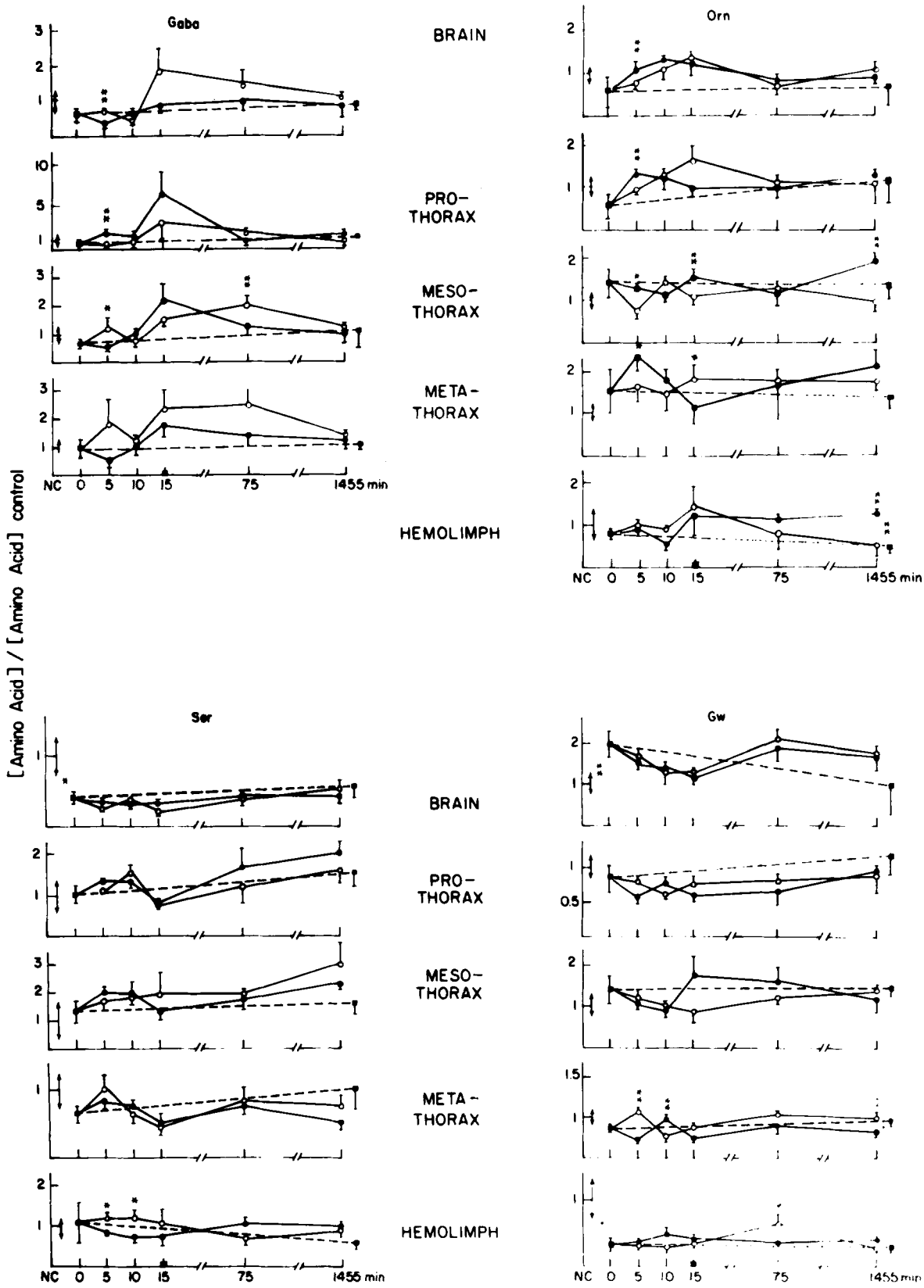

FIG. 6. (See p. 208 for legend.)

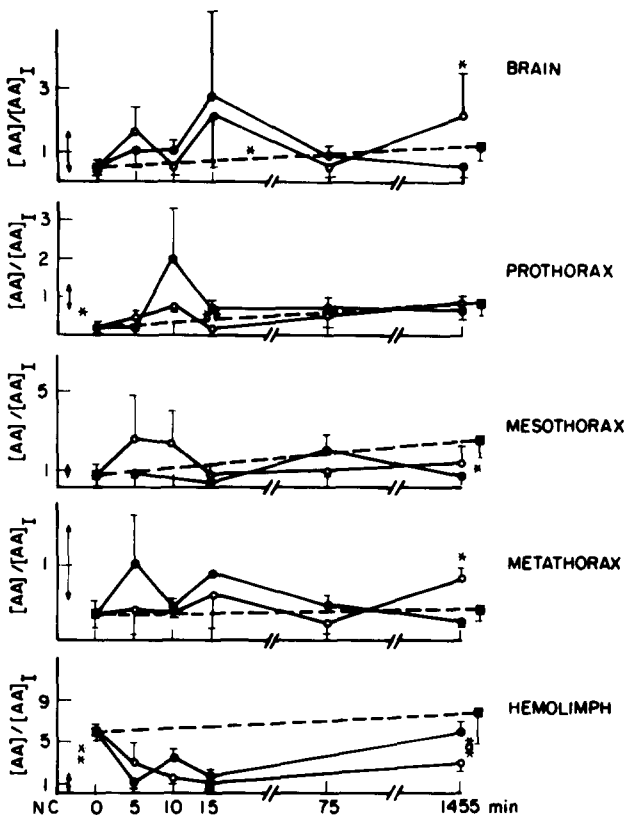

FIG. 7. (See p. 208 for legend.)

(Pearson correlation: $r = -4.0$, $p < 0.001$), but the fact that glutamine also decreased concomitantly to alanine makes us think the decrease of alanine during learning needs an alternative explanation.

- Manipulation of insects induces important biochemical changes. Glutamate and proline show opposite titer changes that differ in the hemolimph and ganglia, suggesting proline is released to the hemolimph whereas glutamate is taken from the hemolimph by the ganglia. The insects' muscles, also activated in PC crickets, are possibly transforming proline into glutamate.

2. *Some amino acids are directly involved in mnemonic processes.*
   - Alanine, arginine, and glutamate decrease their titer during shock avoidance learning. These amino acids also changed their titer during maze learning (19). In addition, injections of these amino acids enhanced memory formation in maze learning (22). By contrast, ornitine and proline—which augmented their titer during shock avoidance learning—inhibit memory formation if injected before maze learning (22). These data suggest a general role for these amino acids in mnemonic processes and that the changes in their titer are not an artifact of specific learning situations. The action of these free amino acids is possibly through the activation of a metabolic pathway, that is, intermediate metabolites are consumed, limiting the speed of the process, and injections of these metabolites may speed up the process. On the contrary, increasing the already high concentrations of accumulated products inhibits the metabolic process. This is in agreement with the NO mechanism: *l*-Arginine is consumed by the NO synthase, which triggers the metabolism to synthesize more *l*-arginine. Also, glutamate reacts to form proline during memory formation, opposite to what happens during energy consumption in the insect's muscle.
   - The different time course for memory consolidation in E and JS is reflected in the time course of titer changes of urea, Orn, Glu, Gln, Ala, and the unidentified compound Q. This fact suggests that these amino acids are involved in memory consolidation processes or in modulating these processes. These results confirm a participation for the urea-Glu-Ala cycle, and possibly of NO, in a general mechanism in memory consolidation.
3. *The brain is involved in mnemonic processes occurring in other ganglia.* Previous works have suggested that shock avoidance learning in insects could be achieved by isolated ganglions [e.g., (11)]. Our results are consistent with an interpretation where all ganglia are involved in memory consolidation, supporting more the results of Chen et al. (6), in which crickets with the connections between brain and prothorax cut were not capable of consolidating memory, that is, the brain is essential, at least for triggering memory consolidation, in insects.

In summary, the results alone do not allow to discriminate univocally which metabolic changes are due to learning and which to electric shock. But, if we compare these results with those obtained with the same species using a completely different training system (19) we may confirm that most metabolic changes reported here reflect a general biochemical phenomena related to learning and memory. Thus, we propose a neuromodulatory role for Ala, Arg, and Gln, and possibly also for Gly, Asn, Pro, and Orn. These neuromodulators could regulate the triggering of the passage of a specific learned information to a more permanent memory (memory consolidation). They should be part of a chain of more complex reactions. Thus, biophysical processes related to memory formation (23) could trigger differential transport, release from pools, or changes in the metabolism of some amino acids. These changes in amino acid concentration would be controlled by the "emotional behavior" (modulated by opiate receptors) responsible for evaluating the effect of specific behaviors on the organism, allowing for a behavioral feedback mechanism that has to include learning. Amino acids could play a role in triggering the passage of labile memory to permanent memory if the "emotions" elicited by specific behaviors are adequate. This could explain the double reactivity of arginine and alanine with mnemonic processes and with the endogenous opioid system, at least in insects. The site where these amino acids would act in triggering memory consolidation is not known, but the passage from labile to consolidated memory involves protein synthesis (1,4,9,18) and other metabolic processes (10,26) probably involved in producing structural changes in specific parts of the neurons. Thus, amino acids could act like growth regulators (30) of neurons involved in memory storage.

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