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Amino Acids and Memory Consolidation in the Cricket I: Changes in the Titer of Free Amino Acids in Nervous Tissue After Learning

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JAFFE, K., A. BAKLIEN, N. A. ZABALA, A. C. FERRER, M. GRANIER, A. TABLANTE, A. RIBBI-JAFFE AND S. BLAU. *Amino acids and memory consolidation in the cricket I: Changes in the titer of free amino acids in nervous tissue after learning.* PHARMACOL BIOCHEM BEHAV **35**(1) 127–131, 1990.—The involvement of certain amino acids in the memory consolidation process was investigated in the cricket *Pteronemobius sp.* Thirsty crickets were trained to constantly turn to one side of a symmetrical Y-shaped maze using reinforcement with water. Controls were trained to turn to both sides of the maze according to a random program. Animals were sacrificed immediately after training and free amino acid fractions were isolated from whole brain, subesophagic, prothoracic, mesothoracic and metathoracic ganglia homogenates and analyzed by high pressure liquid chromatography. A complex pattern of variation in the titer of amino acids emerged after learning, where the changes differed among the various ganglia. The most conspicuous change was an increase in the levels of urea and an amino acid-like compound related to the urea cycle, in all ganglia except the subesophagic one, if compared to controls. Arginine increased in the subesophagic ganglion, but decreased significantly in the metathoracic ganglion. The variation of ganglionic amino acid levels and its possible relation to mnemonic processes is discussed.

Amino acids Memory Learning Cricket Consolidation

THE mnemonic process is certainly based on neural functions. Some of the basic cellular processes in neurons have been related to learning and memory (2,12). In spite of this, very little is known about the physiological and biochemical mechanisms underlying mnemonic processes. A few biochemical correlates of mnemonic processes have been reported [see reviews in (1, 2, 4, 5, 13, 18)]. Some of these correlates are thought to be related to the actual physical site where the mnemonic mark is located (21), whereas others are thought to work as modulators of the learning and memory processes (7,14). Among other substances, amino acids have been indicated as having a modulatory effect on memory formation, at least in insects. Amino acids are known to enhance memory formation, and this enhancement may be blocked by the morphine antagonist naloxone (3, 8, 9, 15, 24). The mechanism of action of these amino acids on memory function is completely unknown, but seems to be very different from that of other mnemonic agents.

This work is part of a series which attempts to find the biochemical events underlying memory formation. Here, we measured the variation of amino acid levels during memory formation in the cricket *Pteronemobius sp.* In another work (11), using the same insect and the same learning paradigms, we analyzed the effect of injections of these amino acids on learning and memory.

In the first work of this series we analyzed the behavioral aspects of a purposely developed learning paradigm (25). It consisted of training thirsty crickets to develop a turning preference for the left (or right) in a symmetrical Y-shaped maze, by giving water droplets as reinforcement after each correct choice, so that all arms of the maze eventually serve as the starting or end point of a trial, with and without reinforcements, thus excluding any possibility of visual or olfactory cues that the cricket could use for finding the water droplets. This training procedure allowed to obtain turning preferences of the crickets detectable up to 72 hr after training. It also had the advantage that learning was independent of the duration of training, but dependent on the number of reinforcements. This spatial position learning could be inverted and extinguished. Memory consolidated into long-term memory (not susceptible to amnesic agents) after training the insects with 10 reinforcements. Control crickets, receiving reinforcements randomly on either side of the maze, did not show any kind of turning preference after training (11,25). Thus, the training procedure seemed appropriate to search for biochemical correlates of learning and memory by comparing changes in the concentra-

tion of amino acids in nervous tissue of experimental crickets with those of controls.

METHOD

Insects

Adult female crickets (*Pteronemobius sp.*), were reared in laboratory cultures at 32°C and 70% RH, following the method described elsewhere (10).

Training

The training procedure described in (25) was used. Insects were placed in a symmetrical plastic Y-shaped maze (size of arms were $20 \times 2 \times 2$ cm), after spending 24 hr in a closed 100-ml glass flask, maintained dry with silica. The cricket was free to walk through the maze. A trail began every time the cricket changed its position in the maze so that it exited one arm and entered another arm of the maze. Each time the experimental cricket (E) entered an arm to the left when exiting any of the 3 arms of the maze, it was offered a 2 μl droplet of water which constituted one reinforcement. Turns to an arm to the right of the cricket were computed as errors and no reinforcements were given. Control crickets (C) received 2 μl droplets of water either after turning to the left or to the right, following a previously established random sequence. Each cricket was trained with a variable number of trails until it made 10 correct choices in the maze and, thus, received 10 reinforcements. Immediately after receiving 10 reinforcements, the insect was killed by plunging it into a mixture of dry ice and acetone. The duration of training varied between 20 and 90 min, but was in mean the same for C and E animals, and so were the number of turns made in the labyrinth. Also, the number of water droplets taken in both cases was the same [see also (25)].

The learning criterion was set as follows: The number of errors made before receiving the sixth reinforcement were divided by the number of errors made after receiving the fifth reinforcement. Thus, values greater than 1 indicated "learning," whereas numbers smaller than 1 indicated "no learning."

Amino Acid Extraction and Analysis

Double-blind experiments were designed in which the experimenter did not know the origin of the sample. The method described in (23) was adapted as follows: 103 nervous ganglia (brain, i.e., proto- deuto- and trito-cerebrum, subesophagic-, prothoracic-, mesothoracic- and metathoracic ganglia from 20 crickets were dissected according to an adapted version of the method in (17). Ganglia were still frozen when dissecting under a stereo-microscope after which they were homogenized for 10 min in 3 ml of 80% ethanol. Homogenates of one ganglion at a time were centrifuged at $5000 \times g$ for 10 min. The precipitate was then washed twice in 80% ethanol and the total supernatant evaporated to dryness under reduced pressure (0.01 atm). The residue was redissolved in 200 μl distilled water added to an equal volume of 0.5 M sodium carbonate (pH 8.5). A 50 μl sample was dansylated with 100 μl dansyl chloride (Pierce) and dissolved in acetone to a final concentration of 2 mg/ml. The reaction which yields fluorescent derivates of amines was allowed to proceed for 25 min at 60°C. In order to suppress the formation of multiple derivates of histidine, tyrosine and lysine, the sample was diluted with 300 μl deionized distilled water containing 10% formic acid before the dansylation. A 25 μl sample of the final product was injected into a high pressure liquid chromatograph (Waters HPLC) consisting of two pumps, injector, solvent programmer, and a data module. The fluorescence was detected using a Waters 420A C fluorescence detector with a standard flow cell (excitation monochromator set at 360 nm and emission at 495 nm). Resolution of the peaks was accomplished using a Radial-Pack μBondapack C18 column (Waters). The mobile phase in pump A consisted of 0.02 N acetic acid and acetonitrile (90:10) and in pump B of the same solution, but in the relation 10:90. The mobile phase started at 15% solution B for 5 min, and then increased linearly over 45 min to a final concentration of 60% solution B, with an isocratic hold for 20 min. The flow rate was set at 1.0 ml/min. This method allowed the detection of pg quantities of amino acids (19).

The identification of the different peaks in the chromatograms was achieved comparing the retention times of each peak with those of standards and then by coinjecting the sample with dansylated standards with each of the 32 different amino acids and amines tested.

Treatment of the Data

Fluorescence for each peak was estimated by integrating the area under the peaks of chromatograms obtained from single ganglia. Comparison with chromatograms of known standards allowed the estimation of the absolute amounts (nmol/mg) for each amino acid represented in Table 1. For Table 2, the mean titer of each amino acid found in experimental animals (E) was expressed as the percentaged change of the titer in relation to the mean of that in control crickets (C); i.e.,

$$\% = 100 \times (\text{mean fluorescence C} - \text{mean fluorescence E})/\text{mean fluorescence C}.$$

Statistics were carried out on untransformed data. With Student's *t*-test we compared the fluorescence of amino acids from control replicates (C) with that of experimental replicates (E) for each type of ganglia (brain, subesophagic-, prothoracic-, mesothoracic-, and metathoracic-ganglia). Pearson correlation analysis, using the SPSS-University of Pittsburgh package, was carried out separately for all the data from each type of ganglia, comparing the fluorescence of the amino acids among themselves and with the learning criterion (Table 3). Stepwise Discriminant Analysis (SPSS) applied to data from each type of ganglia gave the amino acids which, if used to calculate the classification function, predicted correctly the maximum number of C and E insects.

RESULTS

Although the resolution of all amino acids was not possible, our method allowed the identification of 20 different peaks, including GABA, urea and low molecular weight peptides. The absolute concentrations of those amino acids which eluted singly, for each ganglion of nontrained insects, are given in Table 1.

The amino acids which coeluted with others and the unidentified compounds could not be quantified in absolute values. Thus, relative changes of fluorescence in chromatograms of E animals, relative to those of C crickets, were calculated. In Table 2, we represent the percentual change of the mean titer (fluorescence) of the amino acid extracted from the different ganglia of experimental crickets (E) in relation to that of controls (C), which showed some significant changes in their titer. Here, we observed that the titer of most amino acids drops after training, i.e., they are lower in E crickets compared to C animals. Statistically significant changes ($p<0.05$) of E in relation to C, given by Student's *t*-test, were: His and Glu dropped significantly its concentration after training in the prothoracic ganglion and the brain respectively. Urea increased its titer in the brain, meso- and metathorax, and compound Q in all ganglia except the subesophagic ganglion. Amino acids coeluting

TABLE 1

ABSOLUTE AMOUNTS OF FREE AMINO ACIDS IN NERVOUS TISSUE OF CONTROL CRICKETS

	Brain	Prothorax	Mesothorax	Metathorax
	No. of Replicates:			
Amino Acids	7	7	7	9
Tau	14 ± 10	35 ± 13	43 ± 9	14 ± 10
His	5 ± 4	16 ± 7	17 ± 6	10 ± 6
Glu	21 ± 4	38 ± 12	50 ± 17	21 ± 14
Ser	44 ± 16	159 ± 53	199 ± 40	86 ± 36
Asn	7 ± 2	25 ± 4	26 ± 16	19 ± 6
Thr	10 ± 4	33 ± 18	26 ± 13	25 ± 4
Gly	18 ± 9	—	—	27 ± 1
Ala	23 ± 9	98 ± 29	106 ± 10	43 ± 21
Pro	22 ± 10	102 ± 31	99 ± 36	37 ± 19
Val	6 ± 3	37 ± 12	44 ± 6	10 ± 9
Orn	11 ± 6	41 ± 15	79 ± 14	25 ± 15
Lys	5 ± 2	44 ± 16	41 ± 6	16 ± 9
Tyr	<1	<1	5 ± 6	<1
Arg	8 ± 1	36 ± 10	61 ± 14	20 ± 6

Values are expressed as nmol/mg of dry tissue (mean ± sd). Dry weight of the ganglia: brain 0.83 mg: prothorax 0.13 mg; mesothorax 0.073 mg; metathorax 0.14 mg.

with Cys dropped their titer after training in the brain and mesothorax, whereas Tyr increased it in the prothorax. Arg increased significantly its titer in the subesophagic ganglion, but dropped it in the metathorax.

Other amino acids did not show significant changes in their titer due to learning, as measured with a parametric test, i.e., Student's *t*-test. These are Tau, Ser, Hpro, Asn, Gly, Ala, GABA, Pro, Val, Orn and Lys.

Linear correlation (Pearson) between the learning criterion and the concentration of the amino acids in E and C crickets immediately after training gave the results shown in Table 3. We observe that immediately after training, the various ganglia show different amino acids significantly correlated to the learning criterion.

TABLE 2

PERCENTUAL CHANGE IN THE TITER OF AMINO ACIDS EXTRACTED FROM VARIOUS NERVOUS GANGLIA OF EXPERIMENTAL CRICKETS COMPARED TO CONTROLS

	Ganglion				
	Brain	Subesophagic	Prothorax	Mesothorax	Metathorax
	No. of Replicates Exper./Control:				
Amino Acids	8/7	4/5	7/7	8/7	9/9
His	−32	−7	−54*	−33	−19
Glu	−50*	−34	−50	−5	10
Urea	61*	−9	88	190*	240*
Cys, Leu, Phe, Thy, Ile	−50*	3	−44	−68*	−30
Q	333*	−42	1450*	444*	283*
Tyr	360	120	322*	−60	−80
Arg	16	135*	−20	−38	−70*

Only amino acids showing at least one significant change are presented.

Values are expressed as the percentual change respect to the mean titer of control animals. ANOVA: $p<0.01$. *Indicates significant differences ($p<0.05$) if the crude data of C is compared to that of E with Student's *t*-test.

TABLE 3

PEARSON CORRELATIONS BETWEEN THE VALUE OF THE LEARNING CRITERION OF EACH CRICKET AND ITS AMINO ACID CONCENTRATION AFTER TRAINING

Ganglion	n	AA	r	*p*
Brain	14	Ala	−.76	<0.01
		Arg	.51	<0.05
		Asn	−.50	<0.05
Subesophagic	9	Tau	.71	<0.01
		Arg	.86	<0.005
Prothoracic	14	HPro	.62	<0.05
		Val	.52	<0.05
Mesothoracic	15	Gln	−.82	<0.01
		Tau	−.52	<0.05
		Val	−.49	<0.05
Metathoracic	18	Tau	.72	<0.02
		Urea	.92	<0.001
		Q	.73	<0.02

AA indicate the amino acids tested, T the correlation coefficient and *p* gives the significance of the correlation.

Significant values only.

Different statistical analysis gave slightly different results. The Pearson correlation analysis gave significant correlations ($p<0.05$) with the training condition when we assigned C and E groups an arbitrary value for their training condition of 1 and 2 respectively. The following amino acids gave significant correlations: in the brain—Arg, Q, and Urea; none in the subesophagic ganglion; in the prothorax—His, Hpr, GABA and Lys; in the mesothorax—Q; and in the metathorax—Ala and Orn.

A Stepwise Discriminant Analysis, constructing a classification function with the titer of the amino acids in order to discriminate between C and E insects, gave the following results: for the brain with 100% discrimination of C and 100% discrimination of E, Gly, Ala, Pro, Cys complex and Lys where needed; for the subesophagic ganglion, Hpr, urea and Val together discriminated 100% of C and 100% of E; in the prothorax; Lys discriminated 100% of C and 86% of E; in the mesothorax, compound Q discriminated 100% of C and 63% E; whereas for the metathoracic ganglion, urea and Pro together discriminated 100% of C and 75% of E. That is, in all ganglia, the discrimination function constructed for each case could identify correctly the controls (C) in 100% of the cases, and identified experimental crickets (E) in 63–100% of the cases.

None of the 32 commercially available amino acids tested coeluted with compound Q. This compound was not hydrolyzed with 6 M HCl at 110°C for 24 hr, but decomposed at these conditions after 36 hr. IR spectra of the HPLC-purified compound showed free COOH and substituted NH groups. This suggests an amino acid-like structure for compound Q. Finer spectrographic analysis (NMR, Mass-spec., etc.) for the identification of compound Q were not available. Pearson correlations between compound Q and the titer of all other substances in all ganglia showed a highly negative correlation with Orn, $r(76) = -.55$, $p<0.001$, and with Asp, $r(76) = -.61$, $p<0.001$, suggesting a possible

metabolic relation with those amino acids, and, thus, with the urea cycle.

DISCUSSION

Over 120 double-blind experiments performed by 5 different experimenters (11, 20, 25) testing the crickets for turning preference 24 hr after training showed clearly that control crickets are unable to learn, i.e., do not show any kind of turning preference 24 hr after training, making 40 to 60% of the turns to a specific direction. Experimental crickets do show a preference after training, making 50–80% of the turns to the side they received reinforcements during training. This difference is not dramatic due to the way we recorded "learning" in this case, but can be analyzed statistically comparing the outcome of various crickets with a random binomial distribution. The performance during the retention test was significantly correlated with the learning criterion measured during training (11,25). Here, no retention test could be performed due to the fact that crickets were sacrificed immediately after training. Thus, we have to assume that most experimental crickets, but none of the control ones, learned.

Amino acid pools are significantly altered in experimental crickets compared to controls. The changes are different in the various ganglia and more than one amino acid seems to be involved. The different results obtained with the different statistical analysis used tell us that the biochemical changes occurring concomitantly to the mnemonic process are complex and interrelated. Little is known about amino acid metabolism in the insect nervous tissue and the significance of the results obtained can only be visualized with complementary studies, such as reported in (11). However, the following conclusions may be drawn.

Sources of Variation

Our data show important variations in the titer of amino acids in both control and experimental insects. Such variation could be, among others, the result of:

1) Interindividual differences of amino acid levels. Although care was taken to choose individuals of the same sex, age and rearing conditions, considerable interindividual variations among crickets may remain.

2) Differences in the dissection and homogenization of the ganglia and in the extraction of free amino acids. Maximal care was taken in order to achieve a clean dissection of the ganglia, discarding all those ganglia where no assurance could be given as to their complete and clean removal.

3) The heterogeneous learning performance among insects. That is, some crickets may have learned "more" or "better" than others, or may not have learned at all. Also, the memory consolidation process may have occurred with different speeds in different individuals, so that when sacrificed, the insects may have been at different steps of the consolidation process.

Points 1 and 2 may be considered to be a constant error for E and C insects, not affecting the possible conclusions of this work. Point 3 predicts a greater variability among E crickets if compared to C, which seems to be the case as assessed with the Stepwise Discriminant Analysis.

Learning vs. Stress

The changes in amino acid concentration are undoubtedly related to learning and not to some kind of stress, since control animals, which do not learn to preferentially turn to the left, are activated in an equivalent manner. That is, on average, they walk the same distance, are subjected to the same environment and stressful situations, are reared from the same parents and drink the same amount of water, but do not develop a turning preference (25). In experimental animals, immediately after training with 10 reinforcements, memory cannot be further disrupted by anoxia (11, 20, 25) and, therefore, can be considered to have consolidated (8,16). Other control experiments such as training to the right, extinction and inverted learning, etc. (25), have clearly shown that the development of a turning preference in these crickets is a true learning situation. An indirect support to the assumption that the changes in the titer of amino acids is indeed concomitant to learning is the coincidence of percentages of experimental animals predicted by the Stepwise Discriminant Analysis and that of crickets known to have learned the task: the Discriminant Analysis predicted between 63 and 100% of the E crickets, whereas we showed (25) that, on average, 70% of the E crickets learned and 30% did not when trained with only 10 rewards. Clearly, all C insects were unable to learn and the Stepwise Discriminant Analysis was able to recognize them in 100% of the cases, based solely on the amino acid levels in the ganglia.

The Role of Each Ganglion

Clearly, each ganglion suffers different metabolic processes during acquisition and its precise significance cannot be assessed at the moment, but it seems evident from our results that all five ganglia suffer biochemical processes concomitant to learning or memory.

Involvement of the Urea Cycle

The fact that associative learning causes a larger rate of metabolism than simply using the nervous system is consistent with other findings in the literature (2–5, 9, 12, 15, 21). Our results suggest that compound Q, Arg, Ala and urea, all related to the urea cycle, are the amines most strongly correlated with learning. If we compare these with previous results on praying mantis (3, 9, 15), where Arg and Lys increased after learning, it would appear that the urea cycle (or any metabolic product between NH_3 and alanine) is correlated with the memory consolidation process since the levels of urea and its derivates (possibly Arg and Ala) in the cricket and arginine in the mantis change concomitantly to the formation of the mnemonic mark. The difference between the changes in amino acids found in crickets and mantis could be due to a different turnover of the intermediate metabolites of the urea cycle, or to the fact that learning processes may have been interrupted at different points when sacrificing the individuals, thus showing a different picture of the concomitant changes in amino acids levels.

It is, thus, certain that amino acids have a neuromodulatory role on learning and memory in insects and, possibly also in vertebrates (6,22). This opens a new research field in neurosciences, related to anabolism, metabolism and multimodulatory processes in nervous tissue. In a sister paper (11), we continue this research, exploring the effect of externally administered amino acids on the same learning process.

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