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Amino Acids and Memory Consolidation in the Cricket II: Effect of Injected Amino Acids and Opioids on Memory

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JAFFE, K., N. A. ZABALA, M. E. DE BELLARD, M. GRANIER, W. ARAGORT AND A. TABLANTE. *Amino acids and memory consolidation in the cricket II: Effect of injected amino acids and opioids on memory.* PHARMACOL BIOCHEM BEHAV **35**(1) 133–136, 1990.—The effect of injections of selected amino acids on memory, given before a maze-learning, was investigated. Thirsty crickets (*Pteronemobius sp.*) were trained to turn only to one side of a symmetrical Y-shaped maze using reinforcements with water. The insects retained the learned task 24 hr later. N_2 anoxia applied immediately after training produced retrograde amnesia. Injections of Ala, Arg, Gln or morphine prior to training blocked the amnesic action of anoxia, whereas those of Cys, Met, Pro, Orn, octopamine or naloxone did not. Naloxone blocked long-term memory formation, but not learning, whereas Pro and Orn blocked both. The antiamnesic effect of morphine and Arg, but not that of Ala, was blocked by naloxone. A hypothesis assigning a neuromodulatory role to some amino acids is put forward.

Amino acids Memory Learning Cricket Consolidation

PHARMACOLOGICAL approaches to the study of learning and memory have shown the existence of various neuromodulators with an as yet unknown mechanism of action. Few, but recent, experimental evidence suggest that certain amino acids could play an important role in mnemonic processes, but little is known about their mode of action. Amino acids are known to vary their titer during learning. In the nervous tissue of praying mantis, increased levels of arginine after training have been demonstrated (2); whereas in the cricket, an increase in urea levels due to training has been detected (8). In both cases the metabolism of the urea cycle in nervous ganglia seems to be activated during learning. Injections of arginine given prior to training enhance memory formation in the praying mantis (2); the same occurs when injecting pools of amino acids in vertebrates (12). With an imprinting paradigm in chicks (3), it was shown that nonessential amino acids and arginine, phenylalanine, trytophan and tyrosine produced amnesia if injected in excess intracranially. The authors of this work related the effect found to nutritional requirements of the brain during learning and memory. We, however, believe that these amino acids have a rather neuromodulatory function, partly due to the fact that the effect of some amino acids on memory seems to be related to their effects on opiate receptors (13,14).

In this work we explored some of the effects amino acids may have on mnemonic processes. We studied their effect on learning and memory, when injected before training crickets, in a purposely designed and tested Y-maze learning paradigm (15). Those amino acids which were shown to change significantly their concentration in nervous tissue during the same learning process (8) were selected for the test.

METHOD

Animals and Training

Crickets (*Pteronemobius sp.*), reared in the laboratory at 28°C following the method previously described (7), were used. Animals were trained individually using the learning paradigm described extensively elsewhere (8,15). The training consisted of first submitting individual crickets to a dry atmosphere for 24 hr, after which they were introduced individually into a symmetrical Y-shaped maze. The insects were free to move in the maze, but each time an insect exited any of the three arms of the maze and entered an arm to its left, 2 μl of water were offered at the end of the respective arm, and the choice was registered as correct. Any turn to its right was not rewarded and was recorded as an error. Insects were trained in this way until they received 5 or 10 rewards, corresponding to 5 or 10 correct choices, independently of the total number of choices (correct + errors) made.

Experimental Groups

Seven hundred adult female crickets were divided into 27

groups, each with a minimum of 25 animals, in order to test different experimental procedures:

Group A. Consisted of insects trained until receiving 10 rewards. No anoxia was applied.

Group B. Differed from Group A in that fifteen min before training and immediately after receiving their 10th reward, animals were submitted to 3 min of N_2 anoxia.

Group C. Differed from Group B in that animals received rewards, regardless of the errors or correct choices made, but according to a random number sequence.

Group B + Nlx. Differed from Group B in that crickets received an injection of 0.07 μmol of naloxone 5 min before training, i.e., 10 min after the first anoxia.

Group D. Received a 3-min anoxia and 15 min later they were trained until collecting 5 rewards after making the corresponding correct choices. Individuals were then left in their dry cages for 1 hr before starting a second training. The training lasted until they again received 5 rewards, immediately after which the insects received a second 3-min anoxia.

Group E. Trained similar to Group D, except that both anoxias were given after each of the two training sessions, immediately after receiving the fifth reward. The crickets were left in their cages for 1 hr between sessions. The first training session was called F5, whereas the second session was called L5.

Group E + drug. Trained identical to Group E except that injections were given 5 min before starting the first training. This type of training was used to test the antiamnesic effect of amino acids, naloxone and morphine on the retrograde amnesia induced by anoxia.

Injections

A Hamilton syringe was used for injecting 5 μl of the respective solution through the integumentary membrane of the abdomen of imago crickets, between the 6th and 7th tergite. Previous tests with Coomasie Blue showed that this kind of injection produced dispersal of the solution into all body parts in less than 5 min.

All substances were dissolved in distilled water at concentrations of 1 M (unless stated otherwise). Thus, each insect received 5 μmol of the respective substance. The exceptions were: naloxone (0.07 μmol/cricket), morphine (0.5 μmol/cricket), NaCl (2.7 μmol/cricket), and the rather insoluble amino acids Asn, Cys, Met and Gln (1.7 μmol/cricket).

Retention Test (RT)

Twenty-four hours after training, the retention test (RT) was performed. This consisted of releasing the cricket into a new maze and observing its exploratory movements. The first 10 choices made were recorded and no rewards were offered. Each time the cricket exited one of the three arms of the Y-maze and entered an arm to its right or to its left, the experimenter recorded either an "error" or a "correct choice" respectively.

Measurements

During RT we measured the proportion of crickets showing more than 50% of correct choices (Pc), less than 50% of correct choices (Pe) and that showing a percentage of correct choices of exactly 50% (Po). Statistical analysis of the data consisted of comparing Pc/Pe with the proportion predicted by a random binomial distribution. For the comparison, the binomial exact probability test was used.

In order to get an estimate of the amount learned by the insect during the training session, the learning tendency during training (LT) was estimated. That is, the incorrect choices made by each cricket during their training sessions (one or two sessions, depending on the group) were counted before the third reward and after the third reward, for trainings with 5 rewards; and before the fifth and after the sixth reward, for trainings with 10 rewards. If the second number was smaller than the first, the insect was considered to have a positive tendency. LT was expressed as the percentage (%) of the animals from each experimental group with a positive learning tendency. Statistical analysis was performed comparing the proportion of crickets with a positive to that with a negative learning tendency, in the same way as described above.

The time (t) taken to get 10 reinforcements during training of group E was estimated by adding the duration of each of the 5-reward training sessions (F5 and L5). Results were expressed as means with their standard deviations. Statistical analysis consisted in applying an ANOVA to the data and then comparing the means with Student's *t*-test.

Water Intake

Motivation to drink water was estimated by leaving the insect for 24 hr in a dry atmosphere containing water absorbent silica. The insect was injected with 5 μl of the respective solution and reintroduced to its cage. Twenty-four hours later, water was offered through a graduated capillary tube from which crickets could drink directly, and the amount consumed during the first 15 min was measured. Control crickets were not injected and were kept in a humid cage (with RH >70%, similar to that in the rearing cage) during the 24 hr, but with no direct access to water.

RESULTS

As shown in Table 1, trainings with 10 rewards given continuously (Group A and B) resulted in the crickets turning preferentially to the side to which they were trained during RT whereas Controls with 10 reinforcements given randomly did not seem to learn nor retain (Group C). When the training was given in two sessions with 5 rewards each, and anoxias were induced before the first and after the last training session (Group D), animals showed a significant turning preference during RT, suggesting that they were able to learn and remember the position where they received rewards; but if anoxias were given immediately after each training (Group E), animals were unable to retain the learned task 24 hr later, although they did seem to learn during training (significant LT during F5 and L5, Table 1). Thus, only anoxias given after each of the 5-reward trainings inhibited memory consolidation (or recall), but not learning.

Injections of naloxone (Group B + Nlx) inhibited memory formation as measured with RT, but crickets were able to learn during training (significant positive LT, Table 1).

Table 2 shows the amount of water drunk by the crickets 24 hr after receiving an injection. Clearly, crickets kept in a humid environment did not drink water. The effect of all injections tested was similar (ANOVA gave nonsignificant differences between groups when controls were not included). A Duncan test, comparing controls with all other groups, revealed a highly significant difference ($p<0.001$). Injections of distilled water seemed to diminish the motivation for drinking, although no statistically significant results were obtained. Thus, despite a lack of statistical significance, the amount of water consumed 24 hr after the injection could be dependent on the concentration of NaCl injected. Therefore, we used a concentration of 1.5% NaCl for the following experiments, as this concentration gave results similar to those of injections with Arg.

TABLE 1

LEARNING TENDENCIES (LT) DURING TRAINING AND PROPORTION OF CRICKETS THAT SHOWED MAINLY CORRECT TURNINGS (Pc), ERRORS (Pe) OR SHOWED TURNING PREFERENCES AT CHANCE LEVELS (Po) DURING THE RETENTION TEST (RT)

Group	Training Procedure	LT F5	LT L5	RT Pc	RT Pe	RT Po	N
A	10	82†		77	9	14†	35
C	10[c]	43		32	36	30	25
B	10[a]	75†		70	10	20†	30
B + Nlx	10[a]	81†		46	38	10	26
D	[a]5–5[a]	65*	71*	60	29	11*	35
E	5[a]–5[a]	77†	85†	40	40	20	35
E + NaCl	5[a]–5[a]	75†	71†	43	40	17	35

F5 indicates the first five and L5 the last five reward trainings.

* and † indicate $p<0.05$ and 0.01 respectively, of values of LT and Pc/Pe, if compared to a random binomial distribution.

Details of the training procedure are symbolized as follows: [a]Indicates anoxias applied before or after training with 10 or 5 rewards. —Indicates a one-hour rest period between trainings. [c]Indicates random rewards.

Table 3 gives the main results showing the effect of injections with the various substances. Here, we observed different effects on the retention of the learned task. NaCl, Cys, Met, naloxone and octopamine did not inhibit learning nor the amnesic action of anoxia (i.e., significant LT in F5 or L5 and nonsignificant Pc/Pe). Injections of Orn and Pro seemed to inhibit learning during training, and crickets did not show any turning preference during RT. Injections of Arg, Ala, Gln and morphine were shown to have an antiamnesic effect, i.e., they seemed to protect the animal against the induction of retrograde amnesia by anoxia, as the animals retained the learned task 24 hr after training, despite receiving anoxias after each training block (significant Pc/Pe during RT). Octopamine injections were shown to have, in addition, a somewhat different effect as crickets became hyperactive (uncoordinated movements were recorded), but did not perform better during training nor during retention.

Three different classes of substances could be recognized when analyzing statistically the duration of training (t) of the different groups (Table 3). Injections of naloxone, morphine or Arg caused an increased duration of training (animals moved significantly slower or less frequent). Injections of Orn or Ala decreased significantly the duration of training. The effect of injections of all other substances tested did not differ statistically from that induced by NaCl (the control).

When naloxone was injected together with morphine or Arg, the insects behaved similar to those receiving the saline injection, i.e., they did not show turning preferences during RT, and, thus, differed from those insects receiving morphine or Arg alone. Crickets injected with naloxone and Ala continued to show significant turning preferences during RT.

TABLE 2

MEAN AND SD OF THE AMOUNT OF WATER DRUNK BY INSECTS 24 HR AFTER INJECTION

Injection	µl Drunk in 15 Min
Water	10 ± 11
Arg	18 ± 15
Naloxone	16 ± 14
NaCl (1.0%)	15 ± 10
NaCl (1.5%)	17 ± 14
NaCl (2.5%)	21 ± 17

ANOVA (excluding control): Not significant, $p<0.10$. Control: 0.5 ± 0.8. ANOVA (all groups, including control): $p<0.001$.

n = 15 for each group.

TABLE 3

LEARNING TENDENCY (LT) DURING THE FIRST (F5) AND SECOND (L5) TRAINING SESSION AND PROPORTION OF CRICKETS MAKING MAINLY CORRECT TURNINGS (Pc), ERRORS (Pe) OR SHOWING NO SPECIFIC TURNING PREFERENCE (Po) DURING THE RETENTION TEST (RT); AND TIME TAKEN TO GET 10 REINFORCEMENTS DURING TRAINING (MEAN ± SD) USING GROUP E + DRUG

Injection	LT F5	LT L5	RT Pc	RT Pe	RT Po	t of Training (min)	N
NaCl	75†	71†	43	40	17	41 ± 12	35
Cys	74*	86†	39	36	25	39 ± 8	28
Met	72*	79†	45	32	23	40 ± 19	22
Nalox	75†	96†	43	34	23	56 ± 25‡	30
Octopamine	53	81†	30	44	26	37 ± 12	23
Gly	69*	80†	48	29	23	45 ± 11	31
Asn	60	70*	49	25	26	46 ± 13	35
Pro	63	59	36	41	23	35 ± 8	22
Orn	62	62	46	33	21	34 ± 7‡	24
Morph	54	68*	56	29	15*	53 ± 14‡	27
Arg	76†	67	74	15	11†	53 ± 14‡	39
Ala	70*	65	64	24	12*	33 ± 7‡	25
Gln	56	80†	61	21	18*	45 ± 23	29
Morph + Nalox	55	68*	42	45	13	36 ± 10	24
Arg + Nalox	75†	95†	34	42	24	60 ± 19‡	21
Ala + Nalox	59	46	62	24	14*	32 ± 8‡	29

* and † indicate $p<0.05$ and 0.001 respectively, if LT or Pc/Pe are compared with a two-tailed random binomial distribution.

‡Indicates $p<0.05$ using Student's t-test if compared to the control situation with NaCl injections. (ANOVA: $p<0.001$.)

DISCUSSION

The results show that N_2-induced anoxia produced retrograde amnesia, depending if applied before or after training. Anoxia given immediately after each 5-reward training seemed to induce retrograde amnesia, but did not affect learning. By contrast, anoxia given before such trainings did not affect retention. Anoxias given after continuous training with 10 rewards did not induce retrograde amnesia. Thus, we may assume that training with 10 rewards produced consolidated memory, resistant to interference by anoxia, and so do trainings with two sessions of 5 rewards, even if the sessions are separated by intervals of 1 hour. Trainings with only 5 rewards produce a memory which may be interfered with by anoxia.

Chemicals injected before training caused one of two different effects on retention: either they had an antiamnesic effect, i.e., reverted or blocked the amnesic action of the anoxias, or had no effect on RT. In addition, those chemicals showing an antiamnesic effect could be classified, again in different categories, according to their effect on the duration of training: those which increased it (Arg and morphine); those which shortened it (Ala); and those with no effect on the duration of training (Gln).

The effect of naloxone, injected together with the antiamnesic agent, appeared to be consistent with the categories mentioned above. That is, naloxone seemed to inhibit the antiamnesic action of morphine and Arg, but not that of Ala. This suggests that Arg may act through opioid receptors of the insect (14), whereas Ala did not, or at least not through naloxone-susceptible receptors.

Our control experiment estimating motivation to drink water showed that amino acids have an effect on learning and memory which cannot be explained through a change in the motivation of the animal to drink water, as we were unable to detect such a change. The effect of amino acids should be rather directly on memory or on the N_2-induced anoxia.

As shown before (5, 9, 15), nitrogen anoxia seems to interrupt the short-term memory (or labile memory), inhibiting memory consolidation, but does not affect consolidated memory in insects. Thus, any antiamnesic agent, protecting the animal against the amnesic action of anoxia, should act either directly on mnemonic processes by accelerating memory consolidation, for example, and making memory unsusceptible to anoxia or indirectly, by inhibiting the action of anoxia in an unknown way.

Our findings suggest that the effect of antiamnesic agents is even more complex. Some of them seem to act on the endogenous opioid system (i.e., their action is reverted by naloxone) and others do not. This could be explained in two ways: either the agents act on memory processes through two different routes (one of them using systems involving endogenous opioids), or they act in the same way, but some of them have collateral effects on the endogenous opioid system. The fact that morphine and naloxone act on memory with opposite effects, that naloxone blocks the antiamnesic effect of morphine and arginine and that naloxone even blocks long-term memory formation, although not learning, in situations where anoxia does not interfere with memory, would give support to the first hypothesis. This would agree with the independent findings in vertebrates (4), indicating a direct involvement of the endogenous opioid system in mnemonic processes.

In spite of the fact that no clear explanation for the effect of amino acids on memory can be given, the following may be concluded from our results and what is known up to now:

1) It may be strongly suggested that amino acids (probably those involved directly or indirectly with the urea cycle) are involved in mnemonic processes. That is, Arg, Ala and Gln have an antiamnesic effect, possibly accelerating memory consolidation; whereas Orn and Pro seem to inhibit memory formation, as they are unable to improve their performance during training (Table 3). In analogy, vasopressin seems to enhance memory in vertebrates (11). An indirect support for a direct involvement of amino acids in mnemonic processes can be found through the fact that the same amino acids appear to change their concentration in nervous tissue concomitantly to the memory consolidation process (1, 2, 6, 8).

2) Memory in insects is susceptible to opioids, i.e., morphine enhances and naloxone inhibits memory formation (13,14). The phenomenon appears to be similar in vertebrates (4,10).

3) Arginine has a similar effect to that of morphine, not only on memory formation, but also on "pain" (14). Alanine, although it also appears to affect memory processes, does not seem to act on opiate receptors nor on "pain" (in preparation).

Thus, our work confirms a hitherto unrecognized and possibly complex role of amino acids as neuromodulators. The number of amino acids having a neuromodulatory role may be large, but, clearly, further work is required in order to gain some insight into the significance of amino acids in nervous function, not only in insects but also in other phyla.

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