



Involvement of Amino Acids, Opioids, Nitric Oxide, and NMDA Receptors in Learning and Memory Consolidation in Crickets

KLAUS JAFFE¹ AND MARIA ESTHER BLANCO

*Departamento de Biología de Organismos, Universidad Simón Bolívar,
 Apartado 89000, Caracas, 1080, Venezuela*

Received 27 January 1993

JAFFE, K. AND M. E. BLANCO. *Involvement of amino acids, opioids, nitric oxide, and NMDA receptors in learning and memory consolidation in crickets*. PHARMACOL BIOCHEM BEHAV 47(3) 493–496, 1994. — The effect of injections of selected amino acids and of *N*-methyl-D-aspartate (NMDA); morphine; and NMDA, nitric oxide (NO), and opioid inhibitors 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 h later. N₂ anoxia applied immediately after training produced retrograde amnesia. Injections of alanine (Ala), arginine (Arg), glutamine (Gln), morphine, or NMDA prior to training blocked the amnesic action of anoxia. Naloxone, an opioid antagonist, blocked long-term memory formation, but not learning, whereas hemoglobin or 2-amino-5-phosphonovaleric acid (APV), NO and NMDA antagonists respectively, blocked both. The anti-amnesic effect of Morphine and Arg, but not that of Ala or NMDA was blocked by naloxone. The results suggest involvement of NMDA receptors and NO and thus of long-term potentiation phenomena in learning and in memory consolidation, whereas other neuromodulatory systems related to Arg, and opiate receptors, are only involved in memory consolidation.

Amino acids	Insects	Memory consolidation	Long term memory	Crickets	Neuromodulation
NMDA	NO	Opioids	APV	Naloxone	

AMINO acids are increasingly recognized as important neuro-modulators. A few examples may illustrate this point: L-Cysteine destroys neurons in vitro or in vivo (19); glutamate modulates *N*-methyl-D-aspartate (NMDA) receptors (22) and is involved in long range glial signaling (3); and L-aspartate, l-glutamate, and NMDA activate the receptor channel complex of neurons (24). Long-term potentiation, thought to be related to mnemonic processes, is also affected by NMDA (8,9) and nitric oxide (NO) (1,23). In addition, amino acids have been shown to be directly implicated in mnemonic processes: Free arginine increases its titer in the brain of the praying mantis after training (4,14), whereas in the cricket the urea cycle seems to be activated in nervous ganglia during training in an operant conditioned reward task (11) and in an aversive conditioning (12). Injections of arginine enhance memory for-

mation in the praying mantis (4), and injections of arginine, alanine, and glutamine enhance, whereas those of proline and ornithine block, memory formation in the cricket (15). Pools of amino acids enhance memory formation if injected in vertebrates (21). Intracranial injections of single nonessential amino acids and of arginine, phenylalanine, and tryptophan block memory formation in the chick (7). L-Arginine has been shown to be a precursor for NO which activates NMDA receptors (6), and NMDA in turn has been related to long-term potentiation phenomena in neurons (5).

Alternative hypotheses were put forward that tried to explain the action of amino acids on memory consolidation (4,11,12,15) and on opiate receptors (25,26,28) in the context of a two- or more stage model of memory formation. Although no strong conclusions can be drawn for the mo-

¹ To whom requests for reprints should be addressed.

ment, much evidence suggests that some amino acids modulate biochemical reactions leading to memory consolidation (i.e., formation of long-term memory). Amino acids play a role in triggering the processes from labile to consolidated memory (10,16). It has been suggested that this neuromodulation would act by regulating the intracellular L-arginine concentrations, which in turn would modulate NO release (12). To clarify the role NMDA receptors and some free amino acids have in memory consolidation, we evaluated the effect of injections of known neuromodulators and their antagonists (naloxone, an opioid antagonist; hemoglobin and 2-amino-5-phosphonovaleric acid (APV), which are NO and NMDA antagonists, respectively) on learning and memory consolidation of an operant conditioned reward task (11,15, 27) using crickets. This learning system, although too slow for efficiently discerning effects on acquisition from short-term memory (here just mentioned under the broad concept "learning"), is sensitive to effects on memory consolidation.

MATERIALS AND METHODS

Training

Adult female *Pteronemobius sp* crickets were reared in the laboratory at 28°C and 70–85% relative humidity, following the method described before (13). Animals were trained individually using the learning paradigm described extensively elsewhere (11,15,27). The training consisted of first submitting individual crickets to a dry atmosphere for 24 h, 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 μ l 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 made.

Experimental Groups

Six hundred adult female crickets were divided into 20 groups, each with a minimum of 25 animals, to test different experimental procedures:

Group A. Insects were trained until they received 10 rewards. Crickets received an injection of 5 μ l of 1-M solutions (unless stated otherwise) 5 min before training.

Group B. Insects were trained until they received 5 rewards, immediately after which they received 3-min of N₂ anoxia. Individuals were then left in their dry cages for 1 h before starting a second training session, which also lasted until they received 5 rewards, immediately after which insects received 3-min of N₂ a second time. Crickets received an injection of 5 μ l of 1-M solutions (unless stated otherwise) 5 min before the first training session.

Controls. Controls differed from group A in that animals received rewards, regardless of the errors or correct choices made, according to a random number sequence.

Injections

A Hamilton syringe was used for injecting 5 μ l of the respective solution through the integumentary membrane of the abdomen of adult crickets, between the 6th and 7th tergite (15).

All substances were dissolved in distilled water at concentrations of 1 M (unless stated otherwise). Thus, each insect received 5 μ mol of the respective substance. The exceptions were (in μ mol/cricket): naloxone (0.07), morphine (0.5), NaCl (2.7), Gln (1.7), APV (0.6), and hemoglobin (2%). Concentrations of substances injected in solutions different to 1 M were assessed in preliminary trials, so that insects received the highest concentration at which they did not show any conspicuous behavioral abnormality. Concentrations of saline (2.7 μ mol/cricket of NaCl) were chosen so as to not affect the motivation to drink water [see extensive treatment in (15)].

Retention Test

Twenty-four hours after training the retention test 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

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 by counting the incorrect choices made by each cricket during its training sessions before its 5th reward, and before the 10th reward and after the 5th reward. If the second number was smaller than the first, the insect was considered to have a positive tendency. LT was expressed as the proportion of animals from each experimental group with a positive learning tendency. Statistical analysis was performed comparing the performance of each cricket during the first 5 rewards to that during the last 5 rewards using a Wilcoxon's matched pairs test.

During the retention test we measured the proportion of crickets showing more than 50% of correct choices (Pc) and those showing more than 50% of errors (Pe). Those showing equal number of errors and correct choices were not used for analysis (~20% of crickets [see also (11,15,27)]). The "retention index" assessed during the retention test (RI) consisted of the proportion $Pc/(Pc + Pe)$. Statistical analysis was made using the binomial test, comparing the performance of each cricket during the first 5 turns to that of a random distribution, which was the same as comparing them to the controls (RIs of controls were randomly distributed, $p > 0.999$). Only RIs deviating significantly from a random binomial distribution, and which therefore were significantly different from controls, were accepted as showing retention.

RESULTS

Training with 10 rewards given continuously (group A) resulted in the crickets turning preferentially during the retention test to the side to which they had been trained 24 h before (Table 1). Controls with 10 reinforcements given randomly did not seem to learn (nonsignificant LT) or retain (nonsignificant RI) any specific turning preference.

Injections of NaCl and NMDA did not affect learning (LT) or memory (RI), but injections of APV, hemoglobin, or naloxone affected memory consolidation, as insects did not retain the learned task. APV and hemoglobin affected learning as well, as LT were less or not statistically significant compared

TABLE 1

LEARNING TENDENCIES (LT) DURING TRAINING
AND RETENTION INDEX DURING RETENTION TEST
(RI) OF CRICKETS TRAINED CONTINUOUSLY
WITH 10 REWARDS (GROUP A)

Treatment	LT	RI	N
Control (No injection)	.43	.47	25
Experimental (No injection)	.75*	.88†	50
NaCl	.81*	.66†	38
NMDA	.82†	.73†	30
APV	.71†	.47	30
Hb	.71	.53	30
Nx	.81†	.51	26

"Treatment" indicates substances injected prior to training. APV = 2-amino-5-phosphonovaleric acid, Hb = hemoglobin, Nx = naloxone, NMDA = *N*-methyl-D-aspartate, Mor = morphine. * $p < 0.001$, † $p < 0.01$, ‡ $p < 0.05$, applying a Wilcoxon's matched-pairs test for LT or a binomial test for RI.

to crickets injected with NaCl. Saline, NMDA, and naloxone had no apparent effect on learning (Table 1).

Table 2 shows the results of group B. Here, crickets were made anoxic after the first 5 and last 5 rewards during training, blocking memory consolidation so that insects injected with NaCl showed a nonsignificant RI. As expected, injections of the inhibitors APV, hemoglobin, or naloxone did not improve retention. Injections of NMDA, morphine, and the amino acids Gln [which converts to glutamic acid (2)], Arg, and Ala do improve retention, so that crickets showed significant RIs in spite of the anoxia. Naloxone blocked the anti-amnesic effect of morphine and arginine but not that of Ala and NMDA. APV and hemoglobin were not tested further, as they affected learning (Table 1).

DISCUSSION

Our results confirm earlier studies (11,15,27) showing that insects learn to turn preferentially to one side of a maze if rewarded with water. This learning can be retained by the insect for over 24 h. Training crickets with 10 rewards leads normally to memory consolidation immediately after training. This training system can thus be used to evaluate effects of drugs on learning. Anoxia causes the disruption of memory formation, inducing a partial retrograde amnesia if insects are trained in two sessions with 5 rewards. This discontinuous training system is useful for evaluating drugs having anti-amnesic effects [i.e., drugs that potentiate or accelerate memory consolidation (15)].

The substances injected had different effects on learning and/or memory consolidation. APV and hemoglobin interfere with learning, affecting the performance of the crickets during training (LT) and consequently also affecting the performance during the retention test (RI). Naloxone does not seem to affect LT, but affects memory consolidation (RI). NMDA, morphine, Arg, Ala, and Gln accelerate memory consolidation or protect the insect against N_2 anoxias in some other unknown way (11,12,15). This positive effect on memory con-

TABLE 2

LEARNING TENDENCIES (LT) DURING
TRAINING AND RETENTION INDEX DURING
RETENTION TEST (RI) OF CRICKETS TRAINED
IN TWO SESSIONS WITH 5 REWARDS EACH,
RECEIVING AN ANOXIA AFTER EACH
TRAINING (GROUP B)

Treatment	LT	RI	N
NaCl	.75*	.52	38
APV	.68	.47	30
Hb	.60	.53	30
Nx	.96*	.56	30
NMDA	.85†	.70*	30
Mor	.68*	.66*	27
Arg	.76*	.83*	39
Ala	.70*	.73*	25
Gln	.80*	.74*	29
Mor + Nx	.68*	.48	24
Arg + Nx	.95*	.45	21
Ala + Nx	.59	.72*	29
NMDA + Nx	.73†	.70*	30

"Treatment" indicates substances injected prior to training. APV = 2-amino-5-phosphonovaleric acid, Hb = hemoglobin, Nx = naloxone, NMDA = *N*-methyl-D-aspartate, Mor = morphine, Arg = arginine, Ala = alanine, Gln = glutamine. * $p < 0.05$, † $p < 0.01$, applying a Wilcoxon's matched-pairs test for LT or a binomial test for RI.

solidation can be blocked with coinjections of naloxone only in the case of morphine and arginine, but not when NMDA or alanine are used.

These results suggest that at least two different neuromodulatory systems are at work during memory consolidation. One system is modulated by Glu acting on NMDA receptors, and possibly by other amino acids besides glutamate such as Ala, whereas a second system, working independently of the first one, is related to opiate receptors and Arg.

The fact that APV and hemoglobin block learning and memory formation suggests that long-term potentiation phenomena, modulated by NMDA receptors and NO release, are involved in learning and memory formation [see also (17,20)], but the effects on memory consolidation of NMDA, morphine, Arg, Ala, and Naloxone are different from the effect of hemoglobin, suggesting that memory consolidation is in addition regulated by other neuromodulatory systems different from those regulated by NO [see also (18)]. The fact that the action of naloxone on Arg differs from that on NMDA indicates that the effect of Arg on memory consolidation is not due to the fact that Arg is a precursor of NO, but suggests that Arg is a neuromodulator by itself.

In conclusion, memory consolidation, although related to long-term potentiation phenomena triggered by NMDA receptors and NO release, is a more complex phenomena in which other neuromodulatory systems related to opioid receptors and Arg are also at work.

ACKNOWLEDGEMENTS

This work was partially supported by CONICIT-Venezuela through the grant S1-2162.

REFERENCES

- Bohme, G. A.; Bon, C.; Stutzmann, J.; Doble, A.; Blanchard, J. Possible involvement of nitric oxide in long term potentiation. *Eur. J. Pharmacol.* 199:379-381; 1991.
- Chen, P. S. Amino acid and protein metabolism. In: Kerkut, G. A., ed. *Comprehensive insect physiology, biochemistry and pharmacology*, vol. 10. Elmsford, NY: Pergamon Press; 1986: 177-217.
- Cornell, A. H.; Finkbeiner, S. M.; Cooper, M. S.; Smith, S. J. Glutamate induces calcium waves in cultured astrocytes: Long-range glial signaling. *Science* 247:470-473; 1990.
- D'Alessio, G.; Di Donato, A.; Jaffe, K.; Maldonado, H.; Zabala, N. Arginine and memory consolidation in praying mantis. *J. Insect Physiol.* 147:231-235; 1982.
- Fidia Research Foundation. LTP: Presynaptic or postsynaptic? *Neurosci. Facts* 1:2; 1990.
- Fidia Research Foundation. NO and NO synthase in the brain. *Neurosci. Facts* 2:1-2; 1991.
- Gibbs, M. E.; Richdale, A. L.; Ng, K. T. Effect of excess intracranial amino acids on memory: A behavioural survey. *Neurosci. Biobehav. Rev.* 11:331-339; 1987.
- Gustafsson, B.; Wigstrom, H. Physiological mechanisms underlying long-term potentiation. *Trends Neurosci.* 11:156-162; 1988.
- Izumi, Y.; Clifford, D. B.; Zorumski, C. F. Inhibition of long-term potentiation by NMDA-mediated nitric oxide release. *Science* 257:1273-1276; 1992.
- Jaffe, K. Effect of cycloheximide on the learning process in the praying mantis. *Physiol. Behav.* 25:367-372; 1980.
- Jaffe, K.; Baklien, A.; Zabala, N. A.; Ferreras, A. C.; Granier, M.; Ribbi, A. 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.* 35:127-131; 1990.
- Jaffe, K.; Blau, S.; Zabala, N. Amino acids levels during learning and memory consolidation of an aversive conditioning task in crickets. *Pharmacol. Biochem. Behav.* 43:205-214; 1992.
- Jaffe, K.; Granier, M.; Zaldivar, M. E.; Zabala, N. A.; Meza, A.; Lara, W. Un metodo para la cria masiva de grillos *Pteronemobius sp.* *Bol. Entomol. Venez. N. S.* 5:41-56; 1989.
- Jaffe, K.; Maldonado, H. A chemical correlate of learning in the praying mantis. *J. Insect Physiol.* 25:319-325; 1979.
- Jaffe, K.; Zabala, N. A.; De Bellard, M. E.; Granier, M.; Aragort, W.; Tablante, A. Amino acids and memory consolidation in the cricket. II: Effect of injected amino acids and opioids on memory. *Pharmacol. Biochem. Behav.* 35:133-136; 1990.
- Maldonado, H.; Jaffe, K.; Balderrama, N. The dynamics of learning in the praying mantis. *J. Insect Physiol.* 25:525-533; 1979.
- Mathies, C.; DeBarry, J.; Ungerer, A. Memory deficits induced by L-glutamyl-L-aspartate and D₂-amino-5-fosfonovalerate in a Y-maze avoidance task: Relationship to NMDA receptor antagonism. *Psychopharmacology* 105:546-552; 1991.
- Mott, D. D.; Lewis, D. V. Facilitation of the induction of long-term potentiation by BABAb receptors. *Science* 252:1718-1720; 1991.
- Olney, J. W.; Zorumski, C.; Price, M. T.; Labruyere, J. L-Cysteine, a bicarbonate-sensitive endogenous excitotoxin. *Science* 248:596-599; 1990.
- Pontecorvo, M.; Clissold, D. B.; White, M. F.; Ferkany, J. W. N-methyl-D-aspartate antagonists and working memory performance: Comparison with the effects of scopolamine, propranolol, diazepam and phenylisopropyladenosine. *Behav. Neurosci.* 105:521-535; 1991.
- Rosen, P. Amino acids enhancing memory. In: *Proceedings of Congress on Memory and Learning*, California; 1985.
- Sah, P.; Hestrin, S.; Nicoll, R. A. Tonic activation of NMDA receptors by ambient glutamate enhances excitability of neurons. *Science* 246:815-818; 1989.
- Schuman, E. M.; Madison, D. V. A requirement for the intercellular messenger nitric oxide in long-term potentiation. *Science* 254:1503-1506; 1991.
- Vyklicky, L.; Vyklicky, L., Jr.; Vyskocil, F.; Vlachova, V.; Ujec, A.; Michl, J. Evidence that excitatory amino acids not only activate the receptor channel complex but also lead to use-dependent block. *Brain Res.* 363:148-151; 1986.
- Zabala, N. A.; Gomez, M. A. Morphine analgesia, tolerance and addiction in the cricket *Pteronemobius sp.* *Pharmacol. Biochem. Behav.* 40:887-891; 1991.
- Zabala, N. A.; Jaffe, K.; Maldonado, H. Arginine has an opioid like action in insects. *Experientia* 40:733; 1984.
- Zabala, N. A.; Jaffe, K.; Rosas, A.; Zaldivar, M. E. Un aprendizaje operante en grillos *Pteronemobius sp.* *Acta Cient. Venez.* 38:266-273; 1987.
- Zabala, N. A.; Miralto, A.; Maldonado, H.; Nunez, J. A.; Jaffe, K. Opiate receptors in praying mantis; effect of morphine and naloxone. *Pharmacol. Physiol. Behav.* 20:683-687; 1984.