Pharmacology Biochemistry & Behavior, Vol. 21, pp. 467–469, 1984. © Ankho International Inc. Printed in the U.S.A. 0091-3057/84 $3.00 + .00

BRIEF COMMUNICATION

Enhancement of Memory by a Cholinesterase Inhibitor Associated with Muscarinic Receptor Down-Regulation

BERNARD LERER,*‡ HARVEY ALTMAN* AND MICHAEL STANLEY*†

**Department of Psychiatry and †Pharmacology, Wayne State University and Lafayette Clinic*
951 E. Lafayette, Detroit, MI 48207
and ‡Jerusalem Mental Health Center, P.O. Box 140, Jerusalem, Israel

Received 10 January 1984

LERER, B., H. ALTMAN AND M. STANLEY. *Enhancement of memory by a cholinesterase inhibitor associated with muscarinic receptor down-regulation.* PHARMACOL BIOCHEM BEHAV 21(3) 467–469, 1984.—Rats trained on a passive avoidance task 24 hours following a single intraperitoneal injection of diisopropyl fluorophosphate (DFP, 1.2 mg/kg) showed enhanced retention when tested 7 days later. In a parallel group of rats, reduced cortical [^{3}H] quinuclidinyl benzilate binding was demonstrable 24 hours following DFP administration. The association of reduced muscarinic receptor binding and enhanced performance on a memory task contradicts previous reports which suggested that retention was impaired by treatments which down-regulate muscarinic receptors. This contradiction may be reconciled if pre-synaptic factors such as agonist availability are considered in conjunction with post-synaptic receptor effects.

Diisopropyl fluorophosphate Memory [^{3}H] Quinuclidinyl benzilate binding Muscarinic receptors

CHRONIC exposure to agents which increase the synaptic concentration of acetylcholine has been shown to induce a decrease in brain muscarinic receptor density [3,12]. Recent reports have suggested that muscarinic receptor down-regulation may be associated with impairment of memory function [4, 5, 9]. Thus chronic pretreatment with physostigmine was reported to impair subsequent performance by rats on a passive avoidance task [9]. Loullis *et al.* [9] suggested that this impairment may be a function of reduced muscarinic receptor density induced by chronic exposure to acetylcholine.

Association of muscarinic receptor down-regulation with memory impairment has also been reported in relation to electroconvulsive shock (ECS) [4,5]. Single ECS induces immediate reductions in brain acetylcholine levels and increases in acetylcholineesterase activity which are consistent with acute release of the neurotransmitter [2, 8, 13]. Repeated (but not single) ECS induces a significant reduction in cortical muscarinic receptors and an anterograde amnesia characterized by a specific deficit in delayed recall of an aversive stimulus [4,5]. Lerer *et al.* [4,5] have suggested that reduced cortical muscarinic receptor density may underlie the anterograde amnesia induced by repeated ECS.

Diisopropyl fluorophosphate (DFP) is an irreversible inhibitor of acetylcholineesterase which has been shown to enhance memory in rodents following chronic administration [1]. Chronic [12], but also single [6,7] administration of DFP induces a significant reduction in the number of brain muscarinic receptor binding sites. These two phenomena, if studied in conjunction, may shed further light on the role of alterations in muscarinic receptor density in memory function. More specifically, it might be expected that even a single dose of DFP, if sufficient to down-regulate muscarinic receptors, should impair rather than enhance memory function. It was therefore of interest to examine the effect of a dose of DFP previously shown to reduce brain muscarinic receptor density [6] on both cortical [^{3}H] quinuclidinyl benzilate ([^{3}H] QNB) binding and retention following a one-trial passive avoidance task in rats.

METHOD

Animals

Male albino rats (Sprague-Dawley) weighing 175–200 g were housed 2 per cage in a temperature controlled (24°C) environment with a regular 12 hour light-dark cycle. Food and water were continuously available.

Drug Administration

Following a 7 day adaptation to their home cages, rats were administered diisopropyl fluorophosphate (DFP, Sigma) 1.2 mg/kg IP. Control rats received vehicle (saline) injections. Twenty four hours following DFP or vehicle ad-

ministration half the rats in each group were assigned to behavioral training and the other half were decapitated for ^{3}H-QNB binding studies.

Behavioral Testing

Rats were trained 24 hours or 7 days following DFP or vehicle administration and tested 7 or 14 days later. Training and testing were conducted in a two-compartment black Plexiglas shuttle-box (76.2×34.3×50.8 cm high). The smaller (start) compartment of the shuttle-box was illuminated by five (7.5 watt D.C.) bulbs mounted along the top of the rear wall and separated from a larger dark compartment by a door which could be manually opened and closed from above. The floor was constructed of 0.6 cm diameter stainless-steel bars spaced 1.9 cm apart and connected to a Grayson-Stadler (Model 700) scrambled shock source.

During training, each animal was placed in the lighted compartment with the door separating the two chambers closed and allowed 30 seconds to freely explore that side of the apparatus. The door was then raised and the animal was allowed to enter the dark compartment. Once the animal had completely crossed over to the dark side (all four paws) the door was lowered and a 3.0 sec, 0.3 mA inescapable shock was delivered via the grid floor. Immediately after the shock the animal was removed from the dark compartment and returned to his home cage. The latency to cross from the lighted to the dark compartment was recorded for each animal. Testing followed the same procedure except that no shock was applied if and when the animal crossed to the dark side. The initial latency to cross (i.e., step-through latency) from the lighted to the dark chamber was recorded. A test session lasted a maximum of 600 seconds. Any animals which failed to cross within 600 seconds were removed from the apparatus and assigned a maximum score of 600.

^{3}H-QNB Binding

Rats for ^{3}H-QNB binding studies were killed by decapitation 24 hours after the DFP or vehicle administration. Brains were rapidly removed and dissected and tissues were stored at −80°C until assayed. Binding of ^{3}H-QNB was determined in accordance with the methods described by Wastek and Yamamura [14] with modifications. A Brinkman Polytron (setting 7 for 15 sec) was used to homogenize the samples in 50 volumes (w/v) of ice-cold 50 mM tris-HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl. The homogenate was centrifuged at 30,000 g for 10 minutes. The supernatant was decanted and the resulting pellet was washed and centrifuged, as above, twice more. After the last centrifugation, the pellet was resuspended in 33.3 volumes (w/v) of ice cold 50 mM tris-HCl buffer as above. Protein on the final homogenate was determined according to Lowry *et al.* [10] and averaged approximately 0.05 mg/ml protein. Once the protein was determined, an aliquot of the final homogenate was taken and diluted with 50 mM Hepes buffer (pH 7.4) to a final concentration of 0.5 mg/ml protein for the QNB receptor binding assay.

Samples (500 μl) of the homogenate were incubated in duplicate at 37°C with 100 μl of ^{3}H-QNB at 25 pM ^{3}H-QNB concentration (specific activity, 33.1 Ci/mmole from New England Nuclear) and with 100 μl of either 50 mM Hepes buffer or 5 μM final concentration of atropine sulfate. Total assay volume was adjusted to 2 ml with 50 mM Hepes buffer. After 60 minutes the incubate was diluted with 3 ml ice-cold Hepes buffer and filtered through Whatman GF/B glass filters using a modified Brandell cell harvester (Biochemical Research and Development Laboratory, Inc., Gaithersburg, MD). Filters were washed two more times with 3 ml of buffer and placed in glass scintillation vials with 10 ml Aquasol-2 (New England Nuclear) and counted for 10 minutes in a Beckman LS 6800 liquid scintillation counter. Counting efficiency was 45%. Specific binding was defined as that which was displaced by 5 μM atropine sulfate and represented approximately 87% of total binding.

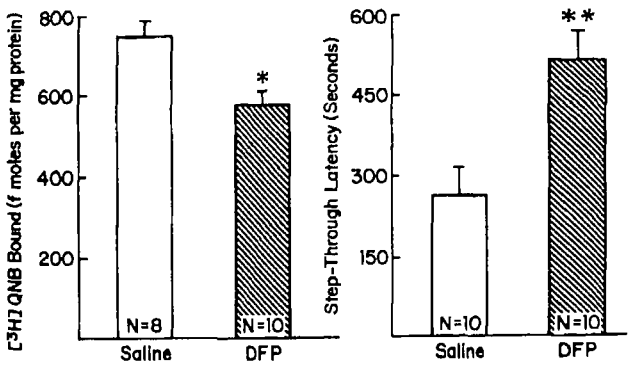

FIG. 1. Effect of DFP on [^{3}H] QNB binding in rat cerebral cortex (left) and on retention following one-trial passive avoidance training (right). Rats were decapitated for [^{3}H] QNB binding or were trained in a shuttlebox 24 hours following administration of DFP (1.2 mg/kg IP) or saline vehicle. Testing was 7 days following the training exposure. *$p<0.01$, DFP vs. saline (Student's t-test, 2-tailed); **$p<0.02$, DFP vs. saline (Student's t-test, 2-tailed).

Statistical Analysis. Two-way ANOVA and Student's t test (two-tailed) were used for statistical analyses.

RESULTS

Administration of DFP 24 hours before decapitation resulted in a 24% ($p<0.01$) reduction in cortical ^{3}H-QNB binding compared to levels in rats pretreated with vehicle (Fig. 1). A parallel group of rats was trained on a passive avoidance task 24 hours following DFP administration and tested 7 days later. Retention was significantly enhanced ($p<0.02$) by DFP pretreatment (Fig. 1). An additional group of rats, trained 24 hours following DFP and tested 14 days later, showed persistence of this effect 14 days after DFP administration (DFP 398±81.20 sec N=10, vs. Saline 193±75.34 sec, N=10. Two-way ANOVA for 7 and 14 day results: $F(1)=9.03$, $p<0.05$ for treatment effect; $F(1)=2.08$, p=NS for time effect; $F(1)=0.03$, p=NS for interaction). Rats trained 7 days following DFP administration and tested 7 days later showed no enhanced retention as compared to vehicle-treated controls (DFP 224±77.82 sec, N=10 vs. Saline 254±80.67 sec, N=10). There were no differences in training latency between the DFP and control groups.

DISCUSSION

The findings reported here confirm previous observations that pretreatment with by DFP enhances performance on memory tasks [1]. In the present study rats were exposed to the training situation 24 hours following DFP administration. Enhancement of retention was demonstrable in the DFP-treated rats when tested 7 or 14 days later. A significant reduction in cortical ^{3}H-QNB binding was present in a parallel group of animals which were decapitated 24 hours following DFP administration. The persistence of enhanced reten-

tion in animals tested 14 days after DFP administration suggests that this enhancement is due to factors influencing the training rather than the testing condition. Although increased presynaptic availability of acetylcholine due to persistent cholinesterase inhibition may still be operative 7 days after DFP administration, this could not be the case 14 days later [11]. Similarly, DFP-induced alterations in [^{3}H] QNB binding are no longer demonstrable by a week following administration of the drug [6]. Finally, the absence of any effect on retention in animals trained 7 days following DFP administration and tested 7 days later, further supports a conclusion linking the significant enhancement observed in animals trained 24 hours after DFP, to effects of the DFP operative at the time of training.

Facilitation of memory in rats trained at a time when cortical muscarinic receptor binding was demonstrably reduced would appear to contradict previous reports which associated muscarinic receptor down-regulation with impairment rather than enhancement of retention [4, 5, 9]. Such an association was reported after both chronic physostigmine [9] and repeated ECS [4,5] administration. This apparent discrepancy may, however, be explained if presynaptic factors are considered in conjunction with post-synaptic effects. Russell *et al.* [11] reported that 24 hours following DFP administration (1.0 mg per kg) brain acetylcholineesterase levels were reduced by 70% and brain acetylcholine levels were increased by 40% over control values. Presynaptic availability of acetylcholine was thus considerably increased 24 hours following a dose of DFP similar to that used in the present study. It is therefore plausible that following DFP administration, increased presynaptic availability of acetylcholine may be sufficient to ensure a net increase in cholinergic neurotransmission in spite of a compensatory reduction in post-synaptic muscarinic receptor number. This would then explain the enhanced memory response in rats pre-treated with DFP, in spite of a reduction in muscarinic binding sites.

Following chronic ECS [4,5] or chronic physostigmine [9] administration, presynaptic agonist availability may not be sufficient to outweigh post-synaptic muscarinic receptor subsensitivity. ECS-induced alterations in acetylcholine turnover are short-lived (present 30 sec after the seizure) and no longer demonstrable by 5 minutes [8]. Physostigmine is a reversible inhibitor of acetylcholine esterase and in the experiment reported by Loullis *et al.* [9], administration was ceased 24 hours prior to training. Following ECS and physostigmine administration, reduced muscarinic receptor density could therefore result in a net reduction in cholinergic neurotransmission which is not balanced or superceded by increased presynaptic availability of the agonist.

Drug-induced alterations in cholinergic neurotransmission should therefore be considered on the background of presynaptic agonist availability in conjunction with changes in post-synaptic receptor sensitivity. The effect of cholinomimetics or cholinergic antagonists on memory is likely to represent the net result of a complex interaction between such presynaptic and postsynaptic factors.

ACKNOWLEDGEMENTS

The authors thank Michael Keegan and Sandra Demetriou for valuable technical assistance and Mary Ratza for typing the manuscript.

REFERENCES

1. Banks, A. and R. W. Russell. Effects of chronic reductions in acetylcholinesterase activity on serial problem solving behavior. *J Comp Physiol Psychol* **64**: 262–267, 1967.
2. Essman, W. B. *Neurochemistry of Cerebral Electroshock*. New York: John Wiley, 1973.
3. Gazit, H., I. Silman and Y. Dudai. Administration of an organophosphate causes a decrease in muscarinic receptor levels in rat brain. *Brain Res* **174**: 351–356, 1979.
4. Lerer, B., M. Stanley and H. Altman. Electroconvulsive shock and brain muscarinic receptors: Relationship to anterograde amnesia. Submitted.
5. Lerer, B., M. Stanley, S. Demetriou and S. Gershon. Effect of electroconvulsive shock on ^{3}H-QNB binding in rat cerebral cortex and hippocampus. *J Neurochem* **41**: 1680–1683, 1983.
6. Levy, A. The effect of central cholinesterase inhibition on the ontogenesis of central muscarinic receptors. *Life Sci* **29**: 1065–1070, 1981.
7. Levy, A., J. Zohar and R. H. Belmaker. The effect of chronic lithium pretreatment on rat brain muscarinic receptor regulation. *Neuropharmacology* **21**: 1199–1201, 1982.
8. Longoni, R., A. Mulas, B. O. Novak, I. M. Pepeu and G. Pepeu. Effect of single and repeated electroconvulsive applications on brain acetylcholine levels and choline acetyltransferase activity in the rat. *Neuropharmacology* **15**: 283–286, 1976.
9. Loullis, C. C., R. L. Dean, A. S. Lippa, L. P. Meyerson, B. Beer and R. T. Bartus. Chronic administration of cholinergic agents: Effects on behavior and calmodinlin. *Pharmacol Biochem Behav* **18**: 601–604, 1983.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Russell, R. W., D. H. Overstreet, C. W. Cotman, V. G. Carson, L. Churchill, F. W. Dalglish and B. J. Vasquez. Experimental tests of hypotheses about neurochemical mechanisms underlying behavioral tolerance to the anticholinesterase, diisopropyl fluorophosphate. *J Pharmacol Exp Ther* **192**: 73–85, 1975.
12. Schiller, G. D. Reduced binding of [^{3}H]-quinuclidinyl benzilate associated with chronically low acetylcholinesterase activity. *Life Sci* **24**: 1159–1164, 1979.
13. Takahashi, R., T. Nasu, T. Tamura and T. Koriya. Relationship of ammonia and acetylcholine levels to brain excitability. *J Neurochem* **7**: 103–112, 1961.
14. Wastek, G. J. and H. I. Yamamura. Biochemical characterization of the muscarinic cholinergic receptor in human brain: Alterations in Huntington's Disease. *Mol Pharmacol* **14**: 768–780, 1978.