BEHAVIORAL BIOLOGY **21**, 358–363 (1977)

Phosphorylation of Proteins of Synaptosome-Enriched Fractions of Brain during Short-Term Training Experience: The Effects of Various Behavioral Treatments[1,2]

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The relationship between the phosphorylation of synaptosomal proteins and the behavior of mice subjected to foot-shock avoidance conditioning has been studied. It appears that the increased phosphorylation of these proteins in the trained mouse is specific to the training, since mice that were merely exposed to the training stimuli or performed the avoidance after they had been previously trained did not show the response. However, mice that extinguished the learned behavior did show such increases. These changes may be relevant to the establishment of neural pathways that enable storage of memory of a novel experience.

As described in the foregoing paper (Perumal *et al.*, 1977), following administration of radioactive orthophosphate there is an increased accumulation of covalently bound radioactivity in mouse brain proteins derived from synaptosome-enriched fractions, apparently induced by a short-term training experience. The present paper attempts to elucidate the behavioral aspect of the experience that leads to the observed increase in the radioactivity of these proteins.

Unless specified otherwise, male C57BL/6J mice (Jackson Laboratories, Maine) 6–8 weeks old, were used. In one series of experiments, female mice of the same strain and age were used. The intracranial

[1] This research was supported by grants from the U.S. Public Health Service (NS-07457, MH-18136), the U.S. National Science Foundation (GB-18551 and BMS72-02341 A02), the Ciba-Geigy Corporation, and the Camille and Henry Dreyfus Foundation, Inc.

[2] We thank A. J. Dunn and D. Entingh for helpful suggestions.

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ISSN 0091-6773

injection of $H_3{}^{32}PO_4$ and $H_3{}^{33}PO_4$, isolation of the synaptosome-enriched fraction and its subsequent chemical fractionation, isolation of AMP, the determination of radioactivity, and the statistical analysis have been described in the previous paper (Perumal *et al.*, 1977).

The training procedure used for mice is described previously (Adair *et al.*, 1968; Perumal *et al.*, 1977). The variations in experience undergone by various groups of animals are: (1) *Quiet animals*. The mice (*Q*) were injected with the radioisotope and returned to their home cages where they remained until sacrifice. (2) *Yoked mice*. Twenty-nine minutes after injection these mice (*Y*) were placed in the compartment of the jump-box without a shelf so that they were subjected to the same amount of conditioned stimuli (light and buzzer) and unconditioned stimulus (foot-shock) as their trained counterparts. However, they could neither escape nor avoid the foot-shock. They were handled to the same extent as the mice undergoing training. (3) *Extinction training of mice*. These were mice (*E*) that had been subjected to one 15-min training session per day on each of 3 successive days, prior to the experiment. On the day of the experiment, 30 min following injection, the mice (*E*) were subjected to a 15-min session in the training compartment of the jump-box during which 30 extinction trials were presented (Coleman *et al.*, 1971a). An extinction trial consisted of presentation of the conditioned stimuli but not the unconditioned stimulus.

The accumulation of radioactive phosphate in proteins obtained from the synaptosome-enriched fractions was studied in pairs of mice of which one was trained for 15 min (*T*) while the other remained in its home cage (*Q*). Figure 1A shows the mean acquisition curve for six naive, injected mice. All animals acquired the avoidance response and made an average of only eight escape responses compared to 22 avoidance responses. Table 1 shows that the amount of radioactive phosphate in the $HClO_4$ residue of the synaptosome-enriched fraction was higher in the trained mice, as the mean ratio of dpm *T*/*Q* in the residue corrected for that in AMP (see Perumal *et al.*, 1975, 1977) was significantly higher than 1.00.

Subsequently, six groups of mice with three mice per group were injected with $H_3{}^{32}PO_4$ and $H_3{}^{33}PO_4$. Two mice of a group received the same isotope, while the third received the alternative. One of the two mice with the same isotope was subjected to training in the jump-box, whereas the other mouse underwent the yoke treatment (*Y*). The remaining animal stayed in the home cage (*Q*). Immediately after the training session, the trained mouse was discarded and the other two were killed and their brains processed together. All the trained animals acquired the avoidance response. Table 1 shows that the amount of radioactive phosphate in the $HClO_4$ residue of the synaptosome-enriched fraction was the same for yoked and quiet mice.

One of a pair of female mice was subjected to training in the jump-box,

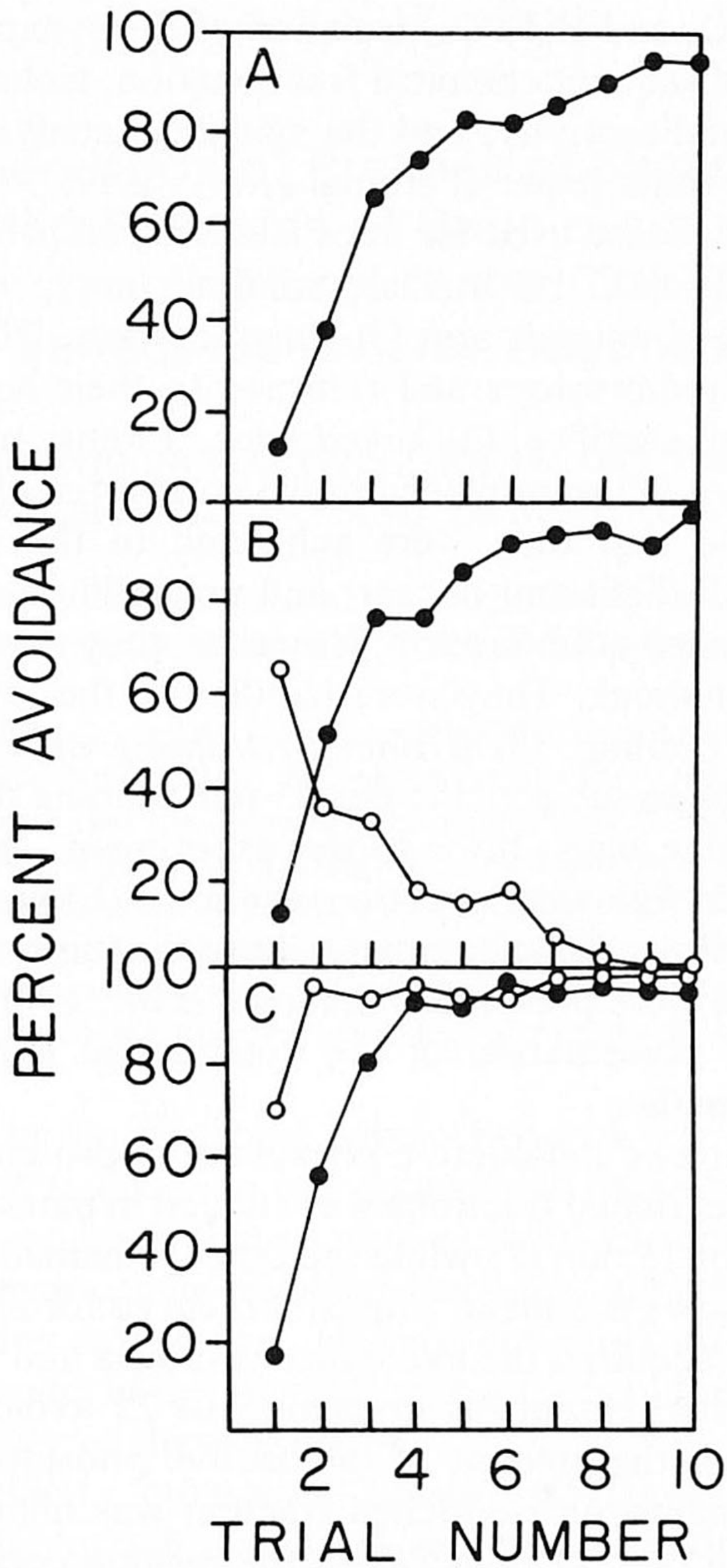

FIG. 1. Acquisition, performance, and extinction of the conditioned avoidance responses. (A) The curve shows the average number of avoidances of 21 naive mice during training. The mice were injected intracranially and 30 min later were trained individually in the jump-box training apparatus for 15 min, as described in the text. (B) The closed circles represent the average avoidance responses of 17 uninjected naive mice trained in the jump-box for 15 min. Following this, they were subjected to a training session per day on 2 successive days. The open circles represent the avoidance responses under extinction conditioning on the fourth day, when the mice were injected, placed in the jump-box 30 min later, and given a training session in which only the unconditioned stimuli (light and buzzer) but not the conditioned stimulus (shocks) were presented. (C) The closed circles show the average number of avoidance responses of 18 uninjected mice trained in the jump-box for 15 min. They were then given a similar training session per day on the 2 successive days. The open circles represent the average of their performance, 30 min after intracranial injections on the day of the experiment (fourth day). Trial numbers refer to the average of blocks of three trials.

TABLE 1
The Effect of Various Behavioral Experiences on Phosphorylation of Proteins of Synaptosome-Enriched Fractions of Mouse Brain

	n	dpm phosphoprotein, experimental/control / dpm AMP, Experimental/control
T/Q[a]	6	1.39 (0.12)[b]*
Y/Q	6	1.00 (0.17)
T/Y[c]	10	1.17 (0.15)*
E/Q	17	1.33 (0.62)*

[a] T = Trained; Q = quiet; Y = yoked; E = extinguished; n = number of animals.
[b] Median ratio, with interquartile range in parentheses.
[c] Female mice.
* $2P < 0.001$ (two-tailed sign test).

while the other served as yoked control (Y). Immediately after training, both the mice were killed and their brains were processed. All the trained animals acquired the avoidance response. Comparison of female trained and yoked mice (Table 1) indicates an increase in the accumulation of radioisotope in synaptosomal proteins in the former ($2P < 0.001$). This indicates that female mice show the same change in synaptosomal phosphoproteins that was previously observed in the male mice, assuming that female mice do not show altered phosphorylation in response to the yoke experience, as is true of males.

One mouse of each pair was subjected to the extinction experience in the jump-box (E), while its naive counterpart remained in the home cage (Q) throughout the entire period. Immediately following the extinction session, each pair was sacrificed and the brains were processed together. Figure 1B shows the record of the mean avoidances during the training and extinction sessions. It is probable that the slightly lower level of avoidance during the first few extinction trials was a result of a traumatic effect of the intracranial injections. All animals extinguished to the 0% avoidance level by the end of the sessions. It can be seen from Table 1 that there was a definite increase in the accumulation of radioisotope in the proteins from the synaptosome-enriched fraction in the extinguishing animals ($2P < 0.001$). The magnitude of the increase is comparable to that found when trained naive mice are compared with quiet.

The results reported here for naive trained animals are comparable to those observed earlier on the incorporation of uridine into brain RNA (Adair *et al.*, 1968; Coleman *et al.*, 1971a, b; Zemp *et al.*, 1966) and to those on the phosphorylation of nucleur nonhistone acid-extractable proteins (NAEP) (Machlus *et al.*, 1974). However, with regard to extinguishing animals, there appear to be differences among the various brain responses studied. The altered incorporation of uridine into RNA and the increased phosphorylation of synaptosomal proteins seem to occur exclu-

sively during novel training or extinction, but not during performance of an already learned response. Increased phosphorylation of NAEP appears to occur not only during the novel training experience but also when the animal is reminded of the training it previously experienced (Dunn *et al.*, 1974; Machlus *et al.*, 1974). Routtenberg *et al.* (1975) have reported that alterations in the *in vitro* incorporation of $^{32}P_i$ into specific membrane proteins from rat brain subcellular fractions can be observed at least 24 hr after a passive avoidance experience.

It would be of importance to know if short-term conditioned avoidance and extinction are the only types of experience to which this phosphorylation of synaptosomal proteins responds. In a series of preliminary experiments, one mouse of each pair was subjected to a prior-trained performing experience (*P*), as described by Adair *et al.* (1968), while the other was a naive animal that remained in its home cage (*Q*). Immediately after the last session, the pairs were killed and both their brains were processed together. Figure 1C shows the mean avoidance acquisition of the mice on the first day of training and performance on the day of the experiment. As was found previously by Adair *et al.* (1968), the injection per se interferes to some extent with the performance of the mice, leading to a lower avoidance level during the first few trials on the day of the experiment as compared with avoidance levels during the preceding days of performance. Nonetheless, the prior-trained performing mice make significantly more avoidances than the naive trained mice. Although this experiment was hampered by a substantial variability in the ratio of dpm *P*/*Q* in protein to that in AMP, the median of the ratios was not significantly different from 1.00, and it is therefore suggested that the phosphorylation response does not occur in the prior-trained mice performing the acquired response.

The fact that there is a difference in phosphorylation between Trained and Quiet, Extinguished and Quiet, and Trained and Yoked, but not between Yoked and Quiet suggests that the response is not triggered by the unconditioned stimuli, the conditioned stimulus, handling, jumping onto the shelf, etc. per se, even though these experiences are undoubtedly part of the training paradigm. The phosphorylation of synaptosomal proteins may be a crucial step in the chain of neural events that underlie acquisition or retention of a novel behavior. Further work is in progress to shed light on the relevance of the present results in view of the hypothesis (Entingh *et al.*, 1975) that such chemical changes, by inducing alterations in the conformation of proteins, may regulate interneuronal connectivity, thus developing new circuitry enabling memory storage or retrieval.

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