Phosphorylation of Proteins from the Brains of Mice Subjected to Short-term Behavioral Experiences

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Over the past decade numerous studies have been performed attempting to relate macromolecular events in the central nervous system to learning and memory (see for instance Glassman, 1969; Gispen and Schotman, 1973; Dunn *et al.*, 1974; Rees *et al.*, 1974). Such studies proved to be very difficult and results obtained by different laboratories often appeared to be contradictory. Presumably, such difficulties occur for at least two reasons. First, most of the molecular events taking place in nerve tissue are not yet elucidated, partly because of the limitations of the neurochemical methods available. Second, there are problems concerning the measurement of learning and memory. Since these processes can only be approached indirectly by analyzing the performance of a given subject in a stimulus situation, other factors such as motivation, fatigue etc., may interfere with the observation. Even if an experimental design could be adopted that would selectively deal with learning and memory, the choice of appropriate experimental groups for comparison would be extremely difficult. In active avoidance conditioning, for instance, animals are subjected to stimuli which by themselves may alter brain metabolism (Jakoubek *et al.*, 1972; Gispen and Schotman, 1975; Glassman, 1974; Rees *et al.*, 1974) and these changes may be totally independent of the processes underlying the acquisition of the new behavior. Appropriate behavioral controls, therefore, are key experiments in studies on molecular processes related to learning and memory.

Throughout the literature one can find the idea that plasticity in the central nervous system may be an important mechanism in the acquisition and storage of behavioral experience in the brain (Horn *et al.*, 1973). Previously, it was suggested that one possible mechanism for molecular control of the interneuronal connectivity that could lead to the formation of new neural pathways or networks would be through conformational changes resulting from an altered phosphorylation of synaptic proteins (Glassman *et al.*, 1973). The present paper describes the phosphorylation of proteins

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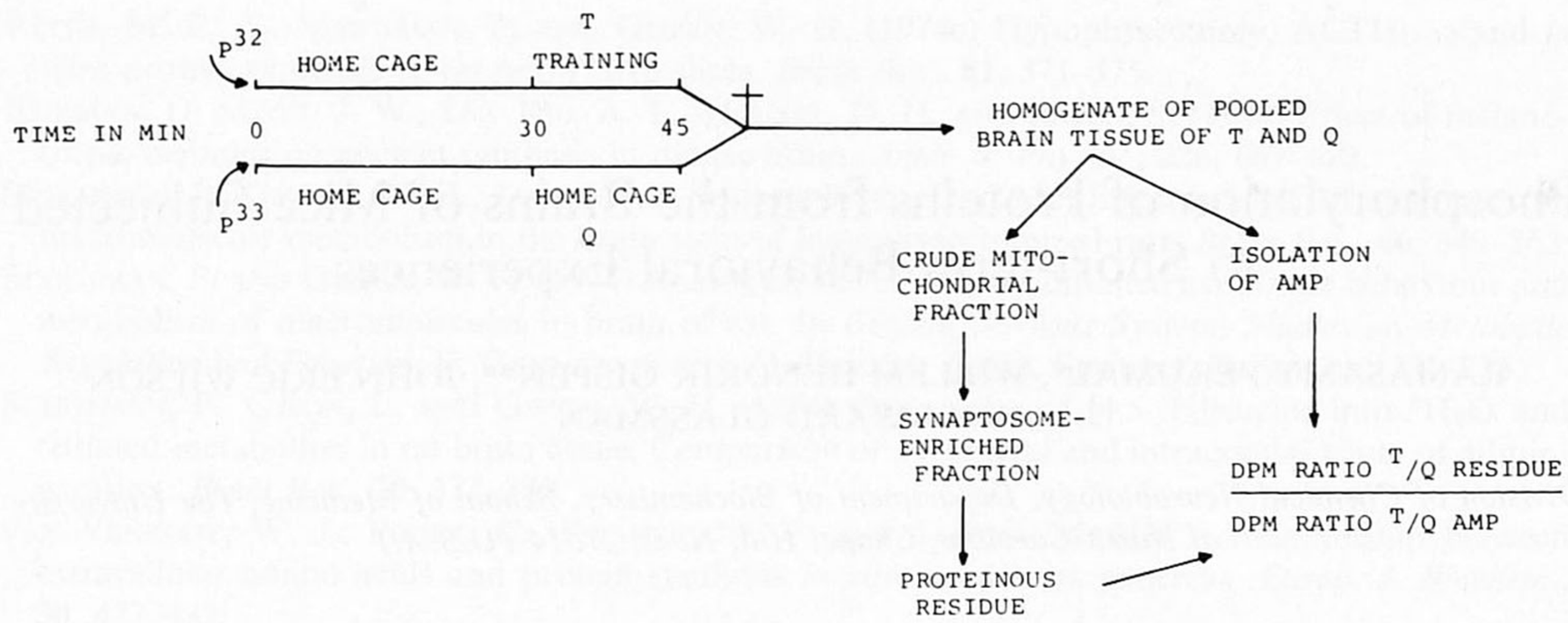

Fig. 1. Experimental design.

obtained from enriched synaptosomal fractions from the brains of mice subjected to short training experiences.

An outline of the experiment is presented in Fig. 1. One of a pair of 6–8-week-old male mice (C57BL/6J from the Jackson Laboratories) was injected intracranially (Adair *et al.*, 1968) with approximately 20 μCi [^{32}P]orthophosphate in 20 μl water, while the other was injected with 20 μCi of carrier-free [^{33}P]orthophosphate from ICN, Irvine, Calif., U.S.A. The mice were coded and placed in their home cages. Twenty-nine minutes after injection one mouse was placed in a jump-box training apparatus and trained as described previously (Zemp *et al.*, 1966). The training lasted 15 min and consisted of about 30 trials. On the average, a mouse made 22 conditioned avoidance responses out of the 30 trials (Fig. 2). The untrained mouse stayed in the home cage during the entire 45 min and will be referred to as Quiet.

At the end of the training, both mice were decapitated after minimal ether anesthetization. The brains were removed and the olfactory bulbs and cerebellum excised. The rest of the brains of both mice were homogenized together at 0–4 °C in 10 ml 0.1 m*M*

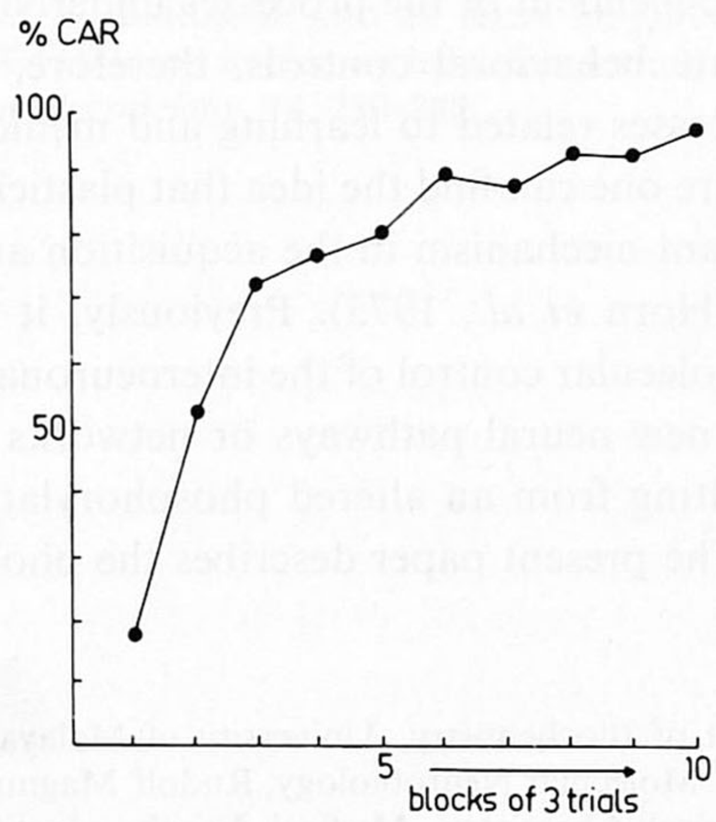

Fig. 2. Mean performance of 6 mice, intracranially injected with radioactive orthophosphate.

sodium phosphate buffer pH 7.6, containing 0.32 *M* sucrose, and a crude mitochondrial pellet was isolated from the homogenate. This was suspended and layered on a discontinuous sucrose gradient (0.8 and 1.2 *M* sucrose), and a synaptosome-enriched fraction was obtained as described by Whittaker (1969). The use of Ficoll–sucrose gradients (Herschman *et al.*, 1972) gave similar results. Electron microscopy confirmed that the preparations used were enriched in intact synaptosomes. The synaptosome-enriched fraction was osmotically shocked in 1 m*M* (sodium) phosphate, pH 7.6 and frozen overnight. Ice-cold $HClO_4$ was added to a final concentration of 0.25 *M*. The residue obtained by centrifugation was then washed twice with ice-cold 0.25 *M* $HClO_4$. Lipids were extracted using acidified 2:1 chloroform–methanol (Bligh and Dyer, 1959). The solid interphase layer was carefully removed and washed twice with ice-cold 0.25 *M* $HClO_4$, then incubated in 0.25 *M* $HClO_4$ at 90 °C for 15 min. Following centrifugation, the sediment was washed twice with cold 0.25 *M* $HClO_4$, and dissolved in Soluene by heating for 2 hr at 50–60 °C. Radioactivity was determined in aliquots from all gradient fractions and extracts. Liquid scintillation counting was performed in a TriCarb Liquid Scintillation Spectrometer Model 3375, using a mixture of toluene–Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (PPO, 4 g/l). For each batch of isotope a set of standards quenched to different degrees with different amounts of chloroform was prepared. The correlation between the automatic external standardization and the counting efficiency of the standards was used to compute the disint./min and the ratio of the disint./min in each channel.

The nucleoside monophosphates in 1 ml of the homogenate were separated by means of thin-layer chromatography (Zemp *et al.*, 1966). The nucleotide spots were scraped off, eluted with 0.1 *N* HCl in scintillation vials, and the radioactivity was determined. The radioisotope ratios of the various brain fractions were corrected for that observed in AMP. The ratio observed in AMP did not differ from that of GMP, UMP or CMP. The radioisotope ratio in AMP was used as a correction factor for the availability of intracellular radioactive phosphate, which varies with the amount of leakage during injection in each brain.

The synaptosome-enriched fraction contained about 27 % of the radioactive phosphorus of the total gradient, and about 75 % of this radioactivity was acid-soluble. About 18 % of the total radioactivity in the synaptosome-enriched fraction was in phospholipid. Almost all of the radioactivity in the final residue was radioactive phosphate covalently bound to proteins (Perumal *et al.*, in prep.). This was shown by the fact that (a) incubation of the residue with alkaline phosphatase rendered over 90 % of the radioactivity acid-soluble; (b) RNAse digestion was ineffective in this respect; (c) electrophoresis of tryptic digests revealed radioactivity coincident with ninhydrin positive material; (d) pronase digestion of the residue resulted in the solubilization of 85–95 % of the radioactivity; and (e) pronase or HCl hydrolysates contained radioactive phosphoserine and phosphothreonine (Perumal *et al.* in prep.).

In 14 out of the 15 mouse pairs, the washed acid-insoluble residue from the synaptosome-enriched fraction from the trained mouse contained more radioactive phosphate than the residue from the quiet mouse, as was apparent from the increase in the ratio

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$\frac{\text{disint./min ratio T/Q residue}}{\text{disint./min ratio T/Q AMP}}$ (see also Fig. 1). The mean relative increase in disint./min in the residue obtained from trained mice was 34.1 %. Although the difference was variable it occurred very consistently throughout our experiments when training was involved (Perumal *et al.*, in prep.; Gispen *et al.*, in prep.). Differences in other fractions obtained during the extraction procedure were much more variable and the average ratios $\frac{\text{disint./min ratio T/Q residue}}{\text{disint./min ratio T/Q AMP}}$ did not differ significantly from 1.00. Furthermore, it was found that this increased phosphorylation occurred mainly in the particulate material from the osmotically shocked synaptosome-enriched fraction, and it was also possible to identify phosphoproteins from isolated membrane fractions on SDS polyacrylamide gels (Perumal *et al.*, in prep.).

To investigate the time relationship between avoidance conditioning and the increased phosphorylation of proteins of the synaptosome-enriched fraction, 35 pairs of mice were injected intracranially with [^{32}P]- and [^{33}P]orthophosphate as described previously. Fifteen minutes after injection, 5 pairs were killed. Three groups of 6 mice were trained 30 min after injection for periods of 5, 10 or 15 min, respectively. Each trained mouse and its quiet control were sacrificed immediately after the training experience. Of the remaining 12 pairs, a mouse of each pair was trained 30 min after injection for 15 min, and then returned to the home cage. Killing was delayed for 15 min (6 pairs) or 30 min (6 pairs). At the end of the 5 min of training, the mice avoided at an average level of 50 % at the end of 10 min, about 80 % and at the end of 15 min, over 90 %. In all cases, quiet mice were used for comparison. The brains of all pairs were processed as described previously.

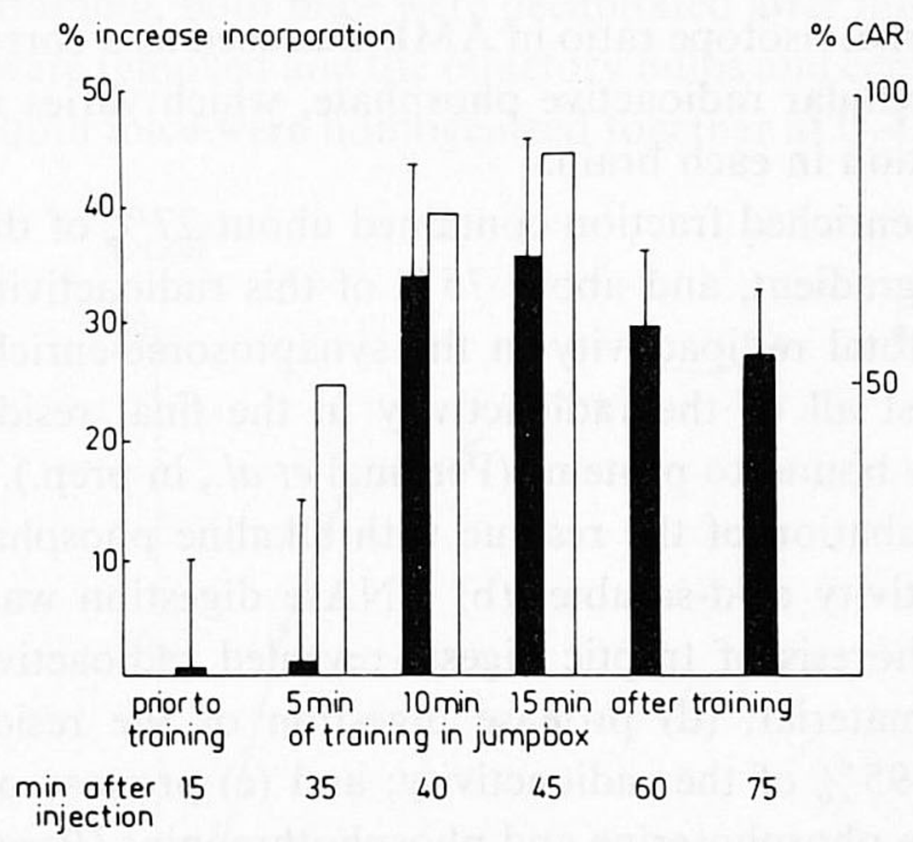

Fig. 3. The percentage increase in phosphate incorporation into proteins from synaptosome-enriched fractions of brains of trained mice. The open bars represent the mean performance of the trained mice. The closed bars represent the mean increase in the ratio $\frac{\text{disint./min ratio T/Q residue}}{\text{disint./min ratio T/Q AMP}}$. On the horizontal axis the time of killing the mice is indicated.

Fig. 3 shows that before training, and even after 5 min of training no difference exists within the pairs with respect to the neurochemical parameter studied. With further training for 10 or 15 min, however, there is a significant increase in the radioactivity in proteins obtained from synaptosome-enriched fractions; this increase lasts at least 30 min after training. The average ratio of the isotopes in AMP did not differ significantly between the various time points. The data therefore suggest that after a certain amount of training experience an increased phosphorylation of proteins obtained from synaptosome-enriched fractions occurs, lasting beyond the actual period of training.

One would very much like to speculate on the mechanism underlying the altered phosphorylation of these proteins. Many important factors are still unclear, however (*e.g.* what is the phosphate donor? Supposing ATP is the donor, how can one routinely isolate ATP in undegraded form from the same homogenate that is further processed to obtain a synaptosome-enriched fraction? Is one dealing with precursor pool compartmentation and what is the specific activity of these pools? etc.). With respect to the time relationship between avoidance conditioning and the altered phosphorylation, it is clear that a great deal of work needs to be done, before one could even begin to speculate on whether the effect is on synthesis or degradation of phosphoproteins. For this reason we do not speculate on the mechanism even though this is a very important, but at the moment unresolvable, point, but merely refer to an increased amount of radioactive phosphate in the synaptosome-enriched fraction.

The experimental mice were subjected to a variety of behavioral conditions in an attempt to assess the specificity of the observed phenomenon. Such experiments were performed according to Adair *et al.* (1968) and included the following experimental groups for comparison: yoked *versus* quiet and prior-trained-performing *versus* quiet. No significant differences existed with respect to the phosphorylation of the protein fractions of these groups. The ineffectiveness of the yoke procedure is puzzling since in this procedure the mice also underwent a new short-term behavioral experience. Since we do not know what clue(s) in the behavioral experience (jump-box conditioning) trigger(s) the neurochemical response, we are still in a state of collecting data and not fully understanding them. However, it should be noted that the experiments on the effects of various behaviors on uridine incorporation into brain RNA also show that the yoked control does not differ from the quiet control, using the same training apparatus (Zemp *et al.*, 1966; Adair *et al.*, 1968). The ineffectiveness of the yoked and prior-trained performing procedure combined with the finding that the T/Q ratios of the mononucleotides were the same make it highly unlikely that the reported increase in ratio would in fact be due to a decrease in adenyl nucleotide concentration as would occur in rat brain after footshock stress (Dickman *et al.*, 1973). Therefore, it was concluded that the stimulation of the conditioning procedure and the motor activity of the mouse are not responsible for the increased phosphorylation of protein obtained from synaptosome-enriched fractions.

At present the biological significance of this effect is not clear. Recently it has been shown that phosphorylation of synaptosomal membrane protein is c-AMP dependent (Johnson *et al.*, 1971; Johnson *et al.*, 1972; Ueda *et al.*, 1973). It has been claimed that

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an altered turnover of these proteins in brain tissue could result from electrical stimulation or stimulation by putative transmitter (Heald, 1957, 1962; Trevor and Rodnight, 1965; Jones and Rodnight, 1971; Reddington and Rodnight, 1972). Moreover, a unique type of synaptic transmission involving transmembrane responses to c-AMP has been suggested (Hoffer *et al.*, 1971). A connection between nerve cell activity, c-AMP levels and phosphorylation of synaptic membrane elements has also been postulated (Greengard *et al.*, 1971). The function of brain phosphoproteins has been thought to be linked to ion transport (Wolff and Siegel, 1970; Jones and Rodnight, 1971; Donella *et al.*, 1972). The above cited literature does in fact underscore how little we know of the function of brain phosphoproteins. Notwithstanding this, it may very well be that phosphoproteins are indeed key elements in the brain's response to behavioral experiences, since the phosphorylation of nuclear nonhistone acid-extractable proteins in rat brain is also affected by various behaviors and reminding experiences (Machlus *et al.*, 1974a and b). Further work is in progress to elucidate the role of synaptosomal membrane phosphoproteins with regard to these ideas.

SUMMARY

Proteins of synaptosome-enriched fractions of mouse brain exhibited an increase in the incorporation of radioactive phosphate during 15 min of avoidance conditioning. The increase was initiated between 5 and 10 min of the training and could be detected 30 min beyond the training period.

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