Effects of Behaviorally Active ACTH Analogs on Brain Protein Metabolism

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Removal of the pituitary in rats impairs acquisition of conditioned avoidance behavior in the shuttle box. The poor performance of these rats is restored towards normal by treatment with ACTH (corticotropin) (de Wied, 1964, 1969, 1974). The influence of ACTH on avoidance acquisition is due to an extra target effect, since the rate of acquisition is also normalized by the ACTH fragments $ACTH_{1-10}$ or $ACTH_{4-10}$ which have no detectable corticotropic, systemic or metabolic effects (de Wied, 1969). Under similar conditions the isomer $ACTH_{1-10}$ or $ACTH_{4-10}$ with D-phenylalanine substituted in the 7th position, has no or even a reversing effect on the already deficient avoidance learning in hypophysectomized rats (de Wied *et al.*, 1972). In intact rats extinction of an avoidance response is sensitive to the treatment with ACTH; administration of this hormone during the extinction period markedly delays extinction of a shuttle box (de Wied, 1967) as well as a pole-jumping avoidance response (de Wied, 1974). This effect again is independent of its action on the adrenal cortex, since smaller fragments of ACTH, devoid of corticotropic activity, also increase resistance to extinction. Furthermore, replacement of the phenylalanine residue in position 7 by the D-isomer reverses the effect of ACTH fragments and induces facilitation of extinction (de Wied, 1974). The behavioral effects of these peptides are caused by a direct action on central nervous structures, presumably at the posterior thalamus level (nucleus parafascicularis) as shown by lesion and implantation studies (Bohus and de Wied, 1967; van Wimersma Greidanus and de Wied, 1971; van Wimersma Greidanus *et al.*, 1974).

Previous work suggests that there are neurochemical correlates in the rat brain to the behavioral effect of these neuropeptides (Gispen and Schotman, 1973). It was found that acquisition of avoidance behavior in the shuttle box in hypophysectomized rats treated with $ACTH_{1-10}$ restores the reduced brain stem polysome population in these rats as measured at the end of the training period (Gispen *et al.*, 1970; Gispen and Schotman, 1970; Gispen *et al.*, 1971). Removal of the pituitary also decreases the incorporation of [^{3}H]leucine into brain stem proteins, measured 5 min after injection of the precursor into the diencephalon (Schotman and Gispen, 1974). Treatment of these rats with $ACTH_{1-10}$-7-L-Phe or $ACTH_{4-10}$-7-L-Phe restores leucine incorporation towards normal values (Schotman *et al.*, 1972; Reith *et al.*,

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1974a). The relationship between the neurochemical and behavioral activities of these ACTH fragments seems rather specific, since the behaviorally inactive sequence $ACTH_{11-24}$ has no effect on the leucine incorporation under similar conditions and the analog $ACTH_{1-10}$-7-D-Phe even further decreases the suppressed incorporation rate in hypophysectomized rats (Schotman *et al.*, 1972). Presumably, these changes in leucine incorporation as found after hypophysectomy and treatment with ACTH fragments reflect alterations in the synthesis of rat brain stem proteins. From a series of experiments on size and metabolism of the precursor pool (Schotman *et al.*, 1972; Reith *et al.*, 1973; Reith *et al.*, 1974b; Schotman *et al.*, 1974) it was concluded that the observed changes in incorporation cannot be accounted for by the effects on the precursor pool. The results with $ACTH_{1-10}$-7-L-Phe or $ACTH_{4-10}$-7-L-Phe on protein synthesis are consistent with those of Reading and Dewar (1971) in intact animals. Rudman *et al.* (1974) studied the effects of melanotropic peptides which share the sequence $ACTH_{4-10}$, on protein synthesis in mouse brain; their observations on the penetration of α-amino isobutyric acid, and the accumulation, the level and incorporation of various amino acids also suggest a stimulatory effect of these peptides on brain protein synthesis.

The data so far do not indicate whether the observed alterations in leucine incorporation are due to a general effect on protein synthesis or to an effect restricted to certain protein species. Previously it was speculated that removal of the pituitary would deplete the rat of pituitary peptides and their breakdown products (Gispen and Schotman, 1973). These peptides (like ACTH fragments) are considered to play a crucial role in avoidance learning of the animal by enhancing the synthesis of certain brain stem proteins. As a result of the lack of these proteins the hypophysectomized rat is unable to store the information necessary to master the task in shuttle box conditioning. Substitution of ACTH-like peptides stimulates the production of such proteins and therefore leads to normal acquisition behavior (Gispen and Schotman, 1973). In subsequent studies on the mechanism of action of these neuropeptides, brain stem proteins were investigated in more detail. Proteins were extracted sequentially with solutions of increasing solubilizing capacity (Grossfeld and Shooter, 1971). By a stepwise treatment with an aqueous buffer, a non-ionic detergent (Triton X-100) and an ionic detergent (SDS), three main protein fractions were obtained. Each fraction was subjected to fractionation on SDS–polyacrylamide gels (Choules and Zimm, 1965; Weber and Osborn, 1969). Both the protein composition and the radioactivity distribution of the gels were determined, the proteins being labeled *in vivo* by a 5-min pulse of [^{3}H]leucine injected into the diencephalon as in the experiments described above (Schotman *et al.*, 1972). The results of this study suggested that hypophysectomy caused an overall decrease in labeling of all protein species, whereas treatment of hypophysectomized rats with $ACTH_{1-10}$-7-L-Phe resulted in a general increase (Reith *et al.*, 1974a; Gispen *et al.*, 1974). These data do not seem to support the view that stimulation of leucine incorporation into proteins by neuropeptides is restricted to certain species of brain stem proteins. In contrast they indicate an influence of the peptide on the mechanism of overall protein synthesis. To substantiate this hypothesis, *in vitro* incorporation of radioactive leucine into

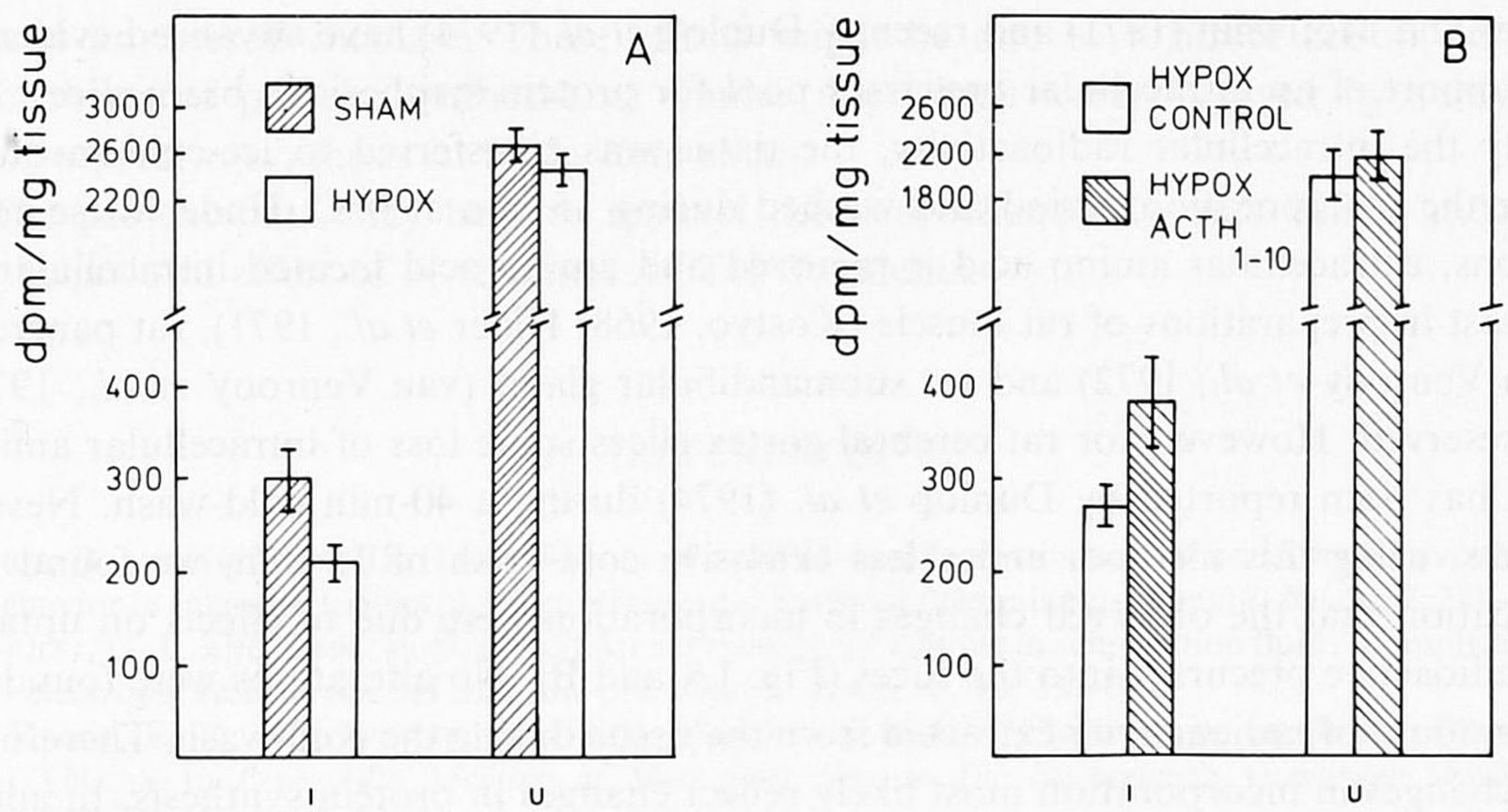

Fig. 1. The *in vitro* uptake (U) and incorporation (I) of [U-^{14}C]leucine in rat brain stem slices. Slices were preincubated for 30 min before adding 1 μCi [^{14}C]leucine and incubation continued for another 30 min. At the end of the incubation, slices were washed three times with ice-cold medium containing unlabeled leucine, each time for 5 min. Results are the mean $\pm$ S.E.M. (vertical bar) for 9 or 10 slices. A: hypophysectomized *versus* sham-operated animals. B: slices, obtained from hypophysectomized rats, incubated with and without 10^{-5} *M* ACTH$_{1-10}$-7-L-Phe.

proteins in slices from the posterior thalamus was studied. Advantages of the *in vitro* system are a controlled supply of the precursor and the possibility to measure precursor uptake simultaneously.

The *in vitro* experiments were carried out 3 weeks after hypophysectomy or sham-operation. The animals were sacrificed by decapitation and a brain stem slice was prepared at about 3400 μm from the frontal zero plane (König and Klippel, 1963) as described elsewhere (Reith *et al.*, 1974c). The slice obtained contained posterior thalamic tissue including the nucleus parafascicularis and weighed approximately 25 mg. This particular brain area was chosen since the nucleus parafascicularis presumably is implicated in the action of ACTH analogs on central nervous structures as pointed out above (van Wimersma Greidanus and de Wied, 1971; van Wimersma Greidanus *et al.*, 1974). After 30 min preincubation, the incorporation of L-[U-^{14}C]-leucine into proteins was measured as described elsewhere (Reith *et al.*, 1974c). The incorporation increased linearly up to at least 60 min after addition of the radioactive precursor. An incorporation period of 30 min was chosen to study the effects of hypophysectomy and ACTH$_{1-10}$-7-L-Phe. Fig. 1A shows the results obtained with slices of hypophysectomized and sham-operated animals. Three weeks after removal of the pituitary, a marked decrease in incorporation was found amounting to approximately 30%. When ACTH$_{1-10}$-7-L-Phe was present in a concentration of 10^{-5} *M* during incubation of slices from hypophysectomized rats, a significant stimulation in incorporation was observed (+ 42%; Fig. 1B). In a first attempt to investigate whether effects on uptake phenomena are involved in these changes in incorporation, an estimation was made of the radioactivity of intracellular amino acid, which has always been considered the source for protein synthesis in brain. Indeed

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Jones and McIlwain (1971) and recently Dunlop *et al.* (1974) have advanced evidence in support of an intracellular precursor pool for protein synthesis in brain slices. To study the intracellular radioactivity, the tissue was transferred to ice-cold medium after the incorporation period and washed during 15 min at 0 °C. Under these conditions, extracellular amino acid is removed and amino acid located intracellularly, at least in preparations of rat muscle (Kostyo, 1968; Hider *et al.*, 1971), rat pancreas (van Venrooy *et al.*, 1972) and rat submandibular gland (van Venrooy *et al.*, 1973) is preserved. However, for rat cerebral cortex slices some loss of intracellular amino acid has been reported by Dunlop *et al.* (1974) during a 40-min cold-wash. Nevertheless, using this method, and a less extensive cold-wash of 15 min, we found no indication that the observed changes in incorporation were due to effects on uptake of radioactive precursor into the slices (Fig. 1A and B). No alterations were found in the amount of radioactivity extracted from the tissue during the cold-wash. Therefore, the changes in incorporation most likely reflect changes in protein synthesis. In addition, the alterations found *in vitro* parallel the observations in *in vivo* experiments on the effects of hypophysectomy and $ACTH_{1-10}$-7-L-Phe treatment of hypophysectomized rats (Schotman *et al.*, 1972; Schotman and Gispen, 1974). The *in vitro* data provide further evidence for a direct action of $ACTH_{1-10}$ on central nervous structures, resulting in a change in protein synthesis.

At various levels of brain cell metabolism, effects of treatment with ACTH or its analogs can be monitored. Not only RNA and protein metabolism, but also glucose metabolism (De Kloet and Witter, 1973) and noradrenaline turnover (Versteeg, 1973; Leonard, 1974) seem to be influenced by treatment with N-terminal fragments of ACTH. At present the relationship of the various effects to one another is not clear and further studies are necessary. Nonetheless we have postulated elsewhere that a peptide–brain membrane interaction should be the crucial signal for the brain cell to respond to peptide treatment (Gispen and Schotman, 1973).

SUMMARY

Hypophysectomy was found to interfere with both long-term active avoidance conditioning and brain stem macromolecule metabolism in rats.

Treatment of these rats with $ACTH_{1-10}$-7-L-Phe restored their poor performance in shuttle box conditioning almost to normal levels; conversely, under similar conditions, the analog $ACTH_{1-10}$-7-D-Phe was ineffective or even showed an opposite effect. It appeared that these peptides also affected leucine incorporation into brain stem proteins. In hypophysectomized rats, $ACTH_{1-10}$-7-L-Phe increased the incorporation rate, whereas $ACTH_{1-10}$-7-D-Phe caused an inhibition; the behaviorally neutral $ACTH_{11-24}$ had no effect. These data raised the question as to whether the observed alterations are general effects, or are restricted to a few protein species. Therefore, brain stem proteins were further analyzed, using techniques of sequential extraction and polyacrylamide gel electrophoresis. Hypophysectomy caused a general decrease in labeling of all protein species studied, whereas $ACTH_{1-10}$-7-L-Phe

caused an overall increase. These effects might be due to a direct action on the mechanism of protein synthesis, as similar alterations were observed by studying *in vitro* protein synthesis in brain stem slices.

The present data may help to unravel the neurochemical events which underlie the behavioral effect of N-terminal ACTH fragments.

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