THE BEHAVIORALLY ACTIVE NEUROPEPTIDE ACTH AS NEUROHORMONE AND NEUROMODULATOR: THE ROLE OF CYCLIC NUCLEOTIDES AND MEMBRANE PHOSPHOPROTEINS

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INTRODUCTION

Investigations on the role of the pituitary-adrenal system in the adaptation of the organism to environmental stimuli, led de Wied to postulate that the pituitary would manufacture peptides which modulate behavior by a direct action on the brain (neuropeptides; DE WIED, 1969). Indeed, fragments of hormones of both the posterior pituitary (vasopressin, oxytocin) and the anterior pituitary (ACTH, MSH, β-LPH) were found to possess strong behavioral activity which was clearly dissociated from effects on the endocrinon. Much effort was given to characterize the effects of the peptides on animal behavior and the significance of the research on behaviorally active peptides became eminent when independent reports in the literature pointed to a physiological role of known and hitherto unknown peptides in normal and abnormal human behavior (DE WIED and GISPEN, 1977; DE WIED, 1978). To date research on neuropeptides is a multidisciplinary struggle to unravel their location in the brain and their mechanism of action in terms of electrophysiological and neurochemical events and to assess their usefulness in the treatment of brain and behavioral diseases.

Acquisition of new behavioral patterns is generally believed to occur through changes in interneuronal connectivity (plasticity) so that (functionally) new pathways are formed. It is difficult to conceive of such permanent connectivity changes occurring in the absence of underlying chemical changes. Phosphorylation could alter the conformation of proteins thus affecting essential membrane properties at the synaptic cleft and hence interneuronal connectivity. Evidence is accumulating that phosphorylation of synaptic proteins may play a key role in nervous system functions (HEALD, 1962; GREENGARD, 1976) and behavior (GLASSMAN, et al., 1973; ROUTTENBERG, et al., 1975; EHRLICH, et al., 1977; PERUMAL, et al., 1977; HOLMES, et al., 1977).

The present paper focuses on the neurochemical mechanism of action of the behaviorally active peptide ACTH with special reference to its effects on brain cyclic nucleotides and synaptosomal plasma membrane phosphorylation.

Origin and function of ACTH in the CNS

Recent evidence indicates that the peptide hormones ACTH and LPH originate from a large molecule (prohormone) with a molecular weight in the range of 31,000 (31K, MAINS et al., 1977; ROBERTS and HERBERT, 1977). Enzymatic cleavage may release $ACTH_{1-39}$ and it is hypothesized that smaller fragments (α-MSH = Ac-$ACTH_{1-13}$ -NH_2, $ACTH_{4-10}$, $ACTH_{4-7}$) may be produced by further enzymatic degradation of the parental molecule. The classical target for pituitary ACTH is the adrenal cortex. Demonstration of its behavioral activity has suggested that the brain also serves as a target for ACTH and its active fragments (DE WIED, 1964; GISPEN, et al., 1977).

Although circulating ACTH reaches brain structures involved in the regulation of adaptive behavior (VERHOEF, et al., 1977a,b), new evidence points to a possible direct transport of ACTH from pituitary to septum (MEZEY, et al., 1978). That the brain itself could be a source of ACTH was first suggested on the basis of data on levels of ACTH in human cerebrospinal fluid (ALLEN, et al., 1974). This suggestion was further supported by the demonstration of immuno-reactive ACTH in several brain structures (KRIEGER, et al., 1977). At present, immunocytochemical studies strongly suggest the presence of a 31K prohormone system in hypothalamic neurons and possibly of ACTH as a free peptide (AKIL, et al., 1978). Thus the regulatory action

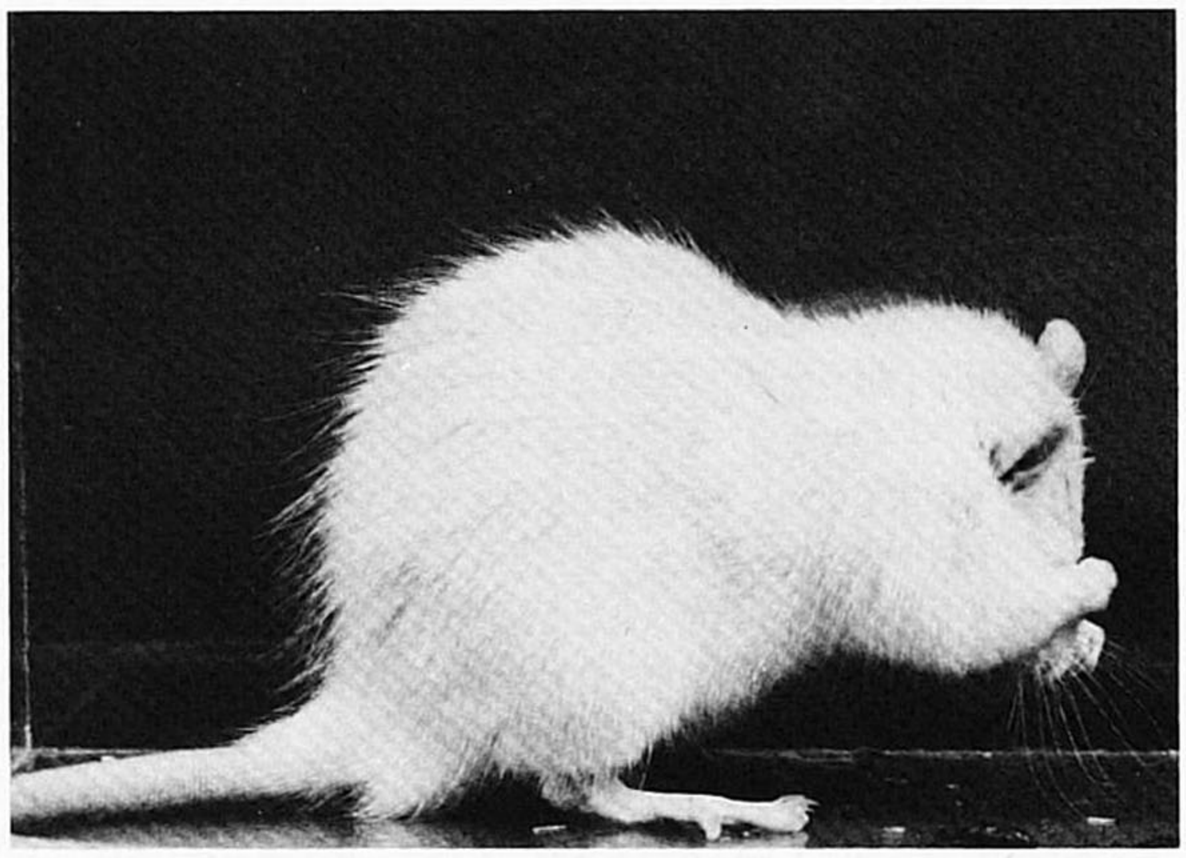
of ACTH on behavior could be envisaged to originate from two alternative peptide sources, i.e. pituitary cells and/or peptidergic neurons.

As pointed out before, ACTH and its fragments are supposed to play an essential role in homeostasis. Its regulatory role in central nervous functions and behavior was discovered in rats whose pituitary-adrenal system was disrupted or functionally suppressed (DE WIED, et al., 1972). Over the years numerous studies using a variety of behavioral paradigms and subjects have added to the knowledge about the functional significance of the regulatory role of ACTH in the brain. In an attempt to unify the known behavioral and neurophysiological effects in one hypothesis, Bohus and de Wied (1978) favor a mechanism involving motivation. They postulate that ACTH and its fragments increase the state of arousal in midbrain limbic structures, which may determine the motivational influence of environmental stimuli and thereby the probability of the generation of stimulus-specific behavioral responses (BOHUS and DE WIED, 1978).

If ACTH, MSH or fragments from LPH are injected directly into the cerebrospinal fluid of mammals, a stretching and yawning syndrome occurs (FERRARI, et al., 1963). In rodents this syndrome is preceded by the display of excessive grooming. The so-called grooming response is characterized by the enhanced display of vibrating of the front paws, washing, grooming, scratching, paw licking, tail licking, etc. In **Fig.** 1 A,B,C three elements of the grooming response are shown: washing, grooming and scratching. Fig. 1D and E show examples of the stretch and yawn usually seen following a long episode of excessive grooming. Fig. 1D depicts a stretch which is usually seen following a long episode of excessive grooming. The behavior is recorded, using a 15 sec sampling technique, establishing whether or not one of these behavioral elements is displayed by the animal. Previously, it has been shown that the data obtained by this procedure are in good agreement with data collected using duration as an Index of activity (GISPEN, et al., 1975). From the behavioral elements constituting the excessive behavioral response, the grooming element itself is by far the predominant seen (70% of total). Therefore, it was decided to refer to excessive grooming in keeping with previous reports.

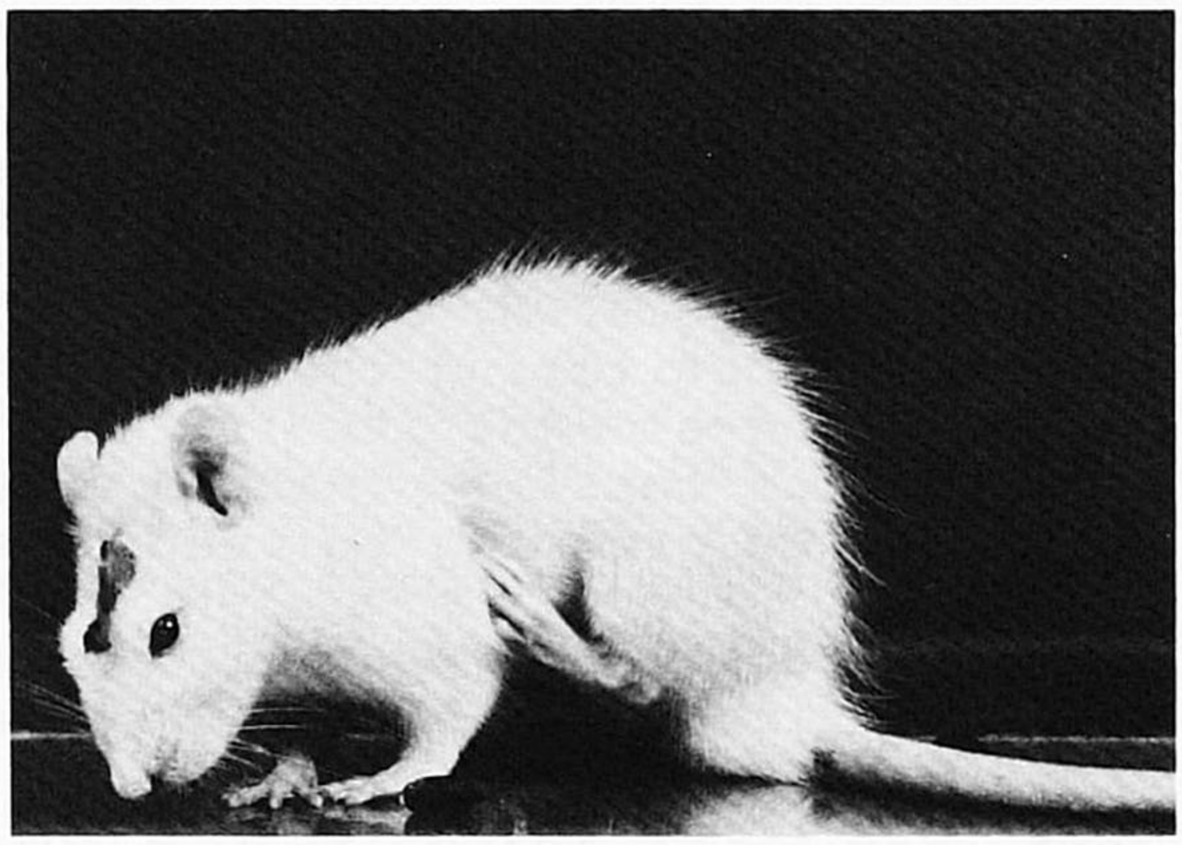
The behavioral significance of the grooming response is not entirely established. Grooming in rodents is sometimes interpreted as representive of "displacement activities" but other investigators think of grooming as a col-

A: Washing

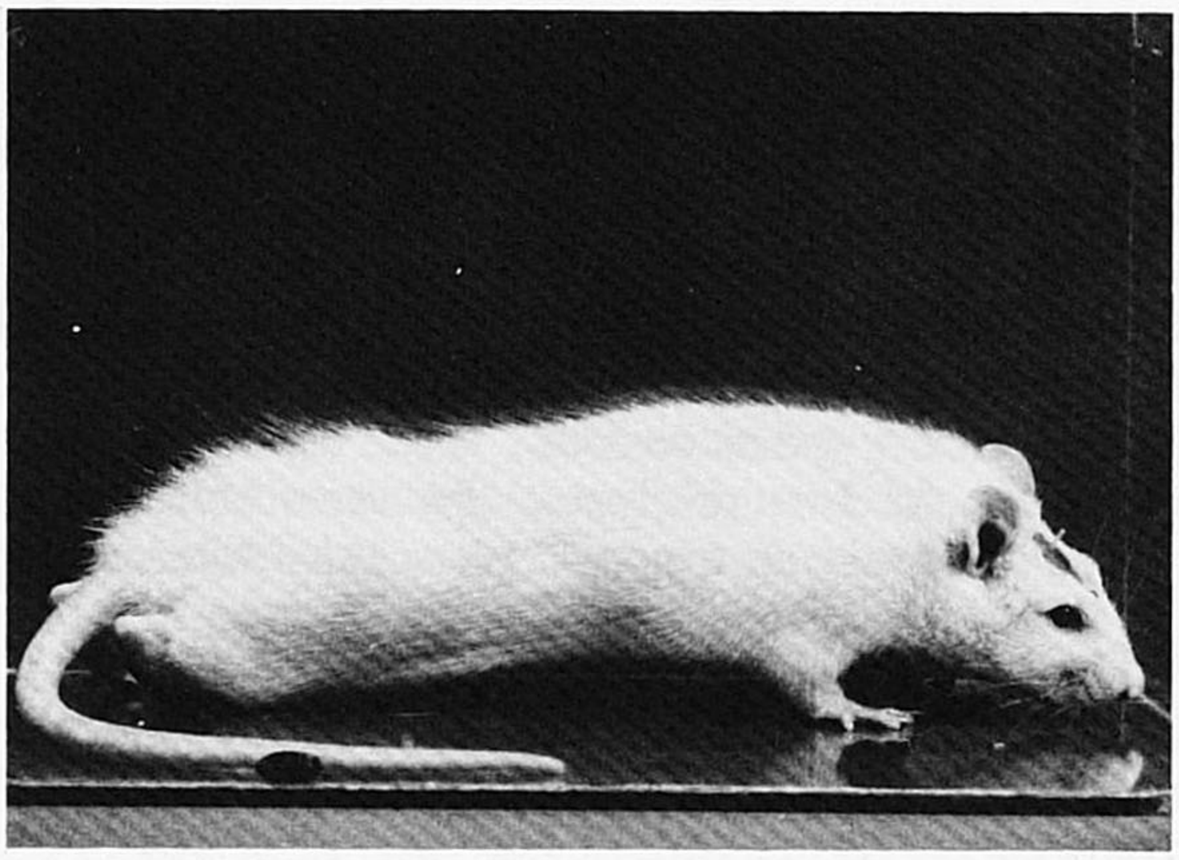


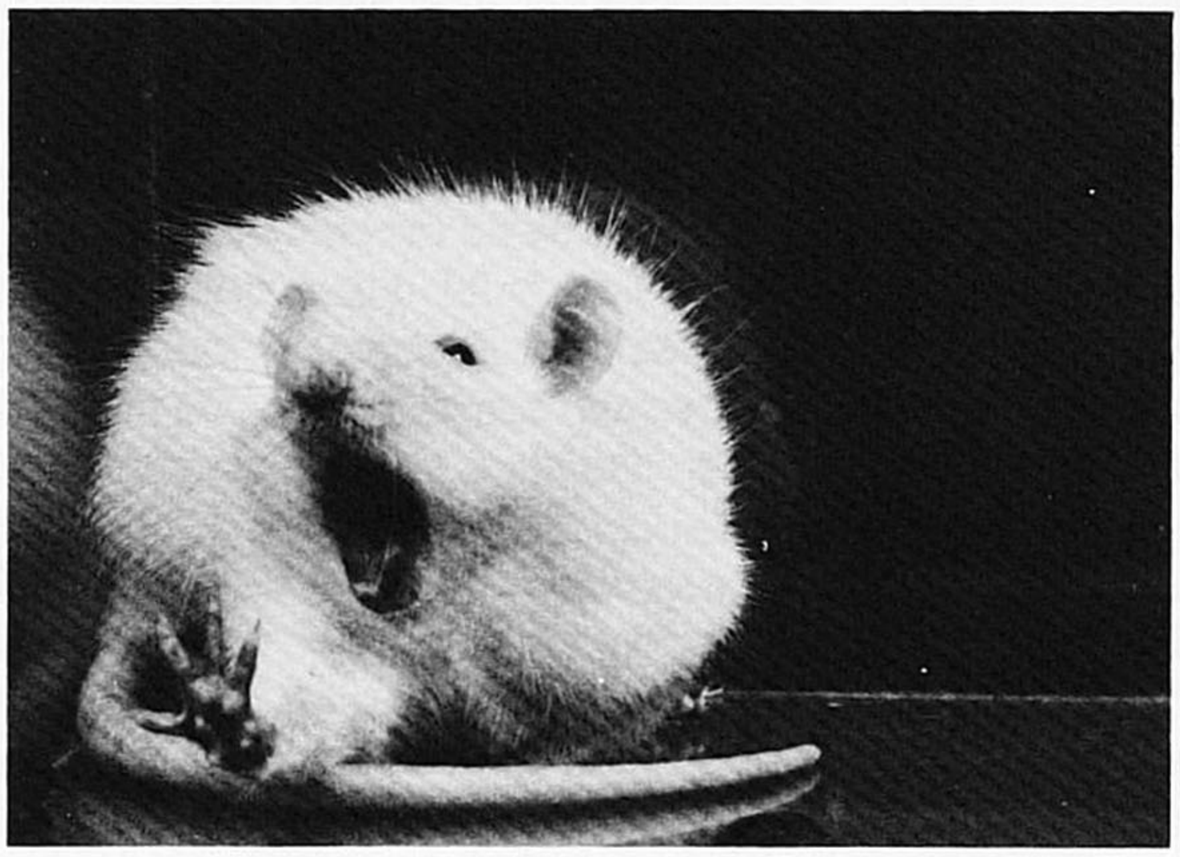
B: Grooming

C: Scratching



D: Stretching





E: Yawning

Fig. 1: The behavioral response after interventricular injection of $ACTH_{1-24}$ in rats.

lateral act, i.e. behavior associated with, but not part of, a goal-directed activity (JOLLES, et al., 1979). While feedback from the periphery is usually important for the development and maintenance of integrated movement repertoire like grooming, there is evidence for a strong internal control of grooming (FENTRESS, 1973). The peptide-induced grooming is not observed when the peptides are administered peripherally. In view of the route of administration, the short onset latency of the effect and its independence of endocrine activity, the induction of excessive grooming behavior seems the result of a direct effect of ACTH on the CNS (GISPEN, et al., 1975).

The induction of excessive grooming by intraventricular administration of $ACTH_{1-24}$ was studied in rats with lesions in midbrain-limbic structures. Such areas have been reported to be implicated in mediating ACTH-induced effects on avoidance behavior, sexual excitement or stretching and yawning, (see COLBERN, et al., 1977). Electrolytic lesions in the septal complex, the anterior hypothalamic/preoptic area, the mammillary bodies, the amygdala, the posterior thalamus and dorsal or ventral hippocampus did not interfere with ACTH-induced excessive grooming. Lesioning of the hippocampal complex by aspiration led to an inhibition of excessive grooming depending on the degree of hippocampal damage. Amygdala and hippocampal lesions enhanced the display of stretching and yawning activity after treatment with the peptide. These data indicate differences in the neural substrates mediating the effect of ACTH on extinction of conditioned avoidance behavior, excessive grooming, sexual excitement and stretching and yawning (COLBERN, et al., 1977).

Neuropharmacological studies also pointed to differences between the effects of ACTH on adaptive processes as discussed above and those on the induction of excessive grooming. It was demonstrated that pre-treatment of rats with the opiate antagonist naltrexone did not abolish the effect of ACTH or β-LPH fragments on avoidance extinction (DE WIED, et al., 1978), but completely suppressed the induction of excessive grooming (GISPEN and WIEGANT, 1976). Further characterization of the neural substrate involved in the induction of excessive grooming pointed to the essential role of dopaminergic fibers originating in the substantia nigra and projecting in the neostriatum and nucleus accumbens (WIEGANT, et al., 1977a; COOLS, et al., 1978). The grooming behavior could be elicited from the substantia nigra by local injections of $ACTH_{1-24}$. In addition local application of dopamine antagonists in the neostriatum and n. accumbens interferred with the induction of excessive grooming. Despite the difference in neural substrate, it was hypothesized that in the case of excessive grooming also peptide-induced arousal is a causative stimulus. Thus, the grooming response would reflect a de-arousal mechanism triggered by the arousal brought about by ACTH (JOLLES, et al., 1979).

Neurochemical mechanism of action of ACTH

There is sufficient evidence available to indicate that ACTH or its fragments affect cerebral RNA and protein synthesis, cyclic nucleotide metabolism, protein phosphorylation and neurotransmitter turnover (acetylcholine,

dopamine and noradrenaline; DUNN and GISPEN, 1977). The scarcity of knowledge about the first steps in the peptide-nerve cell interaction (recognition, activation of receptors, etc.) greatly hampers our understanding of the neurochemical mechanism of ACTH. For instance, knowing the localization of ACTH receptors on nerve cells might help to decide whether ACTH should be considered to be a neuromodulator or a putative neurotransmitter (BARCHAS, et al., 1978). The complexity of the known behavioral, electrophysiological and neurochemical responses to ACTH implies that most likely more than one type of mechanism is involved (differences in receptor, neural substrate, neurochemical response, etc.). In an attempt to bring most of the known neurochemical events in a causal relationship, we took the view that ACTH would not only act as neuromodulator but also would exert trophic influences on its target cells in brain as it does in other tissues (DUNN and GISPEN, 1977; GISPEN, et al., 1977b). By doing so, the peptide would enhance protein metabolism and alter intercellular communication (interneural connectivity), but would not be responsible for direct transynaptic transfer of a nerve signal. The proposed mechanisms of ACTH action on neurons is represented in Fig. 2. ACTH interacts with specific receptors on the outside of the cell membrane, thus activating adenylate cyclase (or perhaps guanylate cyclase). Cyclic nucleotide dependent protein kinases then may activate mRNA in the nucleus, activate protein synthesis by polyribosomes in the perikaryon, modify the properties of post-synaptic receptors, stimulate neurotransmitter synthesis or modulate neurotransmitter release, or any combination of these. Not all cells respond to ACTH and even responsive cells will only show one or a few of these responses (DUNN and GISPEN, 1977).

ACTH and brain nucleotides

Up to now, few reports have been published on the relation of central effects of peptides and the cyclic nucleotide systems of the brain. Occasionally, ACTH has been included in studies on the effects of putative transmitters and hormones on brain adenylate cyclase in cell-free membrane preparations. Burkhard and Gey (1968) and Von Hungen and Roberts (1973) were unable to detect an effect of ACTH on adenylate cyclase in such preparations. Forn and Krishna (1971) did not observe an effect of ACTH on cAMP accumulation in rat brain cerebral cortex slices. On the other hand, indirect indications that ACTH-like peptides might affect brain cyclic nucleotide levels

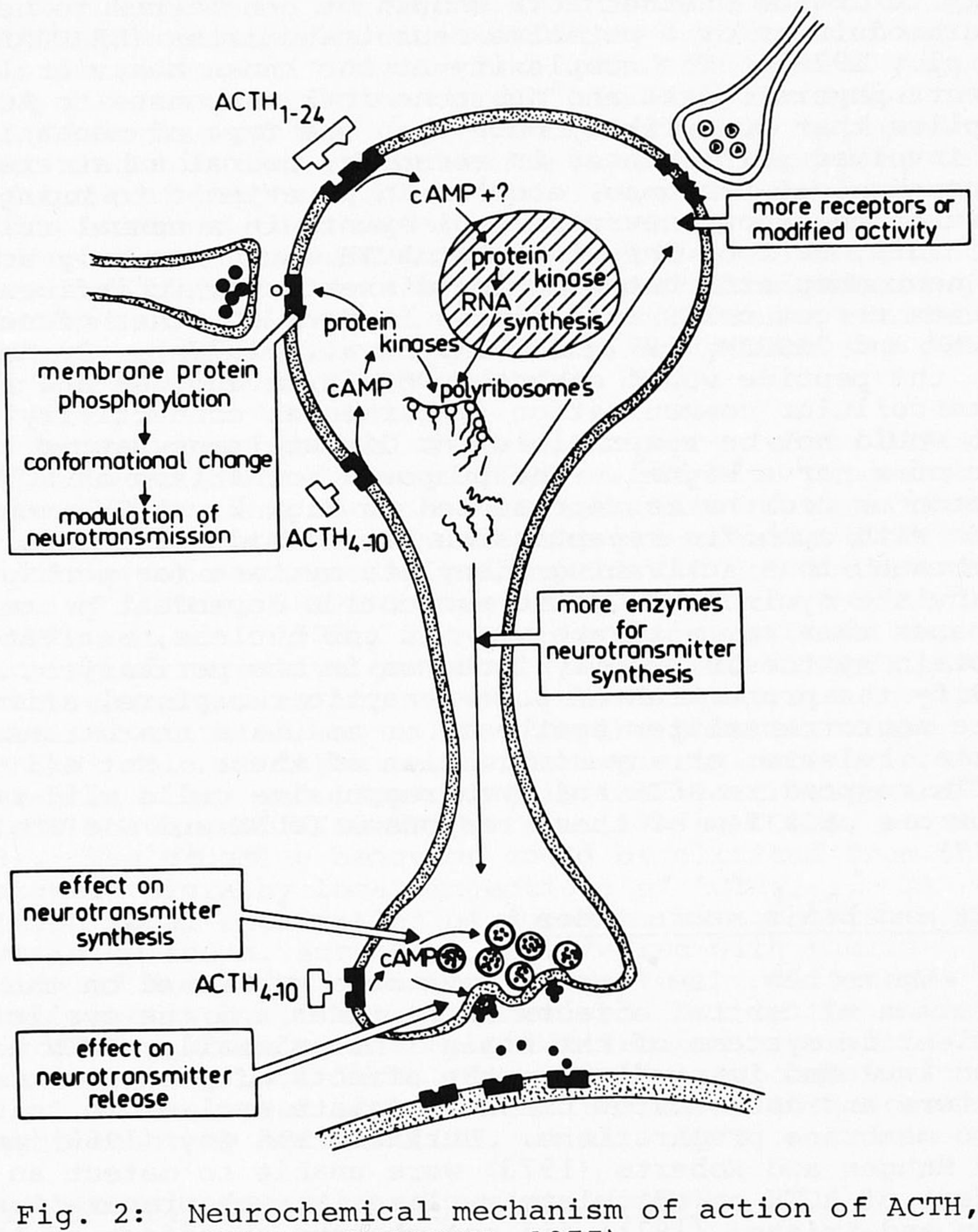


Fig. 2: Neurochemical mechanism of action of ACTH, (see DUNN and GISPEN, 1977).

in vivo, were presented by Rudman and co-workers (RUDMAN and ISAACS, 1975; RUDMAN, 1976). They showed that intracisternal injection of ACTH or β-MSH in rabbits increased the cAMP but not the cGMP concentration in cerebrospinal fluid. In a preliminary study it was reported that chronic treatment of rats with α-MSH (= Ac-Ser^{1}|ACTH$_{1-13}$-NH$_2$) increased the level of cAMP in the occipital cortex (CHRISTENSEN, et al., 1976). Similar treatment left the level of cGMP unaltered in all brain regions studied (SPIRTES, et al., 1978).

Recently, we investigated the influences of N-terminal fragments of ACTH on the accumulation of cAMP in rat brain using three different approaches viz. broken cell preparations (adenylate cyclase activity), slices from posterior thalamus and neostriatum and in vivo levels (WIEGANT and GISPEN, 1975; WIEGANT, et al., 1979). ACTH$_{1-24}$ had a biphasic effect on the activity of adenylate cyclase in broken cell preparations of rat brain subcortical tissue; concentrations below 25 μM stimulated, whereas concentrations of 0.1 mM and higher inhibited adenylate cyclase activity (Fig. 3). The magnitude of the stimulation was dependent on the concentrations of ATP and Mg^{++} in the incubation medium. Under optimal conditions in synaptosomal plasma membrane fractions, maximal stimulation of adenylate cyclase occurred at 0.1 - 1.0 μM ACTH$_{1-24}$. In membrane preparations derived from peripheral target cells (adrenal cortex, fat cells) maximal stimulation of the enzyme also required ACTH-concentrations in the micromolar range (BIRNBAUMER, et al., 1969; GLOSSMAN and GIPS, 1975; LANG, et al., 1976; SCHLEGEL and SCHWYZER, 1977).

In our hands the magnitude of the stimulation was extremely variable. This prevented pharmacological characterization of this stimulatory effect of ACTH$_{1-24}$. The inhibition of brain adenylate cyclase in vitro by concentrations of ACTH$_{1-24}$ higher than 5 μM could not be explained as a secondary effect resulting from an action of the peptide on the residual activity of phosphodiesterase, or from peptide-induced changes in pH or ATP concentration. Therefore, it was concluded that this inhibition resulted from a direct action of ACTH on the rate of formation of cAMP catalyzed by adenylate cyclase. Structure activity studies revealed that in a concentration of 10^{-4} M ACTH$_{1-16}$-NH$_2$ and ACTH$_{4-7}$ also inhibit the activity of adenylate cyclase, whereas ACTH$_{11-24}$, ACTH$_{1-10}$, ACTH$_{4-10}$, |D-Phe^{7}|-ACTH$_{1-10}$ and |D-Phe^{7}| ACTH$_{4-10}$ are inactive in this respect.

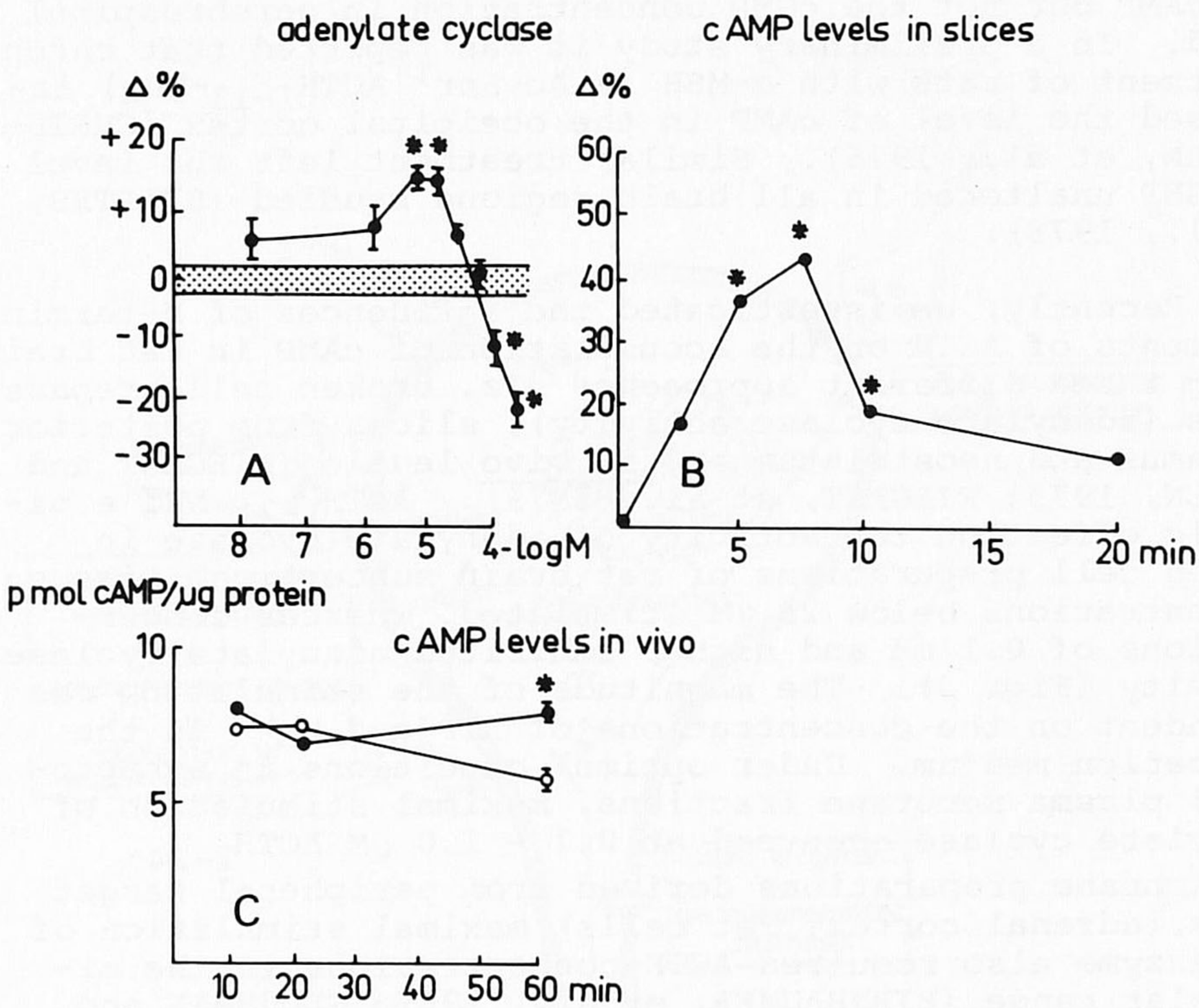


Fig. 3: Effects of ACTH on brain cyclic nucleotides.

A. Dose response curve for the effect of $ACTH_{1-24}$ on the activity of adenylate cyclase in a homogenate of rat brain subcortical tissue (n=5). Basal activity in this preparation was 189 ± 5 pmol cAMP/mg protein/min.

B. Time course of the effect of 10^{-4} M $ACTH_{1-24}$ on the accumulation of cAMP in slices of rat neostriatum (n=5). Basal level = 2 ± 0.1 pmol cAMP/mg protein.

C. The effect of intraventricular injection of $ACTH_{1-16}$ (1 µg) on the level of cAMP in rat septum as determined after microwave fixation of the brain.

* $p < 0.05$ (Student t-test, two-tailed). For further experimental details see WIEGANT, et al., 1979.

$ACTH_{1-24}$ dose-dependently enhanced the accumulation of cAMP in slices from rat brain neostriatum and posterior thalamus, but did not influence the concentration of cGMP in striatal slices. The effect of ACTH on cAMP occurred rapidly, and was of short duration (Fig. 3). Isobutyl-methylxanthine (IBMX), an inhibitor of phosphodiesterase, potentiated the effect of $ACTH_{1-24}$ suggesting that the increase in cAMP concentration in the striatal slices was not the result of inhibition of the hydrolysis of cAMP but rather of an $ACTH_{1-24}$-induced activation of adenylate cyclase activity, perhaps by an interaction of an ACTH receptor with adenylate cyclase.

Intraventricular administration of a low dose of $ACTH_{1-16}\text{-}NH_2$ (1 µg) resulted in a significant 27% increase of septal cAMP concentration, 60 min. after the injection (Fig. 3). No effect of the peptide could be detected in the other brain regions studied, including the neostriatum. Others have also reported relatively small changes in levels of cAMP after electrical stimulation of nerve cell bodies (KORFF, Personal Commun.). Therefore, the increase in septal cAMP was taken to reflect a significant alteration in synaptic activity in this region. In view of the importance of the septal complex to the expression of the behavioral activity of ACTH (VAN WIMERSMA, GREIDANUS, et al., 1975; VERHOEF, et al., 1977a,b), a direct effect of ACTH-like peptides on septal cells resulting in an increase in cAMP seems possible. From the present results, however, we cannot exclude indirect effects such as neurotransmitter mediated effects, etc.

In summary, N terminal fragments of ACTH modulate the activity of brain adenylate cyclase in broken cell preparations, stimulate the accumulation of cAMP in slices of brain tissue containing intact cells through activation of adenylate cyclase, and increase septal cAMP in vivo. These findings evidence a role of ACTH-like neurotrophic peptides as modulators of brain adenylate cyclase although, clearly, cAMP cannot be taken as a possible second messenger for all the effects of ACTH-like peptides on the central nervous system (see below).

Phosphorylation of synaptic plasma membrane proteins

As pointed out in the introduction, phosphorylation of synaptic membrane proteins may affect the transmission of information between neurons. As early as 1962, HEALD speculated that the change in protein phosphorylation that occurred in response to electrical stimulation of respiring brain slices altered the conformation of neuronal

membrane proteins, and that such changes might be involved in the regulation of ion movements through cell membranes (HEALD, 1957, 1962). Trevor and Rodnight (1965) demonstrated that the protein-bound phosphoryl-serine groups that responded to electrical stimulation were indeed in cerebral membrane fractions. In a more defined approach, BROWNING, et al. (1977, 1978) reported that synaptic potentiation of the Schaeffer collaterals in rat hippocampal slices led to an altered Ca^{++}-dependent phosphorylation of a protein band, with a molecular weight of about 40 K, associated with synaptic plasma membrane fraction.

It was recently suggested that phosphorylation of specific protein substrates is involved in the release of neurotransmitters. Such a release is calcium-dependent and experiments were reported describing a calcium-dependent phosphorylation in relation to the exocytosis of neurotransmitters (KATZ and MILEDI, 1967; DOUGLAS, 1973; DE LORENZO, 1976; REDBURN, et al., 1976; DE LORENZO and FREEDMAN, 1977; KRUEGER, et al., 1977; HERSHKOWITZ, 1978; SCHULMAN and GREENGARD, 1978; SIEGHART, et al., 1978).

With respect to cAMP-sensitive brain membrane protein phosphorylation it was hypothesized that the enhanced phosphorylation of the substrate protein accounts for the membrane hyperpolarization observed after the exposure of the nerve cell to dibutyryl cAMP (GREENGARD, 1976; NATHANSON and GREENGARD, 1977). The involvement of protein phosphorylation in brain function is further evidenced by reports on correlative changes in membrane phosphoproteins with a variety of behavioral experiences (GLASSMAN, et al., 1973; PERUMAL, et al., 1975, 1977; EHRLICH, et al., 1977; GISPEN, et al., 1977c; HOLMES, et al., 1977).

The first demonstration of a correlate between behavior and protein phosphorylation concerned the acquisition of a conditional avoidance response in mice and the enhanced incorporation <u>in vivo</u> of radioactive phosphate into total protein of synaptosome-enriched fractions of their brains (GLASSMAN, et al., 1973). The increase could only be detected in the particulate fraction of the synaptosomes. Initial neurochemical characterization confirmed that the radioactivity was covalently bound to amino acids in membrane proteins (PERUMAL, et al., 1975, 1977). Further behavioral studies revealed that the increased phosphorylation of these proteins is specific to the conditioning experience, since mice that were merely

exposed to the conditioned and unconditioned 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 increased phosphorylation (GISPEN, et al., 1977c).

Using a different experimental paradigm Routtenberg and Ehrlich (ROUTTENBERG, et al., 1975; EHRLICH, et al., 1977) have shown that the rate of *in vitro* phosphorylation of rat and mouse brain membrane proteins is altered by behavioral experience. Interestingly, the proteins whose phosphorylation was maximally altered by the preceding training appeared to be different than those whose phosphorylation was maximally stimulated by cAMP *in vitro*. Recently, this *in vivo*/*in vitro* approach was also used to demonstrate that in rats the anxiety accompanying an electroshock procedure can induce an increase in the *in vitro* activity of a membrane-bound protein phosphorylation system, probably involved in synaptic function (HOLMES, et al., 1977). Thus both *in vivo*/*in vitro* studies point to a behaviorally induced alteration of brain membrane protein phosphorylation. Direct comparison of the studies mentioned above remains, however, difficult as it was recently reported that the proteins that preferentially accumulate radioactive phosphate *in vivo* are not necessarily the ones that preferentially accept phosphate in vitro (BERMAN, et al., 1977; WILSON, 1977). Further studies by these authors indicate that proteins associated with the postsynaptic density, although difficult to phosphorylate under *in vitro* conditions, readily incorporate phosphate *in vivo* and thus may represent a major proportion of the labelled proteins of the synaptic membranes (BERMAN, et al., 1978).

We have been studying the endogenous phosphorylation of proteins from rat brain synaptosomal plasma membranes *in vitro* with or without the behaviorally active peptide $ACTH_{1-24}$ in the incubation medium. Many factors may influence this *in vitro* process (RODNIGHT, et al., 1975). Important determinants are: the activity of ATPase(s) controlling the availability of the phosphate donor (ATP), the activity of the protein phosphatase(s) and of course that of the protein kinase(s). As was shown for total membrane protein (RODNIGHT, et al., 1975), the phosphorylation of a given SPM protein band depends very much on the ATP/SPM ratio used in the incubation system (WIEGANT, et al., 1978b). Therefore, as a routine, a low and a high ATP/SPM ratio were used simultaneously, to study the effects of ACTH on endogenous SPM phosphorylation.

At first we reported that $ACTH_{1-24}$ when added *in vitro* reduced the incorporation of phosphate into a number of SPM protein bands which, based on their electrophoretic mobility in SDS-PAGE, have molecular weights smaller than those previously reported to be affected by the *in vitro* addition of cAMP (ZWIERS, et al., 1976). In a number of protein bands a biphasic effect of $ACTH_{1-24}$ was observed; in concentrations of 10^{-4}- 10^{-5} M $ACTH_{1-24}$, a reduced incorporation was apparent, in concentrations of 10^{-6}- 10^{-7}M hardly any effect could be detected, whereas at 10^{-8} M a consistent but small decrease was observed. This latter effect could only be detected when the SPM was freshly prepared (ZWIERS, et al., 1976). It was shown that the effect of ACTH was caused by an inhibition of SPM protein kinase(s) and not by a stimulation of SPM protein phosphatase (ZWIERS, et al., 1976, 1978). Using N-terminal fragments of ACTH, the structure activity requirements for the inhibition were studied. A rather complex interaction of the ACTH fragments with endogenous SPM phosphorylation was observed (ZWIERS, et al., 1978b). The effects were not only dependent on the primary structure of the peptide studied, but also on the ATP/SPM ratio and varied for the various protein bands. In Fig. 4, the structure activity relationships for the inhibition of the endogenous phosphorylation of a phosphoprotein band B-50 (MW 48 K) and those for the induction of excessive grooming in the rat are shown. It is clear that the requirements for both these CNS effects of ACTH are very similar. One of the important findings is that in neither instance does the combination of the sequences $ACTH_{1-10}$ and $ACTH_{11-24}$ act as the total sequence $ACTH_{1-24}$ (GISPEN, et al., 1975; ZWIERS, et al., 1978b).

Furthermore, preliminary evidence indicates that the endogenous *in vitro* phosphorylation of specific SPM proteins prepared from rats treated intraventricularly with $ACTH_{1-24}$ differs from *in vitro* phosphorylation of SPM proteins from saline-treated animals (ZWIERS, et al., 1977). There appeared to be a U-shaped dose-response curve with significantly enhanced incorporation of phosphate into SPM protein bands after injection of 30 or 300 ng $ACTH_{1-24}$. This effect of *in vivo* ACTH treatment on *in vitro* endogenous phosphorylation was not only dose- but also time-dependent, giving the largest effects 30 min. after the peptide injection (ZWIERS, et al., 1977).

In Fig. 5 the effect of magnesium and calcium on the endogenous phosphorylation of protein band B-50 is shown. Using a low ATP/SPM ratio, the B-50 protein kinase is stimulated by both Ca^{++} and Mg^{++}. At relatively high

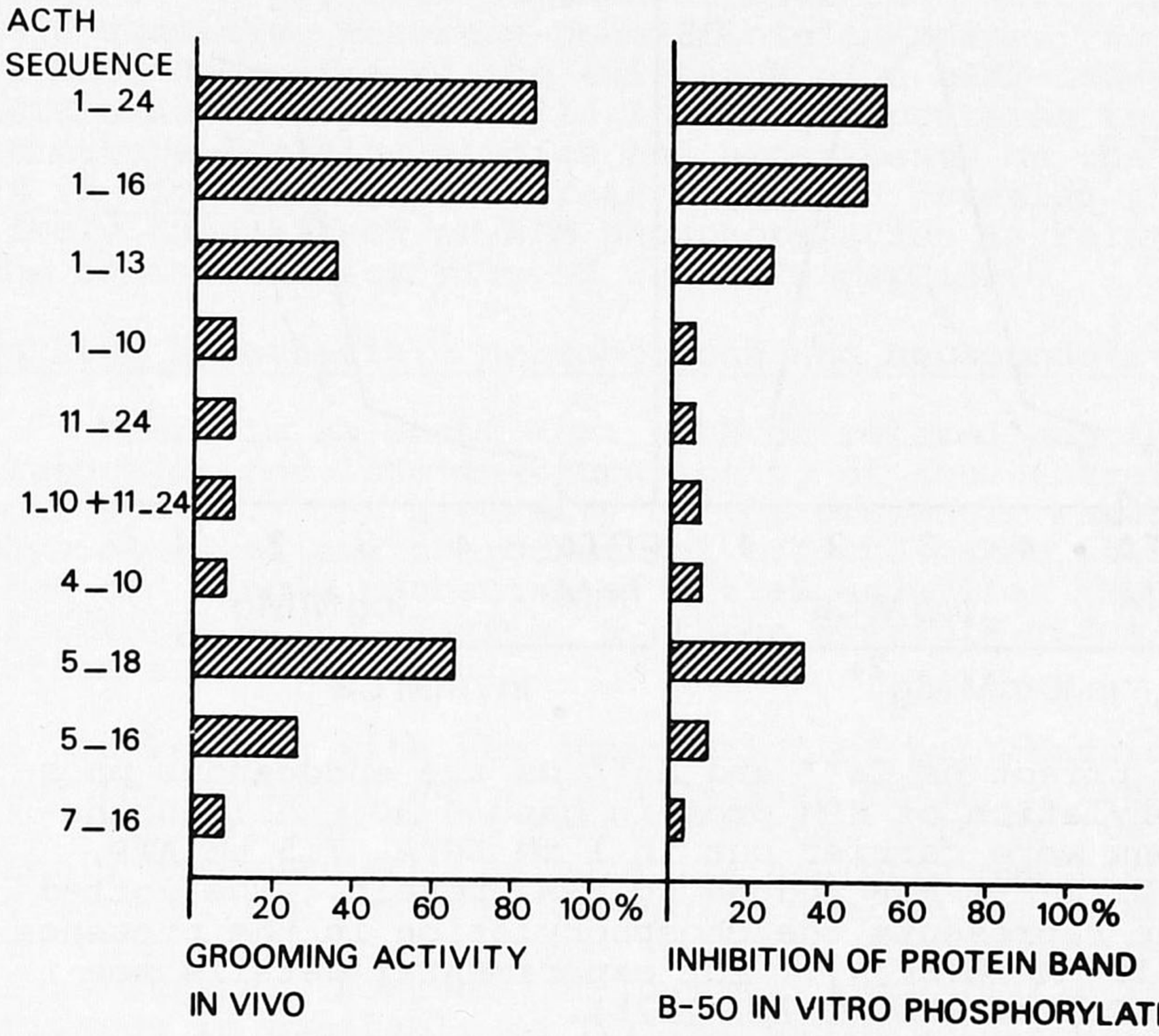


Fig. 4: Structure activity studies on the effect of ACTH on excessive grooming in the rat and inhibition of SPM protein phosphorylation in vitro. The grooming activity was elicited by intraventricular administration of doses equimolar to 3 µg $ACTH_{1-24}$. The inhibition of phosphorylation was studied in the presence of 10^{-4} M peptide.

Ca^{++}a marked drop in enzyme activity was observed. The optimal conditions for Mg^{++} and Ca^{++} are 10^{-2}M and 10^{-3} M respectively. Under optimal conditions also the largest effect of ACTH was observed. Interestingly at 5×10^{-2}M Mg^{++} ACTH was not effective anymore. It may be that ACTH competes with Mg·ATP for a site at the catalytic unit of the B-50 protein kinase. Further study with regard to this aspect is in progress. Although apparently the divalent cation sensitivity of B-50 protein kinase differs from that reported by HERSHKOWITZ (1978) for his protein C,

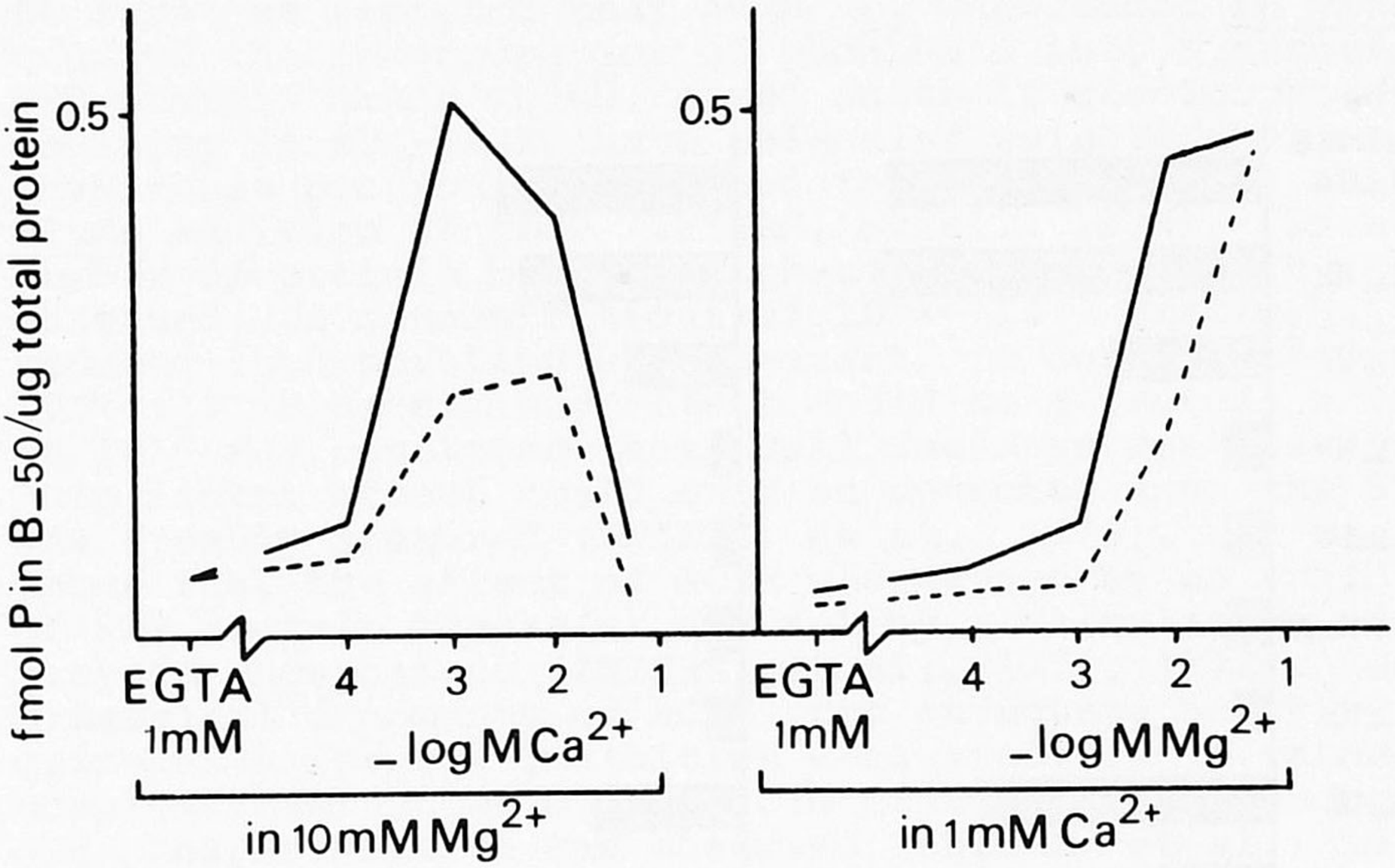


Fig. 5: Effect of Ca^{++} and Mg^{++} on the endogenous phosphorylation of SPM protein band B-50. All incubations were carried out in 1 mM EGTA, 7.5 µM ATP, 3 µCi α-^{32}P ATP and 50 µg SPM protein. The dotted line represents the phosphorylation in the presence of 10^{-5}M $ACTH_{1-24}$. For experimental details see ZWIERS, et al. (1976, 1978).

it is of interest that the phosphorylation of a Ca^{++}-dependent membrane protein band is susceptible to the behaviorally active peptide $ACTH_{1-24}$. It may be that this direct effect on SPM phosphorylation underlies the modulatory role ACTH has on neurotransmission and behavior (see below).

Recently, we demonstrated that treatment of the rat brain SPM with 0.5% Triton X-100 in 75 mM KCl solubilized 15% of the total membrane B-50 protein kinase activity and more importantly, preserved the sensitivity of the enzyme to $ACTH_{1-24}$ (ZWIERS, et al., 1978a). The solubilized membrane material also phosphorylated exogenous B-50 proteins which were previously extracted from similar rat brain membranes. Column chromatography of the solubilized material over DEAE-cellulose pointed to the presence of multiple protein kinase activities in rat brain membranes (MIYAMOTO, et al., 1978), one being the ACTH-sensitive

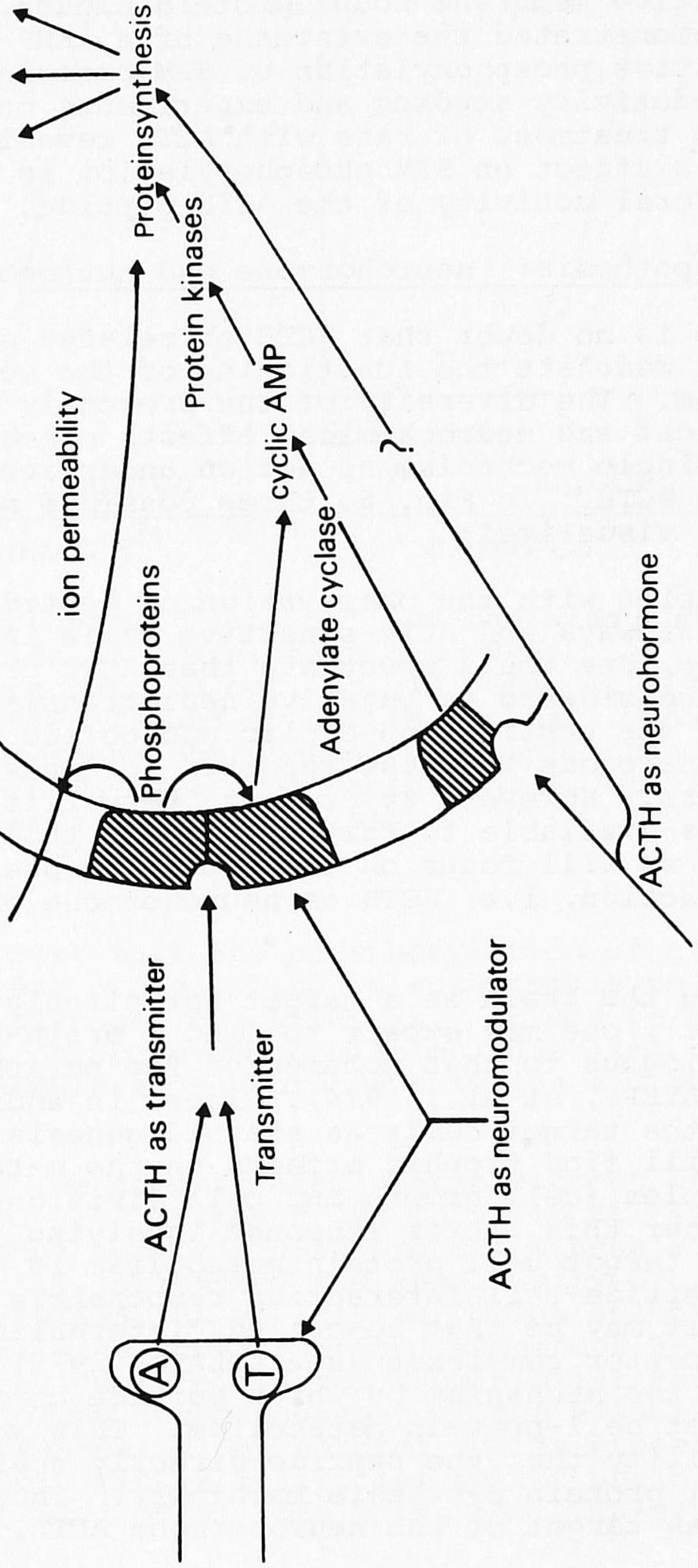
B-50 protein kinase (ZWIERS, et al., 1979). Further study is in progress to characterize and purify this ACTH-sensitive membrane-bound protein kinase. So far, we have demonstrated the existence of a cAMP-independent, ACTH-sensitive phosphorylation of SPM proteins in vitro. Structure-activity studies and experiments on the effect of in vivo treatment of rats with ACTH revealed that most likely this effect on SPM phosphorylation is related to the behavioral activity of the ACTH peptide.

Working hypothesis: neurohormone and neuromodulator

There is no doubt that ACTH or related peptides influence or modulate the functioning of the central nervous system. The diversity of the presently known neurophysiological and neurochemical effects makes it unlikely that one single mechanism of action underlies the CNS effects of ACTH. In Fig. 6, three possible modes of action are visualized:

A. In keeping with the observation of peptidergic (ACTH) neuronal pathways and ACTH-sensitive cells in iontophoretic studies, one could speculate that ACTH or its fragments should be considered as putative neurotransmitters. The effects of the peptides on cyclic nucleotide matabolism are then analogous to those reported for many other neurotransmitters. However, at present, very little if any evidence is available to further support this notion. Therefore, we will focus on the two other possible mechanisms of action, i.e. ACTH as neurohormone or as neuromodulator.

B. Viewing the brain as a target for circulating ACTH (blood, CSF), one may expect to find a brain-ACTH interaction analogous to that documented for peripheral target tissues (SAYERS, et al., 1974). Thus, in addition to responses of the target cells as steroidogenesis or lyposlysis, one will find trophic effects on the metabolism of macromolecules (cell growth and cell division). It is not clear whether this latter response involving, for instance, effects on target cell protein metabolism is mediated by the same peptide-cell interaction responsible for steroidogenesis. It may be that so-called "internalisation" of hormone-receptor complexes (see KOLATA, 1978) is a key element in the mechanism by which peptide hormones affect their target cell-protein metabolism. This would provide the possibility that the peptide directly could affect its target cell protein synthesis machinery. Thus, viewing the brain as target of the neurohormone ACTH, it may be



Three possible modes of action of ACTH: Neurotransmitter, neurohormone and neuromodulator.

Fig. 6:

that the observed effects on brain RNA and protein synthesis represent the neurotrophic influence exerted by ACTH. Whether or not changes in levels of cyclic nucleotides function as regulatory signals in this mechanism, is not clear.

Of course, one should keep in mind that all hitherto described effects of ACTH on brain protein synthesis in vivo and in vitro could have been the result of indirect stimulation through, for instance, the release of neurotransmitters affecting postsynaptic neuronal metabolism. The effects on protein synthesis would then be envisaged to be brought about by ACTH acting as a neuromodulator.

C. Neuromodulators are entities which alter the flow of information between neurons. The mechanism of action should be rapid in view of the physico-chemical events underlying neurotransmission. It may be that they affect processes which are related directly to transmitter availability (release, re-uptake, turn-over, etc.). Modulation of neurotransmission may also be brought about by altering the electrical properties of synaptic membranes. One of the mechanisms by which the latter could be achieved, is the alteration of ion permeability.

We propose that the direct effect of ACTH on a membrane-bound protein kinase may underlie a modulatory role in neurotransmission. Since the membranes used in our studies, mainly will have been of presynaptic origin, it may well be that this effect on membrane phosphorylation represents a presynaptic event. As the B-50 protein kinase is stimulated by magnesium and calcium, it may be that the function of the B-50 substrate protein resembles that described for other Ca-dependent phosphoproteins (see above), i.e., Ca-dependent exocytosis of neurotransmitters. If at the other hand the B-50 protein kinase-substrate protein comples affected by ACTH primarily would be of postsynaptic nature, the observed inhibition of phosphorylation may alter ion permeability and hence sensitivity to presynaptic input as discussed for the role of cAMP in neurotransmission (NATHANSON AND GREENGARD, 1977). Currently we are preparing antibodies to a rather pure protein-B-50 preparation in order to be able to localize B-50 in nerve cells and tissue.

It is clear that more work is necessary before one can start to unravel the mechanism by which ACTH affects the functioning of the central nervous system. The absence of a proven specific binding site (receptor) at the neuron outer membrane greatly hampers our present understanding. Thus it is vital to identify the first step in the ACTH-nerve cell interaction. The important role neuropeptides play in animal and human behavior prompts us to further study their exact mechanism of action.

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