LIPOTROPIN AND THE CENTRAL NERVOUS SYSTEM

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I. Introduction

The lipotropic hormone (β-lipotropin, β-LPH) was first isolated in 1964 from sheep pituitary glands (Li, 1964) and subsequently from the pituitary of a variety of species (see Li and Chung, 1976a). It has been detected in the circulating blood of sheep (Lohmar and Li, 1968) and located in discrete cells of the anterior and intermediate lobes of the pituitary (Moon *et al.*, 1973). Analysis of the amino acid sequence (Fig. 1) of the hormone revealed that this 91-residue polypeptide displays only minor species variances (Li and Chung, 1976a).

Because the amino acid sequence of a part of the β-LPH molecule (residues 41–58) resembles that of the hormone β-melanocyte-stimulating hormone (β-MSH), Li and his colleagues (1965) sug-

β-LIPOTROPIN (PORCINE)

H-Glu Leu Thr Gly Glu Arg Leu Glu Gln Ala Arg Gly Pro Glu Ala Gln Ala (17)
Glu
Ser
Ala γ-LPH (1-58)
Ala Ala Glu Ala Glu Ala Val Leu Gly Tyr Glu Leu Glu Ala Arg Ala Ala (21)
Glu
Lys
Lys β-MSH

(41) Asp Ser Gly Pro Tyr Lys Met Glu His Phe Arg Trp Gly Ser Pro Pro Lys Asp (58)

ACTH 4–10 Lys
Met-enkephalin Arg

(76) Thr Val Leu Pro Thr Gln Ser Lys Glu Ser Thr Met Phe Gly Gly Tyr (61)

Leu α-endorphin β-endorphin
Phe C-fragment
Lys Asn Ala Ile Val Lys Asn Ala His Lys Lys Gly Glu-OH (91)

FIG. 1. Amino acid sequence of the lipotropic hormone.

gested that β-LPH was a possible prohormone of β-MSH. Other peptides could also be formed during the activation of β-LPH and stored in the pituitary. Indeed, it was recently shown that in addition to β-LPH, the peptides β-LPH 1–58 (γ-LPH), β-LPH 1–38 (N-fragment), β-LPH 41–58 (β-MSH, β-LPH 61–87 (C′-fragment), and β-LPH 61–91 (C-fragment) occur in considerable amounts in bovine pituitary glands as intact polypeptides (Bradbury *et al.*, 1976a, 1976c). Each of these peptides seems to be formed by cleavage of the lipotropin chain at the carboxyl side of paired basic residues (Lys–Lys and Lys–Arg) and subsequent removal of the basic amino acids. Thus, the specificity of the pituitary enzymes involved resembles that of other enzymes which generate hormones from their prohormones (e.g., insulin from proinsulin, Kemmler *et al.*, 1972). Studies dealing with the specificity of the pituitary enzymes which activate lipotropin reveal that the first cleavage of β-LPH is at the arginyl–tyrosine bond at positions 60–61, and is apparently caused by a trypsinlike enzyme (Bradbury *et al.*, 1976a). Another pituitary enzyme with a different specificity from trypsin seems to be involved in the cleavage of the bond at positions 39–40 (Lys–Lys) and may therefore be implicated in the *in vivo* release of β-MSH from lipotropin. At the moment, it is not clear whether specific activation of β-LPH leading to the release of LPH 61–91 and β-MSH takes place in one or more separate pituitary compartments. The data nevertheless indicate that β-LPH may serve as a prohormone for other pituitary peptides. Surprisingly, the physiological significance of β-LPH has remained rather

obscure. It possesses lipolytic activity (Yamashiro and Li, 1974) and very recently, morphinelike activity was observed after intracerebroventricular administrations (Ronai *et al.*, 1976). In contrast, no activity could be found when the hormone was tested in *in vitro* preparations reliably affected by morphine (Bradbury *et al.*, 1976b; Cox *et al.*, 1976a; Graf *et al.*, 1976b; Lazarus *et al.*, 1976). This is in accordance with the hypothetical role of β-LPH as a prohormone for other biologically active peptides.

The present chapter deals with the interaction of the lipotropin fragments and related peptides with the central nervous system. For historical reasons β-LPH 41–58 is designated as β-MSH and β-LPH 47–53 as ACTH 4–10.

II. ACTH 4–10

A. ACTH 4–10 and Learned Behavior

Hormones of the pituitary–adrenal system play an essential role in homeostatic functions (see de Wied and Weijnen, 1970; de Wied *et al.*, 1972; Gispen *et al.*, 1975c). Their regulatory role in central nervous functioning and behavior was discovered in rats whose pituitary–adrenal axis was disrupted or functionally suppressed. Over the years, studies have shown that adrenocortotropic hormone (ACTH) and steroids can influence behavior independently. In this review we deal exclusively with the influence of ACTH on the behavior which is brought about by direct peptide–brain interaction and in the majority of instances can be elicited by ACTH 4–10 (de Wied, 1974). Since α-MSH is identical to [Ac–Ser^1] ACTH 1–13–NH_2, it is not surprising that α-MSH has behavioral effects similar to those of ACTH. For an extensive review of the behavioral effects of α-MSH in mammals, the reader is referred to the paper by Kastin, Sandman, Miller and their co-workers (Kastin *et al.*, 1975).

Since a number of studies revealed that either peptides derived from the pituitary (ACTH, MSH, vasopressin, oxytocin, prolactin) or fragments of these peptides may influence behavior extraendocrinally and probably have a direct effect on the brain, such peptides were designated as neuropeptides and the significance of the pituitary for production of these neuropeptides has been discussed previously (de Wied, 1969, 1974; de Wied *et al.*, 1974a; de Wied and Gispen, 1977).

1. *Hypophysectomized Rats*

Hypophysectomized (hypox) rats were used in a number of the studies; such rats are depleted of neuropeptides of pituitary origin and are severely deficient in acquiring a conditioned avoidance response (Applezweig and Baudry, 1955; Applezweig and Moeller, 1959; de

Wied, 1969). Hypophysectomy interferes with passive (Anderson *et al.*, 1968; Weiss *et al.*, 1970; Lissák and Bohus, 1972), one-way (Gispen, 1970; de Wied and Gispen, 1977), and two-way active avoidance behavior (see de Wied *et al.*, 1972). Although general hormone replacement therapy without ACTH (de Wied, 1964, 1971) or an improved food intake (Harris, 1973) can restore the deficient performance to some extent, treatment with ACTH 4–10 alone is sufficient to normalize the avoidance behavior of hypox rats (de Wied, 1974; Bohus and de Wied, 1977). The expression of the behavioral activity of these peptides seems to depend on their presence during the behavioral task to be performed. Termination of treatment with ACTH 4–10 leads to a rapid deterioration of the performance of hypox rats (Bohus *et al.*, 1973).

2. *Intact Rats*

The effect of ACTH on acquisition of aversively motivated behavior is apparently difficult to demonstrate in intact rats presumably due to its dependence on the strength of the aversive stimulus (Murphy and Miller, 1955; Levine and Jones, 1965; Beatty *et al.*, 1970; Ley and Corson, 1971; Kelsey, 1975). Most of our knowledge of the regulatory role of ACTH in aversively motivated behavior was therefore gained in studies concerning the extinction or retention of such behavior. ACTH and ACTH 4–10 delay extinction of avoidance behavior in a two-way shuttle box (de Wied, 1966), in a one-way platform jumping test (Bohus *et al.*, 1968) and in a one-way pole-jumping apparatus (van Wimersma Greidanus, 1970). The effects can be elicited in a dose-dependent manner after both systemic or intracerebral (de Wied, personal communication) administration.

In studies which deal with ACTH fragments and passive-avoidance behavior, a more complicated picture emerges. ACTH 4–10 facilitates retention of passive-avoidance behavior in rats when administered 1 hour prior to a 24-hour retention trial in a step-through, one-trial, passive-avoidance procedure (Ader *et al.*, 1972). However, the improvement of passive-avoidance behavior by ACTH depends on the dose and shock intensity used (Lissák and Bohus, 1972; McGaugh *et al.*, 1975; Gold and van Buskirk, 1976). In a study on the effect of ACTH treatment during both training and testing in a one-trial "step-out" passive-avoidance situation, it was argued that the peptide would in fact enhance state-dependent learning (Gray, 1975). In another aversively motivated behavior, it was shown that ACTH 4–10 delayed extinction of the conditioned taste aversion response induced by pairing sugar water and the unpleasant experience caused by LiCl injection (Rigter and Popping, 1976).

The observations on aversively motivated behavior of rats indicate that ACTH 4–10 can affect both acquisition and extinction processes. The latter seem to be more sensitive to peptide treatment since according to Bohus and de Wied (1977) extinction behavior, thus in the absence of punishment, is more labile than acquisition behavior and thus more sensitive to modulatory influences. Furthermore, it was argued that ACTH 4–10 presumably maintains fear-motivated responses by preserving the motivating value of environmental stimuli. In line with this hypothesis, it was shown that ACTH 4–10 attenuates carbon dioxide-induced amnesia for a passive-avoidance response when administered prior to the retention test (Rigter *et al.*, 1974). As the antiamnesic effect of ACTH 4–10 is independent of the nature of the amnesia-inducing procedure used (Rigter *et al.*, 1975a), it was concluded that ACTH 4–10 facilitates retrieval of stored information.

In addition to the known effect of ACTH on aversively motivated behavior, effects on positively reinforced behavior have been seen in many studies. Guth *et al.* (1971) reported that ACTH increases the rate of bar pressing for water. The effectiveness of the treatment depended on motivation factors. Leonard (1969) found that ACTH partially antagonizes the deleterious effect of sodium barbitone on running time for a food reward in a multiple T-maze. Gray studied the influence of ACTH on the acquisition and extinction of a partially reinforced runway response with food as reward. Partial reinforcement is postulated to induce a behavioral state akin to frustration nonreward (Gray, 1967). Interestingly, partially reinforced rats receiving ACTH (Gray *et al.*, 1971) or ACTH 4–10 (Garrud, 1975) behaved like continuously reinforced rats both during acquisition and extinction. In addition, in an experiment on bar-pressing for food reward it was found that the differential effects of high and low reward on bar press rates were both attenuated by ACTH 4–10 (Garrud, 1975). Recently, Isaacson *et al.* (1976) suggested that in a response rewarded with water, ACTH 4–10 improves correct performance through better use of environmental cues by peptide-treated rats without a general effect on learning itself.

With respect to the extinction of rewarded behavior, it was clearly demonstrated that, in hungry rats, ACTH (Gray, 1971) and ACTH 4–10 (Garrud *et al.*, 1974) delayed extinction of a straight runway response for food. ACTH 4–10 delayed extinction of a sexually motivated approach response of male rats in a straight runway (Bohus *et al.*, 1975). Copulation reward during extinction appeared to be essential for the expression of the behavioral activity of ACTH 4–10. In addition, it was reported that ACTH 4–10 increased the motivation of female rats to seek contact with a sexually active male (Meyerson and Bohus, 1976). In an attempt to

pool the known effects of ACTH 4–10 on animal behavior within one physiological mechanism, Bohus and de Wied (1977) favor a mechanism involving motivation but indicate that more data are needed to assess the validity of such a hypothesis.

3. *ACTH 4–10 in Humans*

a. Volunteers. Repeated auditory stimulation with clicks produced arousal followed by habituation, as was shown by a shift from electroencephalogram (EEG) desynchronization to hypersynchronization (Endröczi *et al.*, 1970). Treatment with ACTH 1–10 (1–2 mg i.v.) restored the initial arousal-induced EEG desynchronization. These data suggest a "disinhibitory" action of ACTH on stimulus-induced EEG synchrony (Endröczi *et al.*, 1970). Both EEG and reaction time were measured in subjects participating in a disjunctive reaction-time task (Miller *et al.*, 1974). Treatment with ACTH 4–10 shifted EEG activity which is assumed to reflect a higher vigilance level. Furthermore, rating scales revealed that ACTH 4–10 treated subjects felt less tense and performed better in the Benton Visual Retention Test, suggestive of increased visual short-term memory. Together, the data imply that ACTH 4–10 raised the level of attention (Miller *et al.*, 1974). Further studies by the same authors support this notion. In a study on emotionality, immediate memory, concept learning, and field dependence, the authors concluded that ACTH 4–10 treatment resulted in a faster intradimensional shift and a slower extradimensional shift in a concept learning task as a consequence of increased dimensional attention (Sandman *et al.*, 1975). Also, in a continuous performance task, in which subjects had to detect the letter X in a series of letters appearing on an oscilloscope screen, ACTH 4–10 improved the performance significantly. Subjects so treated made fewer errors of omission and commission and had improved attention (Miller *et al.*, 1976). There are similar observations by Gaillard who reported that ACTH 4–10 reduced the number of lapses of attention in a self-paced serial reaction-time task. In addition, peptide-treated subjects made significantly less errors than did placebo-treated volunteers (Gaillard and Sanders, 1975a,b).

b. Patients. Retarded adult males were recruited from a workshop for the trainable retarded (Sandman *et al.*, 1976). They were subjected to the same concept learning task as used with healthy volunteers (Sandman *et al.*, 1975). In contrast to the study in volunteers, retarded patients treated with ACTH 4–10 displayed improved performance on both intra- and extradimensional shift. Furthermore, their performance in the Benton Visual Retention Test was also improved. Sandman *et al.* interpreted these results also as an indication of improved attention. A

recent open-safety study in elderly patients failed to show drug-related effects on electrocardiogram (EKG), blood pressure, or EEG parameters after ACTH 4–10 (Ferris *et al.*, 1976). However, the authors reported a significant change in mood, considered to be indicative of a mild antidepressant activity of ACTH 4–10.

Thus, ACTH 4–10 is the first neuropeptide to have been studied in human behavior using double-blind experimental designs. Although far from complete, the available data show increased visual attention and/or motivation in healthy volunteers, a finding which may be of significance to elderly or retarded people.

4. *Structure–Activity*

The behavioral effects of peptides structurally related to ACTH and MSH were first ascribed to the presence of the sequence ACTH 4–10 (Ferrari *et al.*, 1963; de Wied, 1966). There is an extensive series of studies by Greven and de Wied of the structural requirements of ACTH 4–10 necessary to delay extinction of avoidance behavior (Greven and de Wied, 1973, 1977; de Wied *et al.*, 1975). It was found that ACTH 4–7 was the smallest sequence to have essentially the same potency as ACTH 4–10 (Greven and de Wied, 1973). If, however, other fragments, e.g. ACTH 7–10 or ACTH 11–24 or the derivative [AC^{11}] ACTH 11–13–NH_2, were given at 10 times higher dose levels, it appeared that these fragments also induced the behavioral response (Greven and de Wied, 1977). Elongation of ACTH 7–10 to ACTH 7–16 resulted in a peptide as active as ACTH 4–10. It was concluded that there is a redundancy of information within the ACTH molecule with respect to its behavioral activity, supporting the report of two active sites for MSH activity by Eberle and Schwyzer (1975). Information located distal from the C-terminal of ACTH 4–7 may be present in a dormant form and may need to be potentiated by chain elongation in order to be expressed (Greven and de Wied, 1977). Although other ACTH–central nervous system (CNS) structure–activity relationships are not always identical to the one found for ACTH avoidance behavior (excessive grooming, Gispen *et al.*, 1975a, 1976a; Wiegant and Gispen, 1977; opiate receptor binding, Terenius *et al.*, 1975), the general principles of dormant activity and induction of such activity by chain elongation still seem to apply. Thus, if information is indeed encoded in a multiple form, comparison between peptides on the basis of primary structures alone is hazardous.

An important breakthrough in our experimental approach toward the nature of the brain cell–peptide interaction may be the suggestion that at the brain receptor site, ACTH 4–10 assumes an α-helix conformation with the Met^4 and the Arg^8 in close proximity (Greven and de Wied, 1977). Seemingly contradictory structure–activity results based on pri-

mary structures could then be explained by more definite knowledge of the stereoconformation at the receptor site.

When the Phe^7 residue in ACTH 4–10 was replaced by its D-enantiomer, the peptide facilitated rather than delayed extinction of an active avoidance response (Bohus and de Wied, 1966; de Wied *et al.*, 1975). The reversal of the behavioral effect was only found for analogs with the Phe^7 residue in the D-configuration (de Wied *et al.*, 1975). Since Phe^7 seemed to be crucial for the behavioral effect, replacement of this residue with other amino acids was undertaken in subsequent experiments. It was concluded that the electron donor properties of the amino acid residue in position 7 correlate to some extent with the behavioral potency of the peptide (de Wied *et al.*, 1975). It has been suggested that [D-Phe^7] ACTH 4–10 contains a new intrinsic activity with regard to extinction behavior and may thus act at a level different from that of ACTH 4–10 (see Bohus and de Wied, 1977; de Wied and Gispen, 1977).

Using knowledge gained from these detailed studies on structure and behavioral activity, Greven and de Wied (1977) were able to synthesize analogs of ACTH 4–10 which are 10^3 and even 10^6 times more potent than the parent molecule. The peptide [Met^4 (O_2), D-Lys^8, Phe^9] ACTH 4–9 has a 1000-fold potentiated behavioral activity, whereas the MSH activity of the same molecule was reduced by about the same factor, while the steroidogenic activity was significantly reduced. As the *in vitro* half-life of the various substituted ACTH 4–9 analogs in plasma or brain extracts correlated well with their behavioral potency (Witter *et al.*, 1975), a partial explanation for the effectiveness of the various substitutions may be that they provide protection against enzymatic breakdown.

5. *Site of Action; Role of the CSF*

The search for brain structures sensitive to ACTH fragments has been guided by the notion that the limbic system is important for acquisition and extinction of avoidance behavior. Bilateral destruction of the nucleus parafascicularis facilitated extinction of shuttle box avoidance behavior. Rats with lesions in this area did not respond to α-MSH with a delay of extinction of a pole-jump avoidance response (van Wimersma Greidanus *et al.*, 1974; van Wimersma Greidanus and de Wied, 1976). Further lesion studies also implicated the anterodorsal hippocampus as a site of action of ACTH 4–10 for the delay of extinction of avoidance behavior (van Wimersma Greidanus and de Wied, 1976). The results thus obtained were expanded by experiments using microinjection of ACTH 4–10 or ACTH 1–10 in various brain areas and subsequent monitoring of their behavioral effectiveness. These peptides caused inhibition of extinction of the pole-jumping avoidance response when they

were applied locally to the mesencephalic–diencephalic region at the level of the posterior thalamus and in the ventricles (van Wimersma Greidanus and de Wied, 1971). In view of the many negative results obtained in other brain regions, the data were taken as an indication of the importance of the parafascicular area. It is quite possible that, in brain, the neural substrate of these neuropeptides is restricted to a functional rather than to an anatomical unit. It may thus be concluded that the limbic system needs to be intact to permit neuropeptides related to ACTH to exert their behavioral effects (van Wimersma Greidanus and de Wied, 1976).

An interesting finding in the study by van Wimersma Greidanus and de Wied (1971) was that whenever the ACTH 1–10 was applied into the ventricular system, there was a delay of extinction. Recently, it has become more and more evident that the brain ventricular system is an avenue for neuropeptides to reach their site of action (Johnson and Epstein, 1975; de Wied and Gispen, 1977). For instance, intraventricular administration of antibodies against the neurohypophyseal peptide hormone, vasopressin, resulted in a memory deficit most likely due to elimination of the physiologically circulating vasopressin which facilitates memory formation (van Wimersma Greidanus *et al.*, 1975a,b). A variety of behavioral and neurochemical effects have been reported after intraventricular administration of ACTH-like peptides, thus indirectly supporting the notion that the cerebrospinal fluid (CSF) may also serve as a transport system for ACTH-like neuropeptides. There is some indication that ACTH-like immunoassayable material circulates in the CSF (Allen *et al.*, 1974). The actual mode of transport of pituitary hormones to the brain is not known. Various possibilities have been suggested, such as retrograde transport along the pituitary stalk or the existence of basilar cisterns which may be connected with the CSF in the adenohypophysis close to the hormone-producing cells (Allen *et al.*, 1974) or transport via the bloodstream. The latter hypothesis is supported by the recent finding of Ambach and Palkovits (1975) that the nucleus periventricularis receives blood from the anterior hypophyseal artery. There is an urgent need for studies aimed at the elucidation of a pituitary–CSF connection, since such a direct link could explain the role of the pituitary as an important source of behaviorally active neuropeptides.

B. ACTH 4–10 and the Induction of Excessive Grooming

When peptides derived from ACTH, MSH, or LPH are administered intraventricularly, a peculiar phenomenon is observed: a stretching and yawning syndrome (SYS) is induced in a variety of animals (Ferrari *et al.*,

1963; Gessa *et al.*, 1967). Given intrathecally in man, ACTH produces the same type of stretching and vomiting. The latter symptom is thought to be due to impurities in the preparation (Floris, 1963). In rodents, the onset of the syndrome is preceded by a display of excessive grooming (Ferrari *et al.*, 1963; Izumi *et al.*, 1973; Gispen *et al.*, 1975a; Rees *et al.*, 1976). The induction of grooming is independent of the presence of the adrenals, pituitary, or gonads (Gispen *et al.*, 1975a). Evidence is accumulating, however, that the induction of excessive grooming and SYS are mediated by two different CNS mechanisms (Gispen *et al.*, 1975a, 1976c). The biological significance of the behavior elicited after intraventricular administration of ACTH is unclear. Admittedly, the response is not seen exclusively after intraventricular administration of LPH-like peptides (MacLean, 1957; Beagly, 1976; Izumi *et al.*, 1973). Some authors suggest that in addition to SYS, ACTH also induces sexual excitement in rodents (Bertolini *et al.*, 1968, 1969; Baldwin *et al.*, 1974). In pigeons, the behavioral response was compared with displacement behavior as it occurs naturally in these birds (Delius *et al.*, 1976). Grooming is one of the behaviors in rodents often interpreted as representing displacement activities (Fentress, 1968; Hinde, 1970). It is not clear, however, whether the grooming response as discussed here and which lasts for at least 1 hour after the injection of ACTH and is in most instances interrupted only by stretching and yawning is in fact related to the grooming seen in a transitional behavioral state (Fentress, 1968). ACTH 1–24, α-MSH, and β-MSH are equipotent in inducing excessive grooming (Gispen *et al.*, 1975a). In rabbits, ACTH 4–10 was not nearly as active as ACTH 1–24 (Baldwin *et al.*, 1974), whereas in rats (Gispen *et al.*, 1975a) and mice (Rees *et al.*, 1976), ACTH 4–10 was totally inactive even in high doses. If, however, [D-Phe^7] ACTH 4–10 was used, excessive grooming was elicited even with low doses (Gispen *et al.*, 1975a; Rees *et al.*, 1976). This dormant activity of ACTH 4–10 could be potentiated by C-terminal elongation of the fragment (Gispen *et al.*, 1975a) or by shortening of the fragment to ACTH 4–7 which is as active as [D-Phe^7] ACTH 4–10 (Wiegant and Gispen, 1977). Thus, in essence the shortest peptide fragment with full activity on extinction of avoidance behavior (de Wied *et al.*, 1975) is also the shortest which induces excessive grooming (Wiegant and Gispen, 1977).

C. Electrophysiological Correlates of ACTH 4–10

1. *Central Nervous System*

Injections of ACTH increased the electrical activity of the rat brain as was concluded from an increase of voltage, occasional spiking, and

paroxysmal runs of low frequency, high voltage waves. The effect could be demonstrated in intact, hypox, and adrenalectomized rats; it was therefore assumed to reflect a direct effect on the brain (Torda and Wolf, 1952). Kawakami *et al.* (1966) suggested a correlation between circadian peaks in corticosterone and ACTH secretion and changes in multiple unit activity (MUA) in the hypothalamus. Most of the effects of ACTH on brain electrical activity are opposite to those evoked by the administration of adrenal steroids (Feldman *et al.*, 1961; Pfaff *et al.*, 1971). The mechanism by which ACTH induces these neurophysiological effects is independent of the presence of the adrenal cortex. Korányi *et al.* (1971) showed that systemic injection of ACTH resulted in a decrease of spontaneous electrical activity and responsiveness especially of the medial preoptic area of the free-moving cat. Steiner reported that ACTH directly activated neurons in hypothalamic and mesencephalic areas of rat brain (Steiner *et al.*, 1969; Steiner, 1970). In addition, ACTH increased the spike frequency of diencephalic cells (van Delft and Kitay, 1972) and the excitability of cells in the spinal cord (Nicolov, 1967). In addition to adrenal-mediated effects of ACTH in rat diencephalic MUA, some short latency excitatory influences were seen which could also be elicited in adrenalectomized rats (Sawyer *et al.*, 1968). Intraventricular infusion of ACTH 1–24 or ACTH 4–10 in rabbits led to increased electrical activity only in the lateral preoptic diagonal band of Broca and the periventricular preoptic area. Other areas showed little or no change in MUA. The effect was related to the behavioral and neuroendocrine activities seen after intraventricularly administered ACTH (Baldwin *et al.*, 1974). Further support for a direct effect of ACTH-like peptides on brain electrical activity comes from a series of experiments on the effect of α-MSH (= Ac–Ser^1 ACTH 1–13–NH_2) on EEG parameters in rabbits (Dyster-Aas and Krakau, 1965), rats (Sandman *et al.*, 1971), frogs (Denman *et al.*, 1972), and man (Kastin *et al.*, 1975). In view of the limitations of the present chapter the reader is referred to Kastin *et al.* (1975) and Miller *et al.* (1977) for further information.

In a free-moving dog, hippocampal electrical activity was sensitive to ACTH 4–10 and [D-Phe^7] ACTH 4–10. Although both peptides shifted the activity in the theta range to lower frequencies in an operant conditioning situation, the D-analog was less effective (Urban *et al.*, 1974). In rats, when hippocampal theta activity was induced by electrical stimulation of the reticular formation, the administration of ACTH 4–10 produced a shift in peak frequency from 7.0 to 7.5 Hz (Urban and de Wied, 1976). In another study, effects of ACTH 4–10 and [D-Phe^7] ACTH 4–10 on averaged visually evoked responses in cortical area 17 were recorded (Wolthuis and de Wied, 1976). When measured at a wide vari-

ety of light intensities, the amplitudes of the late components of the visually evoked potentials were significantly diminished after ACTH 4–10. In this situation the effect of [D-Phe^7] was in the same direction but again weaker (Wolthuis and de Wied, 1976). In humans, ACTH 4–10 treatment resulted in a statistically significant increase in the power output of the 12+ Hz and the 7–12 Hz frequency bands of occipital EEG, and such subjects did not show habituation of the EEG response to pattern arousal (Miller *et al.*, 1974). This observation is consistent with the findings of Endröczi *et al.* (1970) on the effects of ACTH 1–10 and ACTH 1–24 on human EEG.

In summary, there is ample evidence that ACTH 4–10 directly affects brain electrical activity. Since its effects can in some instances be mimicked by increasing the stimulus strength (Urban and de Wied, 1976), it may be that, in general, ACTH 4–10 facilitates transmission in its neural substrate, i.e., limbic structures (Urban and de Wied, 1975) or visual cortex (Miller *et al.*, 1974; Wolthuis and de Wied, 1976). ACTH 4–10 might increase the state of arousal in such structures, which may determine the motivational influence of environmental stimulus and thereby the probability of the stimulus-specific behavioral response being generated.

2. *Peripheral Nervous System*

Strand *et al.* studied the sciatic nerve–gastrocnemicus muscle preparation and observed that ACTH increased muscle action potential amplitude and contraction height and delays fatigue in normal, adrenalectomized, and hypox rats (Strand *et al.*, 1973–1974). The extraadrenal nature of the peptide–nerve–muscle interaction was corroborated by the finding that α-MSH and ACTH 4–10 also augmented the action potential amplitude. [D-Phe^7] ACTH 4–10 had no effect (Strand and Cayer, 1975). The effect of the peptides is best seen in fatigued and particularly in the hypox, fatigued rat. These observations led Strand *et al.* to perform clinical trials on a patient with myasthenia gravis; a short beneficial effect of ACTH 4–10 treatment was indeed observed (Strand *et al.*, 1975). The data support the hypothesis that such peptides may act as modulators of nerve function (Krivoy, 1970).

D. ACTH 4–10 and Blood Flow

It has been demonstrated in rabbits and cats that ACTH 1–39 and [Gly] ACTH 1–18–NH_2 depressed blood pressure (Korányi and Endröczi, 1967; Nakamura *et al.*, 1976). The same response could be shown in both intact and adrenalectomized rats, suggesting an extraadrenal action of ACTH on blood pressure. Since both α-MSH and human

β-MSH were inactive, it was further concluded that the depressor effect may also be independent of the melanocyte-stimulating properties of the molecule (Ueda *et al.*, 1970). ACTH 4–10 affects the heart rate changes which accompany emotional behavior (Bohus, 1975). Its influence was seen only in situations were certain learning paradigms are involved as in classical conditioning or in passive avoidance behavior. According to Bohus (1975), in this case the primary effect of ACTH 4–10 was on the CNS mechanisms controlling cardiovascular functions.

Goldman *et al.* (1975, 1977) have reported that α-MSH affected regional blood flow of the rat brain. They point out that a variety of drugs which have specific effects on animal behavior act only to redistribute the flow of blood to regions of the brain. There is reason to believe that these alterations in flow reflect the involvement of specific regions of the brain in the effect of these drugs (Goldman *et al.*, 1975). The flow in most areas was reduced within 10 minutes after intravenous administration of α-MSH and only the occipital cortex was spared. The effect was transient for most areas, but perfusion of pons and medulla, cerebellum, hippocampus, and parietal cortex was still low after 20 minutes. According to these authors, this rapid response of brain blood flow may be related to the effects of ACTH/MSH-like peptides on mental performance (Goldman *et al.*, 1977).

E. Brain Uptake of ACTH 4–10

A considerable effort has been made to demonstrate that exogenously administered ACTH-like peptides indeed reach brain structures so as to enable them to exert their regulatory role on behavior. Injection of [^{125}I] α-MSH resulted in a rapid accumulation of the radioactivity in structures such as the pineal gland which are exempted from the blood–brain barrier (Dupont *et al.*, 1975). Much lower concentrations were found within the brain. Only after intraventricular injection did some localization appear in a thalamic nucleus (Pelletier *et al.*, 1975). In view of the loss of biological activity after iodination of α-MSH, these studies could be only of a preliminary nature and were therefore followed up by a study of the distribution of [^{3}H] α-MSH in rat brain (Kastin *et al.*, 1976). In the latter study some accumulation of radioactivity was found in the occipital cortex, cerebellum, and the pons medulla area as compared with other brain parts. Although identification of the labeled material was only tentative, the data support the notion that systemically administered peptides may pass the blood–brain barrier.

Another group of investigators used the analog ^{3}H[Met-O_2-D-Lys^{8}-Phe^{9}] ACTH 4–9 whose 1000-fold higher behavioral potency is most likely related in part to its increased metabolic stability (Witter *et al.*,

1975). After intravenous administration, only about 0.5×10^{-4} of the dose was recovered in the brain as the intact peptide. The recovery was even lower after subcutaneous and oral administration (Verhoef and Witter, 1976). A detailed study of the distribution of this behaviorally potent ACTH 4–10 analog after intraventricular administration revealed that hippocampal and thalamic nuclei had a low or medium uptake of radioactivity (Verhoef *et al.*, 1977a). These areas had previously been found essential for the expression of the behavioral activity of ACTH-like peptides (van Wimersma Greidanus and de Wied, 1971; van Wimersma Greidanus *et al.*, 1975c). However, very high accumulation of radioactivity occurred only in septal nuclei (Verhoef *et al.*, 1977a). It was shown in further experiments that hypophysectomy specifically increased this uptake in the septum, emphasizing the physiological significance of this uptake (Verhoef *et al.*, 1977b). The specificity of the septal uptake system was clearly demonstrated using hypox rats treated with ACTH 1–24, ACTH 4–10, or ACTH 11–24. It was found that only the peptides sharing the 4–9 sequence with the radioactive 4–9 analog (ACTH 1–24 and ACTH 4–10) competed with the septal uptake, whereas the structurally unrelated sequence, 11–24, was ineffective. The specificity of the uptake was further evidenced by the negative data obtained after pretreatment with other neuropeptides including β-LPH 61–76. Interestingly, the septal uptake of the 4–9 analog was not affected by [D-Phe^7] ACTH 4–10 despite its structural resemblance (Verhoef *et al.*, 1977b). It is conceivable, as was pointed out earlier, that ACTH 4–10 and [D-Phe^7] ACTH 4–10 affect behavior at different brain levels. This may account for the ineffectiveness of the [D-Phe^7] ACTH 4–10 analog but also emphasizes the functional significance of the septal uptake of ACTH 4–9. Whether or not this uptake in fact resembles peptide septum–receptor interaction is the object of further research (Verhoef *et al.*, 1977b).

F. Neurochemical Response to ACTH 4–10

1. *Binding to Cell Membranes: Peptide–Cell Communication*

If the interaction of ACTH 4–10 with brain cells resembles that of peptides with their peripheral target cells, the generally accepted hypothesis is that ACTH 4–10 exerts its regulatory influence as first messenger in the manner described for the classical "second messenger" of Sutherland (1972). Binding to a specific receptor at the outside of the plasma membrane triggers adenyl cyclase (and/or guanyl cyclase) resulting in an alteration of the intracellular level of free cyclic nucleotides. The activity of specific cyclic nucleotide-dependent protein kinases is

then altered and a regulatory influence can be measured at various levels in the cell.

No specific binding sites for ACTH 4–10 have been demonstrated, but there is abundant indirect evidence to support the existence of such sites (see below). The paucity of receptors or the limited regions of ACTH-sensitive cells (Motta *et al.*, 1965; Steiner, 1970; van Delft and Kitay, 1972) may be major obstacles to such studies. Alternatively, it is possible that the conventional receptor methodology as applied in the study of specific morphine- (Terenius, 1973) or specific thyrotropin-releasing hormone (TRH)- (Burt and Snyder, 1975) binding to brain membranes is hampered by low affinity of the peptide for its brain receptor.

2. *Cyclic Nucleotides and Protein Phosphorylation*

It has been reported by several investigators that addition of ACTH to rat brain broken cell preparations *in vitro* does not affect adenyl cyclase activity (Burkhard and Gey, 1968; von Hungen and Roberts, 1973). Furthermore, it was found that ACTH, in a concentration of 10^{-4} *M* did not alter the levels of cyclic adenosine 3′-5′-monophosphate (cAMP) in slices of cerebral cortex, cerebellum, and hypothalamus of rabbit, rat, cat, and monkey (Forn and Krishna, 1971). In contrast to these findings, ACTH 1–10 in a dose of 10^{-5} *M* increased the levels of cAMP in slices from rat posterior thalamus by about 40% (Wiegant and Gispen, 1975). Recent data suggest that, in rat striatal slices, ACTH 1–24 increases cAMP and decreases cyclic guanosine 3′-5′-monophosphate (cGMP) levels concomitantly (Wiegant, unpublished observations). Intrathecal administration of high doses of ACTH to rabbits elevated the levels of cAMP in their CSF (Rudman and Isaacs, 1975). A preliminary report on the effect of α-MSH on rat brain cAMP levels *in vivo* states that α-MSH increased the content in occipital cortex of both intact and hypophysectomized rats. Changes in other brain areas were also noted but these did not occur in a consistent manner (Christensen *et al.*, 1976). Another study implicates a modulatory role of the pituitary–adrenal axis in rat cerebral metabolism of cAMP. Although *in vivo* administration of ACTH to hypophysectomized rats restored the decreased activity of adenyl cyclase and subsequent cAMP formation, the authors suggested a steroid–CNS rather than a peptide–CNS interaction (Nakagawa and Kuriyama, 1976). Thus at present no data are available which unequivocally establish an ACTH–cAMP mediated effect in brain. It should be kept in mind, however, that not all peptide–brain membrane interactions necessarily involve cyclic nucleotides.

The effect of cyclic nucleotides and that of ACTH fragments on

endogenous phosphorylation of rat brain synaptosomal plasma membranes *in vitro* has been compared by Zwiers *et al.* (1976). It was shown that even low doses of cAMP and ACTH 1–24 altered phosphorylation, whereas cGMP, ACTH 1–10, and ACTH 11–24 were without effect. However, cAMP-dependent phosphorylation was more or less restricted to an increase in three protein bands (MW 78,000–53,000), whereas ACTH 1–24 diminished the incorporation of radioactive phosphate into five bands of lower molecular weight (Zwiers *et al.*, 1976). Although certainly not conclusive, the data seem to indicate that there may be cAMP-independent effects of ACTH on brain phosphoproteins themselves thought to be important to brain cell physiology (Greengard, 1976) and behavior (Routtenberg *et al.*, 1975; Perumal *et al.*, 1975).

3. *RNA Metabolism*

Pituitary–adrenal hormones may have a regulatory effect on brain macromolecule metabolism as can be concluded from the effectiveness of various stressors to alter the incorporation of labeled precursors *in vivo* or their content in macromolecules (Jakoubek *et al.*, 1970; Rees *et al.*, 1974; Dunn *et al.*, 1976; Dunn and Rees, 1976; Schotman *et al.*, 1977a). The absence of pituitary also affects the metabolism of these macromolecules as can be concluded from the severe deficits in brain RNA and protein metabolism in hypox rats (see Gispen and Schotman, 1973). Yet little is known about the effect of exogenously administered ACTH-like peptides on brain RNA. Giving a single high dose of a purified ACTH preparation (5 I.U./100 gm s.c.) resulted in a transient inhibition of [^{3}H] uridine into mouse (Jakoubek *et al.*, 1972) and rat (Gispen and Schotman, 1976) brain RNA, followed by a small decrease in total RNA. In adrenalectomized rats, there was a marked increase in uridine incorporation (+40%) after similar treatment with ACTH, suggesting a counteraction between ACTH and corticosteroid at the genome level (Gispen and Schotman, 1976). Such counteraction may explain the small effects reported for intact rats or may even totally obscure such effects (Dunn, 1976). The data are sufficient to allow the conclusion that ACTH 4–10 or ACTH 1–10 fails to influence either the labeling of messengerlike and ribosomal brain RNA (Gispen *et al.*, 1970a; Schotman *et al.*, 1972) or the aggregation of brainstem polysomes (Gispen *et al.*, 1971). Furthermore, treatment with ACTH 1–10 did not affect brainstem RNA labeling or content of intact or of adrenalectomized rats (Gispen and Schotman, 1976). No effect of ACTH 4–10 on labeling and content of mouse cerebral RNA was found by Reading and Dewar (1971) or by Dunn (1976). The activity of brain RNAse was not affected by ACTH 4–10 either

(Reading and Dewar, 1971). While the larger sequence ACTH 1–24 may influence brain protein synthesis at the transcriptional level, the short sequence ACTH 4–10, if it were to have such an effect, should exert its action at the translational level (see below). However, in view of the complexity of the methodology involved and of the regulation of mammalian protein synthesis, the significance of this differential effect of ACTH 1–24 and ACTH 4–10 is uncertain (see also Dunn and Gispen, 1977).

4. *Protein Synthesis*

The effects so far reported for neuropeptides on brain protein metabolism are small and the methodology used is open to question. In most studies, the relevant data on precursor pool size, specific activity of the precursor, and of the labeled protein are not presented. The data are, however, treated here as though the incorporation studies indeed dealt with protein synthesis rate as was implied by most authors.

a. Hypox Rats. Chronic treatment of hypox rats with ACTH 1–10 (Schotman *et al.,* 1972) or ACTH 4–10 (Versteeg *et al.,* 1972; Reith, 1975) enhanced the incorporation of [^{3}H] leucine into brainstem proteins. At the end of the short incorporation period used, leucine still retained virtually all the acid-soluble radioactivity (Schotman *et al.,* 1974), whereas the acid-insoluble radioactivity was confined to cytoplasmic proteins (Schotman *et al.,* 1972). The same peptide treatment did not increase aggregation of polyribosomes in brainstem tissue (Gispen *et al.,* 1971). If, however, such hypox rats were subjected to additional daily training in a two-way shuttle box avoidance apparatus, the amount of polyribosomes in their brainstem was increased (Gispen and Schotman, 1970; Gispen *et al.,* 1971). With a 5-minute incorporation pulse and in view of recent calculations by Lajtha *et al.* on the turnover of rat brain proteins (Lajtha *et al.,* 1976), one should expect to have labeling not only of the rapidly turning over proteins but of the slowly turning over proteins as well (Reith *et al.,* 1977). Such a conclusion is consistent with the observation that a whole spectrum of soluble and membrane-bound proteins was labeled (Reith *et al.,* 1974a, 1975b). ACTH 1–10 treatment enhanced the incorporation of radioactive leucine into all proteins. Minor increases in labeling of water-soluble, high-molecular-weight proteins were superimposed on this rather general effect (Reith *et al.,* 1975b). It has been suggested that the enhancement of brain protein synthesis by ACTH-like peptides is specifically related to the behavioral activity of these peptides since, under similar conditions, [D-Phe^7] ACTH

1–10 decreased the labeling of brainstem proteins. In addition, the sequence ACTH 11–24 had no effect on brainstem protein synthesis (Schotman *et al.*, 1972; Reith, 1975).

b. Intact Rodents. In intact rats, ACTH increased the incorporation of amino acids into protein in brain and spinal cord (Semiginovsky and Jakoubek, 1971). Chronic treatment of young rats with high doses of ACTH resulted in a complex pattern of neurochemical changes including a biphasic effect on brain protein content (Palo and Savolainen, 1974). A single high dose of ACTH stimulated the incorporation of various ^{14}C-labeled amino acids into mouse brain by 20–100% 6–24 hours after injection of the precursor. The effect seems to be specific for brain since no change was seen in liver or kidney (Rudman *et al.*, 1974). Using another strain of mice and a shorter incorporation period, others found that ACTH 1–24 increased the incorporation of [^{3}H] lysine into brain and liver proteins, whereas ACTH 4–10 was effective only on brain proteins (Dunn *et al.*, 1976). There is further evidence for this differential effect of ACTH 1–24 and ACTH 4–10 from similar data on chronic treatment of rats with ACTH 4–10 (Reading and Dewar, 1971; Reading, 1972; Lloyd, 1974). Interestingly, such treatment with [D-Phe^{7}] ACTH 4–10 did not influence the incorporation of labeled precursor into brain proteins.

Intraventricular administration of ACTH 1–24 or [D-Phe^{7}] ACTH 4–10 to mice enhanced [^{3}H] lysine incorporation into brain proteins (Rees *et al.*, 1976). ACTH 4–10 was ineffective (Rees *et al.*, 1976). In view of the fact that after intraventricular administration of ACTH-like peptides only the grooming-inducing peptides affected brain proteins, it can be argued that the change in cerebral protein synthesis reported by Rees *et al.* (1976) is related to grooming and to SYS rather than to a peptide–brain protein interaction per se.

There is thus evidence to indicate that in both hypox and intact rodents, ACTH 4–10 can enhance brain protein synthesis *in vivo* but these effects of the neuropeptides are rather slight. It appears, however, that in general, even under severe circumstances such as hypophysectomy (Schotman *et al.*, 1972), undernourishment (Stern *et al.*, 1976), or neurotoxic treatment (Schotman *et al.*, 1977b), brain protein metabolism responds only with changes of the order of 20–30%. The changes of 10–25% in precursor incorporation found after treatment with ACTH 4–10 and related peptides are then as important as could have been expected. To study the possibility that an altered brain uptake of amino acids after peptide treatment (see effect on blood flow) might have contributed to the increased protein labeling, α-amino isobutyric acid was

used as a metabolically inert amino acid analog (Rudman *et al.*, 1974; Schotman *et al.*, 1976). ACTH or ACTH 1–10 did not alter the penetration of this amino acid into brain tissue, suggesting that the observed increase indeed related to some brain cell mechanism. There is further evidence for such a direct effect on brain protein metabolism from studies on the effect of peptide addition on the *in vitro* incorporation of amino acid into brain slice protein (Reith *et al.*, 1974b; Lloyd, 1974). When such slices were taken from hypox rats, ACTH 1–10 and ACTH 1–24 enhanced the incorporation of [^{14}C] leucine (Reith *et al.*, 1974b). Control experiments showed that incubation with the peptides did not alter amino acid uptake (Schotman *et al.*, 1976) or the extracellular space of slices (Reith, 1975). It is therefore unlikely that effects on these parameters underlie the increased protein labeling *in vitro*. In contrast with the *in vivo* observations, it was found that [D-Phe^7] ACTH 1–10 did not affect slice protein synthesis *in vitro* (Reith *et al.*, 1975b). It was found that, in slices from intact rat brain, ACTH 4–10 stimulated the incorporation of [^{14}C] leucine into protein (Lloyd, 1974). Surprisingly, *in vivo* treatment with ACTH was reported to decrease amino acid incorporation into rat brain slices *in vitro* (Jakoubek *et al.*, 1970). This discrepancy has not been explained, but has been confirmed by the recent unpublished observations of Schotman who showed that intraventricular administration of ACTH 1–24 resulted in a decreased incorporation of [^{14}C] leucine into proteins in a cell-free brain system. Apparently, a procedure involving *in vivo* injection followed by an *in vitro* test must be studied more extensively before meaningful conclusions can be drawn.

In conclusion, there is ample evidence that ACTH 4–10 enhances cerebral protein synthesis and that the mechanism underlying this effect involves an extraadrenal, direct CNS effect of the peptide.

5. *Neurotransmitters*

It was reported more than 20 years ago that ACTH increased acetylcholine synthesis in the brains of hypox and intact rats (Torda and Wolf, 1952). In subsequent years, neurophysiological data were collected which suggested that ACTH and melanotropic peptides could modulate neurotransmission (Krivoy, 1970), but surprisingly little is yet known of the transmitters affected.

Stressful stimuli which stimulate that release of ACTH and corticosteroids induced a general increase in brain noradrenaline (NA) turnover (see Versteeg and Wurtman, 1976). Treatment of intact rats with ACTH increased NA turnover in various brain regions (Hökfelt and Fuxe, 1972). Subsequent studies suggested that this effect results from an extraadrenal action of ACTH, since adrenalectomy (high-endogenous

ACTH) increases and hypophysectomy (absence of ACTH) decreases brain NA turnover (Versteeg *et al.*, 1972; Versteeg and Wurtman, 1976). It was recently reported that administration of ACTH to rats 2 days after adrenalectomy resulted in a significant decrease of NA uptake in several brain regions. This suppressive action of ACTH on hippocampal and neocortical NA uptake lasted for at least 12 hours (Endröczi, 1975). In intact rats pretreated with α-MPT to block NA synthesis, ACTH 4–10 accelerated the decline of NA, indicating an increased turnover (Versteeg, 1973). It was also seen in this study that, under similar conditions, treatment with [D-Phe^7] ACTH 4–10 was ineffective. A similar approach but using ACTH 1–10 led to negative results in hypox rats (Versteeg *et al.*, 1972), and it was suggested that steroid hormones might play a permissive role. It was subsequently found that ACTH 4–10 increased the incorporation of [^{3}H] tyrosine into total cerebral catecholamines, and that adrenalectomy blocked this peptide-induced response (Versteeg and Wurtman, 1975). ACTH 4–10 and [D-Phe^7] ACTH 4–10 increased the turnover of NA in rat forebrain and hindbrain, while ACTH 4–10 increased dopamine (DA) turnover in midbrain (Leonard, 1974). Others showed that α-MSH (Ac–Ser^1 ACTH 1–13–NH_2) increased the turnover of NA, but not of DA in intact rats (Kostrzewa *et al.*, 1975). Recent experiments in mice showed that ACTH 1–24, ACTH 4–10, or [D-Phe^7] ACTH 4–10 all increased the conversion of [^{3}H] tyrosine to DA, but not to NA (Dunn *et al.*, 1976) and again, adrenalectomy blocked the effect of the ACTH-like peptides. The fluorescence intensity of DA neurons in the substantia nigra was increased after treatment of rats with α-MSH and ACTH 1–24 (Lichtensteiger and Lienhart, 1975). Interestingly, peripheral administration of DA antagonists (haloperidol, fluphenazine) or DA-receptor blockade in the neostriatum suppressed ACTH-induced excessive grooming (Wiegant *et al.*, 1977a). Since ACTH 1–24 was ineffective when injected into the neostriatum but effective when applied in the substantia nigra, it was speculated that a nigrostriatal DA pathway was involved (Wiegant *et al.*, 1977a).

Few investigations have measured the effect of ACTH on the enzymes involved in catecholamine matabolism. In hypox rats, the activity of dopamine-β-hydroxylase (DBH) was decreased throughout the brain and the effect was only reversed by treatment with ACTH or ACTH 1–10 in hypothalamus and brainstem (van Loon and Mascardo, 1975). In intact rats, ACTH increased hypothalamic and decreased cortical DBH (van Loon and Mascardo, 1975). Other preliminary data suggest that ACTH and ACTH 1–10 administered *in vivo* can increase the *in vitro* activity of striatal tyrosine hydroxylase (Dunn *et al.*, 1976). With respect to brain 5-hydroxytryptamine (5-HT) there is one report of an acceleration of 5-HT turnover in hypox rats after α-MSH (Spirtes *et al.*,

1975). In intact rats, ACTH 4–10 and [D-Phe^7] ACTH decreased 5-HT content and turnover (Leonard, 1974), whereas α-MSH had no effect (Spirtes *et al.*, 1975). Rigter *et al.* (1977) studied the relation between ACTH 4–10, retrieval of a passive-avoidance response, and hippocampal 5-HT content. They had shown previously that application of footshock during the acquisition of a one trial passive-avoidance test was associated with a rise in hippocampal 5-HT concentration during the retention test 24 hours later (Leonard and Rigter, 1975; Rigter *et al.*, 1975b). Retrograde amnesia can be produced by treating the rats with CO_2. Treatment with ACTH 4–10 1 hour prior to the retrieval test alleviated the CO_2-induced amnesia. The antiamnesic effect of ACTH 4–10 was paralleled by a rise in the hippocampal 5-HT concentration while preacquisition treatment with ACTH 4–10 did not affect hippocampal 5-HT (Rigter *et al.*, 1977). The biochemical response therefore seems more likely to be the result of a behavioral change than of a peptide action.

In summary, the data on brain neurotransmitters are scarce and often confusing. No clear-cut evidence is available regarding the mechanism of action (modulator putative neurotransmitter, synthesis, release, etc.), the nature of the neurotransmitter system involved, and the specificity of the observed effects.

G. ACTH 4–10 AND MORPHINE

1. *Brain Opiate Receptors*

In the course of search for the identity of the endogenous ligand for morphine receptors, Terenius tested a variety of peptides for their capacity to inhibit binding of [^{3}H] dihydromorphine to rat brain opiate receptors. Substance P, lysine vasopressin (LVP), desglycinamide lysine vasopressin (DG-LVP), bradykinin, TRH, and prolyl-leycyl-glycine amide (PLG) were inactive (Terenius, 1975; Terenius *et al.*, 1975), while ACTH 1–28 and ACTH 4–10 had an affinity for these receptors (Terenius, 1975). Structure–activity studies pointed to an active site within ACTH 4–10 with some indication that a second affinity sight might be present in a sequence which is distal to the C-terminal (Terenius *et al.*, 1975; Gispen *et al.*, 1976a). [D-Phe^7] ACTH 4–10 and ACTH 4–10 were equally active. ACTH 4–7, the shortest sequence able to delay avoidance extinction (de Wied *et al.*, 1975) or to induce excessive grooming (Wiegant and Gispen, 1977), was inactive in the dose used. Analysis of the binding characteristics of ACTH-like peptides revealed a relatively low selectivity of these peptides for agonist or antagonist binding

sites, comparable to nalorphine, a partial agonist–antagonist (Terenius *et al.*, 1975; Terenius, 1976). In view of the relatively low affinity of ACTH 4–10 for the opiate receptor (IC_{50} in the order of 10^{-6}–10^{-5} M), it seems unlikely that it is an important endogenous opiate receptor ligand under physiological conditions. Since fragments from LPH 61–91 have a high affinity for such receptors, the low-affinity site of LPH 47–53 reflects the redundant manner in which information is encoded in LPH. The fact that ACTH 4–10 and ACTH 1–24 have affinity for CNS opiate receptors may, as discussed below, explain the observed interaction of these peptides with morphine at various levels of nervous system function (Wiegant *et al.*, 1977b).

2. *Morphine-Induced Analgesia*

Morphine has profound effects on pituitary–adrenal function (Selye, 1936; Briggs and Munson, 1955; de Wied *et al.*, 1974b; van Ree *et al.*, 1977a,b), and evidence is accumulating to suggest an interaction of ACTH with the CNS effects of morphine, e.g., counteraction of morphine-induced spinal reflex activity *in vivo* and *in vitro* (Zimmermann and Krivoy, 1973). In studies on the analgesic action of morphine in rodents, it was observed that ACTH could counteract this response (Winter and Flataker, 1951), although evidence was obtained that such an antagonism occurred only in the presence of the adrenal gland (Paroli, 1967; Gispen *et al.*, 1975b). Furthermore, glucocorticoids have been shown to alter certain actions of morphine on the CNS (Gispen *et al.*, 1975b; Brown and Garret, 1972; Zimmermann *et al.*, 1974). In a recent study, ACTH-like peptides, devoid of corticotrophic activity, were used to investigate the counteraction by peptides of morphine-induced analgesia as measured by a hot-plate technique (Gispen *et al.*, 1976a). The peptides inhibited the analgesic response by 50–60% with [D-Phe^7] ACTH 4–10 more potent than ACTH 4–10 (Gispen *et al.*, 1976a). The peptides were without a detectable action on the response behavior of saline-treated control rats, (Gispen *et al.*, 1970b, 1973, 1976a). Thus, the structure–activity relationship observed for ACTH–morphine interaction *in vivo* resembles that for ACTH affinity to rat brain opiate binding sites *in vitro*.

III. β-MSH

It is most likely because of the sequence 47–53 (ACTH 4–10) that β-MSH has effects on the CNS which in many cases are identical to those observed with ACTH or ACTH 4–10. We will thus briefly review relevant data on CNS effects of β-MSH.

In mice, chronic subcutaneous treatment with β-MSH results in hyperexcitability and hypersensitivity which could not be mimicked by α-MSH (Sakamoto, 1966; Segawa *et al.*, 1973). In contrast, repeated injections of β-MSH in rats induce excessive drowsiness (Sakamoto, 1966). For a variety of species, intracranial application of β-MSH elicits the SYS. (Ferrari *et al.*, 1963). In rats, this syndrome is preceded by display of excessive grooming (Izumi *et al.*, 1973; Gispen *et al.*, 1975a).

In the majority of instances, the effects of β-MSH on learned behavior resemble those brought about by α-MSH. Although most studies deal with α-MSH, many state that similar results have been obtained with β-MSH in the same paradigm (Kastin *et al.*, 1975; van Wimersma Greidanus, 1977). In short, α- and β-MSH increase the rate of avoidance acquisition in hypophysectomized (de Wied, 1969; Gispen and Schotman, 1970) and intact rats (Stratton and Kastin, 1974). Furthermore, these melanotropic peptides inhibit the extinction of active and passive avoidance behavior (de Wied, 1966, 1969; Sandman *et al.*, 1971; Dempsey *et al.*, 1972; Greven and de Wied, 1973; van Wimersma Greidanus, *et al.*, 1975d; van Wimersma Greidanus, 1977) and appetitive behavior (Sandman *et al.*, 1969; Kastin *et al.*, 1974).

On the basis of the few studies on neurochemical correlates of β-MSH activity, it seems conceivable that β-MSH influences brain protein metabolism in a manner similar to ACTH 4–10. β-MSH 6–24 hours after being injected increases the rate of incorporation of [^{14}C] valine into mouse brain proteins without affecting the acid-soluble radioactivity (amino acid precursor pool) (Rudman *et al.*, 1974). Furthermore, after treatment with β-MSH and conditioning in the shuttle box, hypox rats who had acquired the response as a result of peptide treatment had more of the large polysomal aggregates in their brainstem than did the controls (Gispen, 1970).

In a study of the ventral root potential evoked by stimulation of the dorsal root of cat spinal cord it was found that intravenously administered β-MSH facilitated the ventral root response (Guillemin and Krivoy, 1960; Krivoy and Guillemin, 1961), and this action could not be mimicked by ACTH or α-MSH. It has been demonstrated that β-MSH altered the recovery period of nerve cells, causing these cells to remain longer in a hyperexcitable state (Krivoy *et al.*, 1963). Further study of the facilitating effect of β-MSH on postfiring recovery of synaptic transmission in cat spinal cord revealed that α-motor neurons are specially sensitive to β-MSH and the the action of the peptide is probably post-synaptic (Krivoy and Zimmermann, 1977). The existence of β-MSH-degrading enzymatic activity in the brain may further support the hypothesis of a physiological role for this peptide in CNS function (Long *et al.*, 1961).

Krivoy proposed that β-MSH should be regarded as a modulator of synaptic transmission (Krivoy, 1970; Zimmermann and Krivoy, 1973); the report by Strand and Cayer (1975) of an increased amplitude of sciatic nerve potentials (hypox rats) and gastrocnemicus muscle (intact and hypox rats) after treatment with β-MSH fits the concept of a modulator.

Krivoy and co-workers have shown that morphine reduced the amplitude of evoked mono- and polysynaptic reflex activity in the decerebrated cat (Krivoy *et al.*, 1973). When cats were injected with β-MSH before morphine, the depressant action of morphine was not seen (Zimmermann and Krivoy, 1973), probably as a result of the stimulatory effect of β-MSH itself (Krivoy *et al.*, 1974). If this counteraction was not caused by competition of β-MSH and morphine for the same receptor site, it is not surprising that β-MSH did not alter morphine-induced analgesia as manifested in reduced response of rats to inescapable footshock (Gispen *et al.*, 1975b).

IV. β-Lipotropin 61–91

Mild digestion of β-LPH by trypsin led to specific cleavage at the peptide bond 60–61 and as a consequence, to release of the C-terminal part of β-LPH representing residues 61–91 (C-fragment, β-endorphin) (Bradbury *et al.*, 1976d). β-LPH 61–91 was isolated in large quantities as an intact polypeptide from pig and camel pituitary glands (Bradbury *et al.*, 1976; Li and Chung, 1976b) and has since been shown to be endogenous to the brain (Bradbury *et al.*, 1976c). However, the calculated amount of β-LPH 61–91 in a single brain is approximately one-eighth the amount present in a single pituitary (Bradbury *et al.*, 1976c).

In addition, smaller peptides which share amino acid sequences with β-LPH 61–91 have been isolated from whole brain (Hughes *et al.*, 1975) or from hypothalamus–neurohypophysis extracts (Guillemin *et al.*, 1976). These peptides have in common at least the sequence Tyr(61)–Phe(64) of the NH_2-terminus of β-LPH 61–91. Tyr–Gly–Gly–Phe–Met (sequence 61–65, Met^5-enkephalin) and Tyr–Gly–Gly–Phe–Leu (Leu^5-enkephalin) were isolated from pig and bovine brain tissue (Hughes *et al.*, 1975; Simantov and Snyder, 1976). Longer peptides were obtained from a crude extract of porcine hypothalamus–neurohypophysis, e.g. β-LPH 61–76 (α-endorphin) and β-LPH 61–77 (γ-endorphin) (Guillemin *et al.*, 1976; Ling and Guillemin, 1976) and β-LPH 61–87 (C′-fragment) occurred as an intact polypeptide in hypophyseal extracts (Bradbury *et al.*, 1976c).

A. Morphinelike Activity

The striking feature of these peptides, and one which in part led to the discovery, is that they mimic certain actions of morphine. The affinity to stereospecific receptors of narcotic analgesics in brain and peripheral tissue preparations has been used as a guide to isolate most of these peptides. Their morphinelike action was assessed *in vitro* with preparations of the guinea pig ileum longitudinal muscle and of mouse vas deferens (Cox *et al.,* 1975; Hughes, 1975; Hughes *et al.,* 1975; Guillemin *et al.,* 1976) and in displacement studies using specific binding to high-affinity opiate binding sites in brain subcellular fractions (Terenius and Wahlström, 1975; Pasternak *et al.,* 1975). Such endogenous substances with opioid activity have been designated generally as endorphins. To date, β-LPH 61–91 appears to be the most active of the endorphins, especially in the binding assay (Ling and Guillemin, 1976; Bradbury *et al.,* 1976d). All the endorphins presently available act as full morphine agonists on the guinea pig ileum and mouse vas deferens; the same holds for the binding assay with respect to the enkephalins. β-LPH, however, has the binding properties expected for a morphine antagonist or mixed agonist–antagonist (Bradbury *et al.,* 1976d; Birdsall *et al.,* 1976). In view of the *in vivo* activity of this peptide (see below), generalizations derived from studies with morphinelike drugs are of little value in predicting the characteristics of a peptide in this respect.

When endorphins were administered *in vivo,* their morphinomimetic action could be determined. Antinociceptive effects of intraventricularly injected Met^5-enkephalin have been reported for rats and mice, but these effects were rather weak and transient (Belluzzi *et al.,* 1976; Büscher *et al.,* 1976), probably due to rapid enzymatic degradation of Met^5-enkephalin *in vivo* (Hambrook *et al.,* 1976). Analogs of enkephalin resistant to degradation by brain enzymatic activity can indeed produce long-lasting analgesia (Hambrook *et al.,* 1976; Pert *et al.,* 1976, Pert, 1976). Profound analgesia was found after intraventricular injection of β-LPH 61–91 in cats, rats, and mice (Feldberg and Smyth, 1976; Graf *et al.,* 1976a; Loh *et al.,* 1976; van Ree *et al.,* 1976), suggesting that this longer peptide is more resistant to the metabolizing enzymes. On a molar basis, β-LPH 61–91 appeared to be about 100 times more potent than morphine in cats (Feldberg and Smyth, 1976). The antinociceptive activity of this peptide was slightly lower (30–40) and 15–35 times more potent than morphine in rats and mice (Graf *et al.,* 1976a; Loh *et al.,* 1976; van Ree *et al.,* 1976). In addition, it was found that this peptide produced analgesic effects when it was injected intravenously in mice (Tseng *et al.,* 1976). After five intraventricular injections of β-LPH 61–91 in a 3-day treatment schedule, hardly any analgesia could be detected using a hot-

plate test procedure. In these "tolerant" animals, the analgesic response to intraventricularly administered morphine was also diminished, indicating that cross-tolerance between β-LPH 61–91 and morphine had occurred (van Ree *et al.*, 1976). Cross-tolerance between morphine and enkephalin has been established in the same manner. Rats made tolerant by implantation of morphine pellets showed a diminished analgesic response to both intraventricularly administered morphine and Met^5-enkephalin (Bläsig and Herz, 1976). The enkephalin-induced inhibition of cortical single neurons was abolished in morphine-tolerant animals (Zieglgänsberger *et al.*, 1976). Furthermore, cross-tolerance between morphine and Met^5-enkephalin appeared to occur in peripheral preparations obtained from morphine-tolerant animals (Waterfield *et al.*, 1976).

β-LPH 61–91 and morphine share similar dependence properties as assessed by naloxone-induced withdrawal signs (Wei and Loh, 1976a,b; Loh *et al.*, 1976). Continuous infusion of β-LPH 61–91 directly into periaqueductal gray fourth ventricular spaces or repeated intraventricular injections of purified pituitary material induced physical dependence as evidenced by the occurrence of a typical morphinelike withdrawal syndrome after treatment with naloxone (Wei and Loh, 1976a,b; Loh *et al.*, 1976; Bläsig and Herz, 1976). Physical dependence was also present after infusion of considerable amounts of Met^5-enkephalin into brain tissue. In contrast, Leu^5-enkephalin appeared to be devoid of dependence-producing properties (Wei and Loh, 1976a,b).

Assuming that tolerance and physical dependence may be due to a biochemical response subsequent to the activation of the recognition of the receptor complex, these data provide further evidence that morphine and endorphins interact with similar receptor sites as had been proposed on the basis of affinity studies. Structure comparisons between morphine and enkephalin reveal that the primary attachment of enkephalin to the receptor may involve the aromatic hydroxyl moiety of the tyrosine residue (Roques *et al.*, 1976; Jones *et al.*, 1976). Indeed, substitution of the tyrosine residue in endorphins leads to substantial loss of affinity for the receptor (Ling and Guillemin, 1976; Chang *et al.*, 1976).

B. Grooming Activity

As already mentioned, rats displayed excessive grooming after intraventricular administration of ACTH-like peptides (Ferrari *et al.*, 1963; Izumi *et al.*, 1973; Gispen *et al.*, 1975a). This behavioral response was completely suppressed by specific opiate antagonists (Gispen and

Wiegant, 1976), suggesting that these peptides interact with brain opiate receptors. There is more evidence for a specific interaction from the findings that: ACTH-like peptides have an appreciable affinity for brain opiate binding sites *in vitro* (Terenius, 1976; Terenius *et al.*, 1975); they counteract the analgesic effect of morphine (Gispen *et al.*, 1976a); and they exhibit a morphinelike action in preparations of mouse vas deferens (van Ree, unpublished observations). The data obtained with the binding assay, the mouse vas deferens, and the morphineinhibiting action *in vivo* show that the active core of ACTH with respect to affinity and intrinsic activity to opiate receptors is located in the sequence 4–10. However, relatively large quantities of ACTH-like peptides are needed to demonstrate these activities.

If the induction of excessive grooming by ACTH-like peptides is mediated by opiate receptors, endorphins should be more potent in this respect. Indeed, β-LPH 61–91 elicited grooming activity and was somewhat more potent than ACTH-like peptides. An intraventricular injection of as little as 10 ng of β-LPH 61–91 significantly induced excessive grooming (Gispen *et al.*, 1976c). β-LPH 61–76 was much less active than β-LPH 61–91. The sequence β-LPH 61–91 was slightly less potent than β-LPH 61–76, while Met^5- and Leu^5-enkephalin could hardly elicit grooming activity over the wide dose range tested. These results agree with the data given above concerning the analgesic action of these peptides *in vivo*. The rapid enzymatic deactivation of enkephalin might also be responsible for the low activity of these shorter peptides in inducing excessive grooming.

C. Avoidance Behavior

Interestingly, peptides derived from β-LPH 61–91 and ACTH share another specific interaction with CNS processes. Extinction of active avoidance behavior (pole-jumping test) can be delayed by a single systemic injection of ACTH 4–10 (see above). Using a similar test procedure, it was found that a subcutaneous injection of 3 μg Met^5-enkephalin, 0.3 μg LPH 61–76, or 0.3 μg β-LPH 61–91 had behavioral effects identical to those of 3 μg ACTH 4–10 (de Wied, 1977). Peptides derived from β-LPH 61–91 are thus approximately 10 times more potent in inhibiting the extinction of active avoidance behavior. It is unlikely that this particular action of the peptides is due to their morphinelike activity, since morphine is unable to induce similar effects (de Wied, unpublished observations). Several years ago, peptides with high behavioral potency as assessed by their effect on the extinction rate of active avoidance behavior were isolated from pituitary glands (de

Wied *et al.*, 1970; Lande *et al.*, 1973). Tryptic digestion of one of these peptides yielded three major components after subsequent electrophoresis. The behavioral activity appeared to be restricted to one component. Although the amount of material was not sufficient to permit complete chemical identification or structure elucidation, the amino acid composition of the behaviorally active component was similar to that of β-LPH 61–69. One of the inactive components resembled the residues found in β-LPH 70–79. Therefore, it might be assumed that the behaviorally active peptide isolated from the pituitary gland shares at least the sequence 61–79 with β-LPH.

Evidence has been presented that ACTH 4–10 and related neuropeptides are involved in motivational processes (de Wied, 1974). Since neuropeptides related to β-LPH 61–91 have behavioral effects identical to those of ACTH 4–10, and since β-LPH 61–91 and ACTH-like neuropeptides have an affinity for brain opiate binding sites, it is tempting to speculate that peptides of pituitary origin which mimic or antagonize the acute action of morphine may modulate pain motivation (van Ree and de Wied, 1976).

D. Electrophysiology

Iontophoretic application of Met^5- Leu^5-enkephalin to single neurons in the medial brainstem (i.e., reticular neurons) of decerebrated cats revealed that these peptides depressed the firing rate of most neurons (Gent and Wolstencroft, 1976a,b). β-LPH 61–76 exerted the same overall inhibitory effect as enkephalin. Similar results were obtained when Met^5-enkephalin was applied to single neurons of rat brainstem (Bradley *et al.*, 1976; Gayton and Bradley, 1976). Morphine mimicked the response of enkephalin in both species. The specific opiate antagonist naloxone inhibited the action of both morphine and enkephalin in the rat (Bradley *et al.*, 1976). Because reticular neurons are implicated in the neuronal transfer of noxious stimulation (Casey, 1971; Wolstencroft, 1964; Haigler, 1976), it may be that enkephalin is physiologically involved in the pathway mediating pain perception. This hypothesis is supported by the finding that the excitatory response of thalamic neurons to peripheral noxious stimuli was completely blocked by iontophoretic application of Met^5-enkephalin (Hill *et al.*, 1976a,b). Although iontophoresis of Met^5-enkephalin depressed the firing rate of single neurons regardless of which brain area was studied (cerebral cortex, thalamus, medulla), the short latency, synaptically evoked firing of cuneate nucleus and ventrobasal thalamus sensory neurons was not blocked by Met^5-enkephalin (Hill *et al.*, 1976b).

Evidence has been presented that enkephalin can affect the postsynaptic cell membrane (Zieglgänberger and Fry, 1976; Satoh *et al.*, 1976). This peptide delayed and finally blocked the late response of cortical neurons to transcallosal stimulation. Intracellular recordings from cat spinal neurons revealed that iontophoresis of enkephalin antagonized l-glutamate-induced depolarisation without causing detectable changes in membrane potential or membrane resistance. The data from microiontophoretic application of enkephalin to single neurons, together with the rapid deactivation of these peptides when they are administered *in vivo*, point to the possibility that these substances are inhibitory neurotransmitters (Gent and Wolstencroft, 1976a; Bradley *et al.*, 1976; Hill *et al.*, 1976a; Frederickson *et al.*, 1976; Kosterlitz and Hughes, 1975; Zieglgänsberger and Fry, 1976). The pre- or postsynaptic receptors of these putative transmitters could be sites where the opiate alkaloids exert their pharmacologically morphinomimetic action.

E. Cyclic Nucleotides

There is increasing evidence to suggest that cyclic nucleotides are involved as mediators of the postsynaptic action of some neurotransmitters (Greengard, 1976). The hypothesis that enkephalins may act as brain neurotransmitters and the observation that these peptides may be endogenous ligands for "opiate receptors" have raised the question whether enkephalins influence the metabolism of cyclic nucleotides in preparations in which morphine-induced changes have been seen. In cultured neuroblastoma X glioma hybrid cells which display many properties characteristic of neurons and which have a high density of opiate receptor binding sites, morphine elicits an increase in cGMP content, a decrease in cAMP content, an inhibition of basal adenylcyclase activity, and a blockade of prostaglandin E_1-induced rise in cAMP (Traber *et al.*, 1975a; Sharma *et al.*, 1975a,b; Gullis *et al.*, 1975). Similar effects were observed when enkephalins were added to the incubation medium (Gullis *et al.*, 1976; Brandt *et al.*, 1976; Klee *et al.*, 1976; Klee and Nirenberg, 1976). Both these effects of morphine and of enkephalins can be completely antagonized by naloxone. In this particular assay, as in the mouse vas deferens preparation (Hughes *et al.*, 1975), the enkephalins showed a very high potency in comparison with morphine. Prolonged exposure of the cells to morphine caused an increase in adenyl cyclase activity, which was interpreted to reflect a biochemical correlate of tolerance and dependence (Traber *et al.*, 1975b; Klee *et al.*, 1975). The same effects were observed when the cells were incubated with Met^5-enkephalin for 12 or more hours (Klee *et al.*, 1976; Lampert *et al.*, 1976).

Morphine increased the *in vitro* accumulation of cGMP and decreased that of cAMP in slices of rat neostriatum, an area containing a very high density of opiate binding sites (Minneman and Iversen, 1976a,b; Kuhar *et al.*, 1973; Snyder, 1975). The addition of enkephalins to these slices had similar effects. High concentrations of the enkephalins were needed, however, even after the addition of a peptidase inhibitor, in contrast to the neuroblastoma X glioma hybrid cells preparations. Again, these data are suggestive of the instability of the enkephalins in mammalian tissue. It is nevertheless concluded that the enkephalins affect the brain cyclic nucleotides in a manner similar to morphine. Whether such changes can account for the postulated action of enkephalins on postsynaptic cell membranes is still open to question.

F. Biological Significance

The origin of endorphin is not yet known with certainty. The identity of Met^5-enkephalin with β-LPH 61–65 indicates a possible pituitary origin for this peptide. However, no evidence was found for the existence of Met^5-enkephalin in pituitary material. It is possible that larger endorphins, specially β-LPH 61–91, are physiologically active and that Met^5-enkephalin is merely a degradation product with high receptor affinity. Indeed, when β-LPH was incubated with extracts of rat brain, peptide fragments with opiatelike activity were generated (Lazarus *et al.*, 1976). This finding suggests that the enkephalin found in brain extracts arose from breakdown of larger sequences during isolation procedures. The first cleavage of β-LPH to liberate β-LPH 61–91 can occur in the pituitary. This fragment might then reach the brain via the CSF or other routes and interact with specific receptors in the brain.

At the moment, one can only speculate about the physiological significance of β-LPH 61–91 and related peptides. Most of the present research seems to be concentrated on the enkephalins. Whether the morphinelike action of these peptides really is their most important biological function is questionable as, even in very low doses, the endorphins affect brain processes involved in conditioned avoidance behavior. In contrast, relatively high amounts of the peptides were needed for morphinelike activity *in vivo*, especially when the enkephalins were tested. The lower potency of enkephalins cannot solely be explained by their assumed higher sensitivity to enzymatic degradation, since such a mechanism is also present in the avoidance behavior test.

Other peptides with morphinelike activity and with characteristics which differentiate them from peptides related to β-LPH 61–91 have been isolated from the pituitary (Cox *et al.*, 1976b; Gentleman *et al.*,

1976; Goldstein, 1976) suggesting that enkephalin need not be the only endogenous ligand for opiate receptors. The present confusion extends to the physiological role of the endorphins as well. No conclusions could be drawn from experiments involving naloxone treatment to block the receptors for these ligands in test situations reliably affected by morphine. Naloxone did not change the sensitivity threshold for foot-shocks (Goldstein *et al.*, 1976) or affect temperature control under cold stress (Goldstein and Lowery, 1975). Yet, using inescapable electric foot-shock (EFS) to measure the animal's response to pain, it was found that hypox rats—thus depleted from pituitary endorphins—displayed an increased responsiveness to the aversive stimulus (Gispen *et al.*, 1970b). Although developed for the purpose (Evans, 1961), it is unlikely that EFS exclusively measures the animal's response to pain: Other factors such as motivation, locomotor activity, and integrity of reflex pathways also influence the response to the EFS (Gispen *et al.*, 1973).

It is interesting that naloxone blocked the hyperthermia produced by conditional stimuli (Lal *et al.*, 1976) and food- and water-seeking behavior in hungry or thirsty rats (Holtzman, 1974, 1975). A study of the regional distribution of the opiate binding sites showed many such sites in structures of the limbic system (Kuhar *et al.*, 1973; Snyder, 1975). Therefore, it can be inferred that peptides which have specific affinity for opiate binding sites play an essential role in limbic system functions. This assumption is supported by the effects of naloxone on food- and water-seeking behavior and by the marked effect of endorphins and ACTH 4–10 on extinction of active avoidance behavior. The activity of limbic structures might be involved in all these test situations.

V. Concluding Remarks

Since the discovery of β-LPH in 1964 by C. H. Li, this polypeptide has been a hormone in search of a function. At the moment, the picture emerges that peptides derived from β-LPH strongly influence nerve cell functioning. ACTH 4–10 (=β-LPH 47–53) and related peptides (ACTH, β-MSH, α-MSH) are thought to facilitate acquisition and retention of learned behavior by increasing the motivation value of environmental stimuli. In man, the effects are defined in terms of increased attention, in both volunteers and retarded patients. At the cellular level, these peptides exert a trophic response which had previously been interpreted as reflecting the activity necessary to alter neuronal connectivity. This latter process then may underlie the storage of newly acquired information. Thus, a behavioral experience may activate the pituitary to

release ACTH 4–10-containing neuropeptides which reach the brain by a still unknown mechanism and then exert their action.

Peptides derived from the C-terminus of β-LPH have a similar or higher potency than ACTH 4–10 in behavioral tests. Therefore, the physiological role of these peptides is more likely to be the regulation of behavioral mechanisms than the surprising morphinelike activity. If the morphinelike activity is meaningful in this respect it may be that these peptides affect pain motivation rather than pain perception. It still remains to be proven that the effects of LPH fragments *in vivo* are due to peptide action alone. It may be that part of the biological activity results from an interference with normal pituitary function due to the effects of the fragments on neuroendocrine mechanisms. Thus, some of the effects of β-LPH fragments may serve only as trigger signals, whereas a mediating principle (hormone) in fact underlies the functional response. The recognition that both ACTH 4–10, β-MSH, and several endorphins are identical to certain sequences in the peptide β-LPH may be crucial for understanding their mechanism of action. Increasingly, β-LPH is viewed as a prohormone for β-MSH and endorphins. Prohormones are not uncommon in mammalian physiology: A peptide–prohormone usually generates an active peptide by enzymatic cleavage of the inactive parental molecule. β-LPH seems to fit such a precursor role since, in many tests, the molecule does not mimic the effect of its active fragments. LPH has no affinity for the opiate receptor, has no morphinelike activity, and is inactive in the behavioral tests studied so far. In the case of β-LPH, it is postulated that a behavioral experience triggers the production of LPH fragments. Knowledge of the enzymatic activity involved (enzymes, cellular/regional localization, regulation, etc.) is a prerequisite for the understanding of the biological significance of β-LPH.

It seems not unlikely that β-LPH generates at least two classes of neuropeptides (ACTH 4–10/β-MSH and endorphins). In view of the similarities between the activities at least as they are known at present of these two classes (affinity for opiate receptors, effects on avoidance and grooming behavior) it can be argued that their existence merely reflects the redundant way in which information is encoded in biologically active peptides. According to this view the C-terminal peptides would contain the true active site, whereas the N-terminal peptides would reflect the dormant activity sites. However, we would like to propose that the difference between the two classes resides not in their potency but rather in the direction in which they influence brain mechanisms. There is a variety of evidence to support the notion that two opposite messages are encoded in LPH, one, N-terminal from Tyr^{61} (ACTH 4–10/β-MSH), and the other, C-terminal, with the Tyr^{61} as N-terminal (endorphins). A known

but still debated parallel is the case of oxytocin which does not itself affect MSH-release but appears to influence this release in opposite ways by means of its two fragments, ring (tocinamide) and tail (PLG).

In summary (see Table I), peptides containing ACTH 4–10, in general, stimulate firing rate, excitability of neurons and neurotransmission, increase cAMP levels, and counteract some morphine-induced CNS effects (inhibition of spinal reflex activity, analgesia). Effects opposite to these have been reported for LPH–endorphins, i.e., the inhibitory influence on neurophysiological processes and cAMP levels and the morphinomimetic action (*in vitro*, analgesia, tolerance, etc.). Thus, proper enzymatic cleavage could release both an excitatory and an inhibitory influence on nerve tissue. The actual processing of such signals should not necessarily be as "black and white" as the release of opposing information suggests. The differences known to exist between the two classes of LPH fragments in, e.g., metabolic stability, intrinsic activity, and receptor affinity can be regarded as tools which allow, upon cleavage of LPH, a release of information to neurons in a subtle manner. If indeed, as already suggested, there is more than one type of opiate or peptide receptor in the central nervous system, the two classes of LPH fragments need not affect behavioral processes through identical receptors but may influence identical brain structures (limbic system?) via binding to different receptor sites. Indeed after intraventricular administration of low doses of β-MSH or β-LPH 61–91 to rats, a complex behav-

TABLE I
SUMMARY OF CNS ACTIVITIES OF LPH FRAGMENTS[a]

CNS activity	β-LPH	
	β-MSH/ACTH 4–10	Endorphins
Electrical activity	↑	↓
cAMP levels	↑	↓
Opiate receptor affinity *in vitro*	+	+++
Opiatelike activity *in vivo*	0/−	+
Avoidance extinction	+++	+++
Excessive grooming	++	+++

[a] The evidence summarized in this table is discussed in the various sections of this chapter. The arrows indicate increase or decrease of activity. The + rating system compares the potency of the two classes of fragments for a given parameter. 0 stands for no effect and − for counteraction of effect.

ioral response is observed, which is qualitatively and quantitatively different for the two classes of LPH peptides despite an overlap with respect to excessive grooming behavior.

It is clear that, in 1975/1976, neurobiology has arrived at the brink of a new neuropeptide era. From the abundance of preliminary communications in recent months it is not difficult to predict that, in the near future, much research will be undertaken to elucidate the role of endorphins in the CNS, behavior, and even abnormal behavior. Previously, the delay of extinction of a learned response, thus the perseverating performance in the absence of punishment or reward, has already been considered to reflect a maladapted behavioral response to an altered environment. Exogenously administered ACTH 4–10 enhances this process and it was therefore proposed that one of the endogenous factors involved in the onset or maintenance of neuroses related to a dysfunction in the production of pituitary ACTH 4–10-containing neuropeptides (Gispen *et al.*, 1976b). Attention has recently been called to a possible role of LPH–endorphins in schizophrenia and psychoses (Terenius *et al.*, 1976; Bloom *et al.*, 1976, Jacquet and Marks, 1976). Amidst the turbulent development of current LPH–neuropeptide research into putative neurotransmitters, modulators, peptidergic neurons, pain perception/motivation, etc., the major effort should not be a single-minded approach but a broad and critical one based on the enormous potential of such peptides in brain and behavior processes.

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