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CORTICOSTERONE MODULATION OF NORADRENALINE STIMULATED CYCLIC AMP FORMATION IN HIPPOCAMPUS

A Thesis
Presented to the
School of Graduate Studies

by

Veronica J. Roberts

In partial fulfilment of
requirements for the degree of
Master of Science

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ABSTRACT

Corticosterone modulation of the noradrenaline-responsive cyclic AMP generating system was examined in rat hippocampus in order to investigate some of the possible mechanisms underlying such conditions as behavioral disorders, learning and memory impairments, and stress. These conditions have been linked to alterations in brain noradrenergic mechanisms and to changes in circulating steroid levels. The hippocampus was the focus of attention here because it retains more corticosterone than any other part of the brain.

Adrenalectomy was found to produce a small but significant elevation in the rate of cyclic AMP formation in response to noradrenaline. Implantation of corticosterone pellets 5 days prior to sacrifice prevented this adrenalectomy-induced increase. Metopirone, an inhibitor of corticosterone synthesis, was also observed to increase noradrenaline-responsive cyclic AMP formation. This elevation was seen 2 hours following a 50 mg/kg i.p. injection and was completely prevented by corticosterone pellet implantation. Metopirone had no significant effect on cyclic AMP production after 1 hour, while a slight but non-significant elevation remained at 4 hours. These observations parallel the inhibitory effect of Metopirone on corticosterone synthesis as determined by serum corticosterone levels.

Metopirone had no significant effect on the cyclic AMP generating system <u>in vitro</u>. This suggests an indirect action of the drug which presumably is due to its inhibitory effect on

corticosterone synthesis. However, corticosterone and two *in vitro* corticosterone antagonists (progesterone and 11-deoxycortisol) did not alter noradrenaline stimulated cyclic AMP formation *in vitro*. These results, and results from hypophysectomized animals, suggest that the observed increase in cyclic AMP formation following steroid reduction may be due to secondary changes such as increased ACTH levels.

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Unquestionably, the pursuit of my graduate career would not have been the same without my husband, Dave. I thank him for helping me learn.

Finally, in appreciation of my parents who have supported all of my adventures, I dedicate this thesis.

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I. INTRODUCTION

ROBERTS

INTRODUCTION

Chemical Messengers

The literature of a decade ago highlighted the differences between neurons and endocrine cells, but recently the similarities have been the focus of attention. Both the endocrine system and the nervous system use chemical substances to transfer information from cell to cell and thus create an intricate communication network. In the endocrine system these substances are called hormones and consist of steroids, peptides, and amino acids. They are secreted by specialized cells into the bloodstream, and generally act some distance from where they are released. In the nervous system, the chemical messengers are called neurotransmitters. They are released from presynaptic neurons directly onto the site of action. The known neurotransmitters include acetylcholine, biogenic amines, neuroactive peptides, and amino acid transmitters.

Many substances have been found to act as both hormones and neurotransmitters. For example, amines such as serotonin, dopamine, and histamine, and peptides such as substance P and vasoactive intestinal polypeptide (VIP) are found in the paracrine-endocrine cells of the gut and act as hormones. They are also released from neurons in the brain into the synaptic cleft and act in a manner analogous to transmitter substances. In addition, many neurons in the brain are both endocrine and neuronal. The cells of the hypothalamus, for example, release

peptides into the local or systemic circulation where they act as hormones on distant cells. Other substances are released into the synaptic cleft where they serve as neurotransmitters.

Hormones derived from amino-acids cannot enter the cell. Instead, they exert their action through specific receptors located on the cell membrane. As with amino-acid-derived endocrine hormones, neurotransmitters also act by binding to cell receptors. These receptors are located on the postsynaptic receiving neuronal membrane and in some cases on the presynaptic secreting neuronal membrane as well. Receptors are specialized protein molecules capable of binding select hormones and neurotransmitters. Membranes of different tissues and neurons contain different receptors. These receptors are classified according to the potency order of substances to which they respond and by the ability of specific drugs to block the response. The response to neurotransmitters or hormones is determined by the type of receptor complex present on the receptive cell.

The Cyclic AMP Generating System

The receptors for many endocrine and transmitter systems are coupled to an adenyl cyclase enzyme which converts ATP to cyclic AMP. In 1957, Sutherland and his co-workers were able to show that the application of adrenaline to liver cells resulted in the increased formation of cyclic AMP within the cell. The membranes of these cells were found to contain adenyl cyclase, an enzyme that converts ATP to cyclic AMP (Sutherland, et al., 1962).

Adrenaline, glucagon, corticotropin and some other hormones have been shown to activate adenyl cyclase through specific receptors in certain tissues. Of the neurotransmitters noradrenaline, dopamine, histamine, serotonin, and even some opiates may mediate some of their effects through receptor coupled adenyl cyclase.

Intracellular cyclic AMP has been shown to mediate catecholamine stimulated gluconeogenesis, the formation of glucose from lactate, and glycogenolysis, the breaking down of glycogen to glucose, in liver cells (Exton et al., 1970). Lipolysis, the degradation of stored triglycerides into fatty acids, is also stimulated via the actions of catecholamines on adipose tissue. This effect involves catecholamine stimulated cyclic AMP as the mediator of the response as well (Butcher et al., 1965).

The primary effect of most neurotransmitters is believed to take the form of an alteration in the electrical excitability of the cell. The membrane of the neuron is capable of integrating the net effect of many transmitters as their inhibitory or excitatory effects summate or cancel. Therefore, in a broad sense, there may be an interaction between all neurotransmitters. There may also occur more restricted or specialized interactions where the effects of stimulation of two receptors is more than additive (synergistic) or antagonistic. Examples of such interactions can be found in alpha-receptor modulation of beta-adrenergic or VIP responses.

Steroid Hormones

Steroid hormones can also achieve such specialized interaction with other endocrine or transmitter systems, however in contrast to the actions of the other transmitters, steroids act intracellularly. The cholesterol derived steroid hormones are relatively small and lipid soluble. They are able to penetrate the cell membrane and act directly within the target cell. Here, they regulate DNA transcription and protein synthesis. The resulting changes in enzyme levels and other cellular components can modify the effects of other hormones and neurotransmitters. It has been observed that adrenal corticosteroids regulate many of the physiological effects of catecholamines, such as carbohydrate, protein, and fat metabolism (Ramey and Goldstein, 1957).

Corticosterone Modulation of Noradrenergic Mechanisms

Alterations in brain noradrenergic mechanisms and changes in circulating steroid levels have been shown to occur under such conditions as stress, learning and memory impairments, and mood disorders. This thesis examines the intracellular interactions between noradrenaline and corticosterone in the brain to help discover how such alterations are linked to these conditions. However, before proceeding to this evidence it is instructive to review the earlier observations which were derived in peripheral tissue.

LIVER AND ADIPOSE TISSUE

In examining the effect of adrenal steroids on adrenaline stimulated lipolysis in adipose tissue, Reshef and Shapiro

(1960) and Shafrir et al. (1960) found that free fatty acid release by fat pads from adrenaline treated animals was greatly reduced in steroid deficient (adrenalectomized) rats. This effect was prevented by pre-treating the animals with cortisone. Adrenaline was shown to increase lipolysis in adipose tissue and to enhance free fatty acid release. While cortisone itself had no effect on the rate of free fatty acid release it significantly enhanced this effect of adrenaline on adipose tissue. Experiments by Exton et al. (1972) revealed that adrenaline stimulated cyclic AMP levels, expressed as nmoles/mg DNA, were higher in the fat pads of adrenalectomized rats compared to the controls. These results suggested that changes in the levels of corticosteroids could affect adrenaline stimulated lipolysis and alter the sensitivity of catecholamine sensitive adenyl cyclase systems in rat adipose tissue.

Exton and his co-workers (1972) also examined the effect of corticosteroids on noradrenaline stimulated gluconeogenesis and cyclic AMP accumulation in rat liver. Adrenaline stimulated glucose production in livers from adrenalectomized rats was greatly reduced compared to glucose production in the controls.

Adrenaline stimulated cyclic AMP levels were also examined in the livers of normal and adrenalectomized rats (Exton et al., 1972). The basal level of cyclic AMP production was the same in normal and adrenalectomized animals. However,

adrenaline stimulated cyclic AMP production in adrenalectomized rats was greater than adrenaline stimulated cyclic AMP production in normal rats.

Bitensky et al. (1970) also found that when endogenous steroid levels were reduced by adrenalectomy, adrenaline-responsive hepatic adenyl cyclase activity increased. The replacement of the steroids by subcutaneous injections, twice a day for nine days, reversed the adrenalectomy induced rise in cyclic AMP levels back toward control values. He was also able to show that the addition of exogenous steroids in normal animals resulted in a slight decrease in adrenaline-responsive hepatic adenyl cyclase activity.

Bitensky et al. (1970) also measured cyclic AMP formation in the livers of hypophysectomized rats. Hypophysectomy is the surgical removal of the pituitary. This gland releases adrenocorticotrophic hormone (ACTH) which stimulates the synthesis and release of adrenal corticosteroids. The results were parallel to the effects of adrenalectomy. Likewise, the effect of hypophysectomy could be reversed by the chronic administration of ACTH.

These results suggested that noradrenaline stimulated cyclic AMP formation in liver and fat tissue could be modified by changes in steroid levels. It is likely that an increase in adrenaline stimulated cyclic AMP formation, following a decrease in steroid levels, could be an adaptive response to maintain cellular mechanisms.

BRAIN

Catecholamine sensitive adenyl cyclase systems have also been found in brain tissue (see Daly, 1977). Very recently, Mobley and Sulser examined the effect of corticosteroids on the noradrenaline receptor-coupled adenyl cyclase system in rat frontal cortex (Mobley and Sulser, 1980a) and rat limbic forebrain (Mobley and Sulser, 1980b).

Mobley and Sulser found that the cyclic AMP accumulation was significantly higher in the frontal cortex and limbic forebrain from steroid deficient animals as compared to sham operated rats at a noradrenaline concentration of 100 μM. The dose response curve indicated that the increase in cyclic AMP accumulation was due to an enhanced maximal responsiveness of the system, not to an altered affinity for noradrenaline.

This adrenalectomy induced increase in cyclic AMP formation could be reversed by administering corticosterone daily for 5 days before sacrifice. Steroid administration in control animals did not significantly decrease the cyclic AMP response to noradrenaline.

It was later shown that the administration of corticosterone for only three days would also effectively reverse the increased response to noradrenaline following adrenalectomy (Mobley et al., 1983). In this same publication, it was reported that medullectomy, the selective removal of the catecholamine producing region of the adrenal gland, did not alter noradrenaline stimulated cyclic AMP production in rat

brain. This ruled out the possibility that decreased plasma catecholamine levels were responsible for the observed alterations in the noradrenaline sensitive adenyl cyclase system. These results suggest that adrenal steroid reduction enhances the activity of the noradrenaline sensitive adenyl cyclase system in brain. This result is parallel to that found in liver and adipose tissue.

MECHANISM

Beta-adrenergic receptors

Evidence from a number of sources suggests that alterations in the availability of hormones or neurotransmitters can modify the sensitivity of the cyclic AMP generating system. Much of this work has been done on the catecholamine sensitive beta-adrenergic receptors. For example, a decrease in the availability of noradrenaline at postsynaptic sites in the brain, through neuronal lesions (Huang et al., 1973; Kalisker et al., 1973; Palmer, 1972) or with various drugs that deplete noradrenergic stores, such as reserpine (Dismukes and Daly, 1974), results in a gradual up-regulation or increased sensitivity of the cyclic AMP generating system to noradrenaline. Presumably this "supersensitivity" is a compensation for the decreased availability of the neurotransmitter. The opposite effect "subsensitivity" occurs when the availability of noradrenaline is increased. For example, tricyclic antidepressants, which inhibit the reuptake of noradrenaline, and amphetamine, which inhances its release, both increase the amount of noradrenaline

in the synaptic cleft. As a compensatory response, the activity of the cyclic AMP generating system is decreased (Vetulani and Sulser, 1975; Schultz, 1976; Baudry et al., 1976; Martres et al., 1975).

The cellular mechanism for steroid-induced modulation of adrenergic mechanisms is not clear. In the liver, the increase in cyclic AMP production following adrenalectomy is accompanied by a 3 to 5 fold increase in the number of beta adrenergic receptors (Wolfe et al., 1976). This increase was reversed by cortisone replacement. It has been suggested that this is the mechanism by which the activity of the cyclic AMP response is increased in adrenalectomized animals. However, following adrenalectomy, no significant alteration in the affinity of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) for beta receptors or in the number of these receptors was noted in either rat frontal cortex (Mobley and Sulser, 1980a) or hippocampus (Roberts and Bloom, 1981).

This raises the possibility that different mechanisms are involved in the effect of adrenalectomy on liver and brain. It is possible that the increase in beta receptors in the liver, following adrenalectomy, may actually occur because adrenalectomy not only removes the source of corticosteroids, but it also deprives the liver of endogenous adrenaline. This is not, however, the case in the brain. Adrenalectomy removes a peripheral source of adrenaline and noradrenaline, but in the brain, both adrenaline and noradrenaline are synthesized in

various brainstem nuclei. Thus, the source of these catecholamines to the brain is not depleted by adrenalectomy and no increase in either the B_{max} or K_D of [^{3}H]-DHA binding (supersensitivity) develops.

Bilateral 6-hydroxydopamine lesions of the dorsal noradrenergic bundle have been shown to produce near total depletions of forebrain noradrenaline (Roberts et al.,1976). This treatment results in 62% and 34% increases in the number of beta-receptor sites in the frontal cerebral cortex and hippocampus respectively, as measured by [^{3}H]-DHA binding (U'Pritchard et al., 1980). Roberts and Bloom (1981) also reported a 41% increase in maximum [^{3}H]-DHA binding in the hippocampus four weeks following the 6-hydroxydopamine treatment. Thus, following forebrain noradrenaline depletion, there is a marked and persisting proliferation of beta-adrenergic receptors.

As mentioned earlier, adrenalectomy alone does not increase beta-adrenergic receptor binding in the brain. However, when adrenalectomy is combined with injections of 6-hydroxydopamine into the the dorsal noradrenergic bundle, maximum [^{3}H]-DHA receptor binding increases by 68% (Roberts and Bloom, 1981). This is significantly greater than the increase (41%) that was observed following 6-hydroxydopamine lesions alone. This effect of adrenalectomy could be reversed by treating rats for one week with twice daily corticosterone injections.

In conclusion, when the source of brain noradrenaline is

removed, steroid reduction results in an increased density of beta-adrenergic receptors in this tissue. Thus, the brain and liver may be similar in their regulation of cellular mechanisms by adrenal steroids.

Alpha-adrenergic receptors

There is evidence that the activation of alpha-adrenergic receptors in brain tissue facilitates the beta-adrenergic stimulation of the cyclic AMP generating system. The alpha-adrenergic agonists phenylephrine, oxymetazoline, and clonidine have been shown to potentiate the adenyl cyclase stimulatory effects of submaximal concentrations (0.1 μM) of isoproterenol, a beta-adrenergic agonist, in rat cerebral cortical slices (Skolnick and Daly, 1975).

In contrast, Vetulani et al. (1977) reported that clonidine did not affect the cyclic AMP response to 10 μM of the beta-adrenergic agonist isoprenaline. It should be noted however, that Skolnick and Daly (1975) had already shown that clonidine did not affect stimulatory concentrations of isoproterenol greater than 1 μM. Vetulani's experiments were also done in a different brain region, limbic forebrain, and with a different species of rat, adult, male Sprague-Dawley, than those of Skolnick and Daly who used cerebral cortical slices and adult, male F-344 rats.

Schultz and Kleefeld (1979) also failed to find an effect of the alpha-adrenergic agonists, clonidine and methoxamine, on the cyclic AMP response to low or high doses of isoproterenol.

Experiments were done in cerebral cortical slices of adult male Sprague-Dawley rats. These results are in agreement with Vetulani et al. (1977) who also used Sprague-Dawley rats but a different brain region. Daly et al. (1981) found that the cyclic AMP accumulation in response to the beta-adrenergic agonist, 2-fluoronoradrenaline, was not as great as the response to noradrenaline, in all eight brain regions examined. The alpha-adrenergic agonist, 6-fluoronoradrenaline, produced a relatively small accumulation of cyclic AMP but when combined with the 2-fluoronoradrenaline the response was often more than additive. The response to the combination of the two fluoronoradrenalines was similar to that elicited by noradrenaline. These experiments were done in adult, male Sprague-Dawley rats, but they support the findings of Skolnick and Daly (1975) that the activation of alpha-adrenergic receptors in brain tissue facilitates the beta-adrenergic stimulation of the cyclic AMP generating system.

It is difficult to draw conclusions from these results, due to the inconsistencies of the observations. However, if facilitory alpha-adrenergic receptors are coupled to the intracellular cyclic AMP system, then it is possible that the increase in forebrain cyclic AMP formation following adrenal steroid reduction could be due to an enhanced responsiveness of this alpha-adrenergic facilitation. However, this seems unlikely because adrenalectomy has no detectable effect on alpha-adrenergic receptors. No changes were found in either the affinity of [^{3}H]-clonidine for alpha-2-noradrenergic

receptors or [^{3}H]-WB-4101 for alpha-1-noradrenergic receptors, nor was there a change in the number of these receptors in rat frontal cortex following adrenalectomy (Mobley et al., 1983). Nevertheless, it is still conceivable that steroid regulation of the alpha-adrenergic system may be affected by mechanisms other than the control of receptor density.

The non-beta-component

Mobley et al. (1983) made the following observations: The response of the noradrenaline stimulated cyclic AMP generating system in rat frontal cortex was increased 2 weeks following bilateral adrenalectomy. This alteration appeared to be restricted to the non-beta component of the system because the cyclic AMP response to isoproterenol, a beta-adrenergic agonist, was not significantly altered following adrenalectomy.

While it has been reported that alpha agonists enhance beta-adrenergic stimulated cyclic AMP formation (above), these agonists have also been shown to inhibit the increase in the non-beta-component of noradrenaline stimulated cyclic AMP production. The alpha-adrenergic agonists clonidine, oxymetazoline, and methoxamine have been reported to inhibit noradrenaline stimulated cyclic AMP accumulation in rat cerebral cortical slices (Skolnick and Daly, 1975; Schultz and Kleefeld, 1979). The alpha agonists reduced noradrenaline stimulated cyclic AMP accumulation to levels comparable to those reached with maximal beta-adrenergic receptor stimulation. The combination of clonidine and the alpha-

adrenergic antagonist phenoxybenzamine did not reduce noradrenaline stimulated cyclic AMP levels ky any more than either agent alone, while clonidine and the beta-adrenergic antagonist propranolol reduced these levels to non-stimulated values. These results are indicative of an alpha-adrenergic inhibition on the non-beta component of noradrenaline stimulated cyclic AMP formation.

Similar effects of alpha-adrenergic agonists have been observed in rat limbic forebrain. Vetulani et al. (1977) reported that both clonidine (50 μM) and phenylephrine (50 μM) significantly reduced cyclic AMP accumulation in response to 5 μM noradrenaline. Likewise, Mobley and Sulser (1979) found that the addition of various concentrations of the alpha-adrenergic agonist methoxamine, to rat limbic forebrain slices, resulted in a dose-dependent inhibition of noradrenaline stimulated cyclic AMP accumulation. It remains to be determined whether this inhibition is due to a competitive interaction at the alpha-receptor.

These results suggest that alpha-adrenergic agonists inhibit the noradrenaline stimulated cyclic AMP generating system in brain. In addition, it seems that this alpha-adrenergic induced inhibition may be restriced to the non-beta-component of the system. Thus, it is possible that the increased responsiveness of the noradrenaline sensitive adenyl cyclase system following adrenalectomy might be due to a decrease in the inhibitory influence of the alpha-adrenoceptor system on noradrenaline stimulated cyclic AMP formation. While

no significant changes in alpha-adrenergic receptor binding were observed following adrenalectomy (Mobley et al., 1983), it is possible that steroid induced changes in the alpha-adrenoceptor system may not be measurable at the receptor level.

Regulatory enzymes

The increase in noradrenaline stimulated cyclic AMP production in brain following adrenalectomy does not appear to be due to alterations in the activities of the regulatory enzymes. Mobley et al. (1983) found no change in basal and NaF-stimulated adenyl cyclase activity in rat frontal cortex two weeks after adrenalectomy. Likewise, no differences in the activity of phosphodiesterase, the inactivating enzyme of cyclic AMP, were observed between adrenalectomized animals and the controls.

Protein synthesis

Steroid hormones have been shown to regulate the synthesis and degradation of specific proteins in a variety of peripheral tissues. Reif-Lehrer and Chader (1969) reported a cortisol induced synthesis of glutamine synthetase in embryonic chick retina while McCoy et al., (1966) demonstrated glucocorticoid induced increases in alkaline phosphatase activity in human leukocytes. Baxter and Tomkins (1971) have found that specific glucocorticoid hormone receptors in hepatoma tissue culture cells mediate the induction of tyrosine aminotransferase by dexamethasone. These are just a few examples of the many

enzymes in various tissues whose activity is altered by glucocorticoids (see Thompson and Lippman, 1974 for review). These tissues all have specific, high-affinity, limited-capacity cytoplasmic and nuclear glucocorticoid receptors.

A free exchange of corticosterone into all parts of the brain and the specific retention of this hormone by the hippocampus has also been demonstrated. Using i.p. injections of [^{3}H]-corticosterone in adrenalectomized rats, McEwen and his colleagues (1969) were able to show that the concentration of corticosterone in the brain increases with increasing concentrations of the hormone in the blood. Likewise, the fall in circulating corticosteroids is rapidly followed by a decrease in brain corticosteroids. It appears that there is a very rapid exchange between the steroid in the blood and that in the brain. This non-specific entry of corticosterone from the blood into the brain is accompanied by the specific retention of the hormone by the hippocampus and the septum.

It appears that corticosterone retention by the septum is only saturated at high and unphysiological concentrations of the hormone, while uptake and retention by the hippocampus is saturated at physiological concentrations of corticosterone found in normal rats (McEwen et al., 1969). In addition, McEwen and his co-workers have isolated and characterized nuclear and cytoplasmic corticosterone receptor proteins from the rat hippocampus (McEwen et al., 1975; McEwen and Plapinger, 1970).

The parallels between the hippocampus and corticosterone

target organs in the periphery prompted investigations into the effect of adrenal glucocorticoids on protein synthesis modulation in the hippocampus. Etgen et al. (1979), found that the incubation of hippocampal slices in 10^{-9} M corticosterone for one hour significantly increased the incorporation of labeled amino acid into a specific region of the soluble cytoplasmic fraction of hippocampal slices. The total amount of protein synthesis in the slices was not altered by the hormone treatment as determined by [^{3}H]-leucine incorporation into acid-precipitable protein. This suggests that glucocorticoid modulation of specific hippocampal proteins occurs without altering the overall protein metabolism of the cell.

It is interesting to note that identical hormone treatment did not alter protein synthesis in neocortical slices, a brain region containing few glucocorticoid receptors (Etgen et al., 1979). In further experiments it was shown that progesterone, a steroid which does not translocate hippocampal corticosterone receptors to the cell nucleus (McEwen et al., 1976), did not induce hippocampal protein synthesis (Etgen et al., 1980). This hormone competes with hippocampal corticosterone receptor binding and a 10-fold excess of progesterone effectively blocks corticosterone induced protein synthesis (Etgen et al., 1980).

These results provide evidence that corticosterone may regulate gene expression in the brain as well as in peripheral target tissues. In addition, it appears that this modulation

may occur through the interaction of corticosterone with specific, high-affinity steroid hormone receptors.

Corticosterone induced protein synthesis or degradation could lead to the modulation of many cellular mechanisms. Noradrenergic receptor protein numbers or their binding properties could be altered as well as various intracellular enzymes. Membrane proteins could also be affected resulting in membrane fluidity and permeability changes. Such changes could explain the mechanism by which corticosterone modulates noradrenergic stimulated cyclic AMP production.

Conclusion

Adrenal steroids regulate noradrenergic stimulated cyclic AMP formation in the brain. This effect is similar to that found in peripheral tissues such as the liver and fat cells. At present, we can only speculate as to the nature of this interaction. It is possible that the increase in cyclic AMP production following steroid reduction may be due to an alteration in beta-adrenergic receptors which directly stimulate the cyclic AMP generating system. However, no significant alteration in either the B_{max} or the K_d of these receptors has been noted following adrenalectomy. Results suggest that only after the source of noradrenaline is removed, is the effect of adrenal steroids measurable at the receptor level.

There is evidence that the activation of alpha-adrenergic receptors in brain facilitates the beta-adrenergic stimulation of the cyclic AMP generating system. Alpha-adrenergic agonists

have also been reported to inhibit the non-beta-component of noradrenaline stimulated cyclic AMP production. The increased responsiveness of the noradrenaline sensitive adenyl cylase system following adrenalectomy could be attributed to alterations in the facilitory and/or inhibitory mechanisms of the alpha-adrenoceptor system. While no significant changes in alpha-adrenergic receptor binding were observed following adrenalectomy it is possible that steroid induced changes in the alpha-adrenoceptor system may not be measurable at the receptor level.

The increase in noradrenaline stimulated cyclic AMP production following adrenalectomy does not appear to be due to alterations in the activities of the regulatory enzymes. No differences in the activity of brain adenyl cyclase or phosphodiesterase were observed between adrenalectomized and sham-operated animals.

Steroid induced changes in protein synthesis have also been demonstrated in brain tissue. Such changes in gene expression could result in alterations in membrane proteins, various enzymes, as well as the binding properties of the noradrenergic receptors. Such modulations could again explain the effect of corticosterone on the noradrenergic cyclic AMP generating system.

In the present study, corticosterone modulation of the noradrenaline-responsive cyclic AMP generating system was examined in rat hippocampus, a brain region which selectively

binds corticosterone. Because many hormonal responses are affected by adrenalectomy, convergent evidence for the role of corticosterone on noradrenaline receptor mechanisms was sought using Metopirone, a drug which inhibits the synthesis of corticosterone.

Review of Metopirone

Metopirone was synthesized in 1959 by Bencze and Allen and shown to inhibit the secretion of 17-hydroxycorticosteroids by the adrenal glands in dogs (Chart et al., 1958) and in humans (Liddle et al., 1958). Metopirone also inhibited 17-hydroxycorticoid synthesis in adrenal preparations from rats, dogs and guinea pigs (Chart et al., 1958). Further studies showed that this drug was an inhibitor of 11-beta-hydroxylase thus preventing the conversion of 11-deoxycortisol to cortisol and of deoxycorticosterone to corticosterone (Jones, 1969; Griffiths and Glick, 1966). Metopirone was originally thought to inhibit 11-beta-hydroxylase specifically, but additional studies showed that Metopirone also affected the activity of other enzymes involved in steroid metabolism namely 17-, 18- and 19-hydroxylase (for review see Neher and Kahnt, 1965).

The mechanism of the inhibition of 11-beta-hydroxylase by Metopirone was examined by Williamson and O'Donnell in 1969. They found that both Metopirone and deoxycorticosterone compete for the same binding site on mitochondrial cytochrome P-450, the oxygen activating and steroid binding hemoprotein of the 11-beta-hydroxylase system. This competition decreased the deoxycorticosterone induced shift in the cytochrome spectrum.

It was suggested that the inhibition of the 11-beta-hydroxylating enzyme by Metopirone is a result of this drug's ability to competitively compete for binding sites on cytochrome P-450.

The Metopirone induced suppression of 11-beta-hydroxylation was found to produce an increase in 11-deoxycortisol and deoxycorticosterone (Jenkins et al., 1958; Liddle et al., 1958). This effect was originally thought to be a result of a compensatory increase in ACTH secretion. However, Matsuki et al. (1982) reported, that this increase may also be due to some other mechanism. A significant increase of 11-deoxycortisol was seen 1 hour after Metopirone treatment in man, but ACTH levels did not increase until 3 hours. Cortisol was decreased at 1 hour and reached its lowest level 2 hours following Metopirone treatment. Thus 11-deoxycortisol levels may originally increase following Metopirone treatment as a result of its decreased conversion to cortisol.

Metopirone has been used clinically to test the pituitary-adrenal axis. In normal individuals, the Metopirone-induced decrease in steroid levels is followed within a few hours by a feedback stimulation of ACTH (Liddle et al., 1958). Schoneshofer et al. (1983), however, have recently reported a dual effect of Metopirone on plasma ACTH. In addition to the known feedback effect, Metopirone appears to have a suppressive effect on plasma levels of ACTH. The mechanism of this effect is not known but may explain the frequent "failures" of the

Metopirone test for evaluation of the pituitary-adrenal axis.

Collectively, the inhibition of 11-beta-hydroxylase by Metopirone results in a decrease in plasma cortisol and corticosterone. A compensatory increase in ACTH secretion occurs but may be complicated by a suppressive effect of Metopirone. The rise in plasma 11-deoxycortisol and deoxycorticosterone could be linked to increased ACTH levels as well as to a shift from cortisol and corticosterone to 11-deoxycortisol and deoxycorticosterone respectively.

For the purpose of the present study, Metopirone will be used to inhibit the synthesis of corticosterone. The effect of these pharmacologically altered steroid levels on noradrenergic systems in the brain will be assessed.

II. METHODS AND MATERIALS

METHODS AND MATERIALS

Animal Preparation

Male Wistar rats (175-225 g) were maintained on a constant 12 hour light, 12 hour dark cycle at 23 °C. Animals were given free access to food and water. In the case of adrenalectomized (ADX) rats, a 0.9% NaCl solution was substituted for water. Bilateral adrenalectomies were performed 9 days prior to sacrifice. In sham-operated rats, adrenals were located but not removed. Hypophysectomized male Wistar rats were purchased from Charles River Canada Inc. Upon arrival, a 5% glucose solution was provided in substitution for water. Animals receiving corticosterone pellets were anesthetized with ether and pellets were implanted subcutaneously in the back of the neck 5 days prior to sacrifice. Sham-operated rats received an incision but no pellet. Rats treated with Metopirone were given a 50 mg/kg i.p. injection approximately 3 hours into the light cycle and sacrificed 1, 2, or 4 hours later. Injection volume was 1 ml/kg body wt. Animals treated chronically were injected once a day for 6 days with the inhibitor, then sacrificed 2 hours after the last injection. All control animals received an equal volume of the vehicle (40% propylene glycol in water).

Tissue Preparation

Tissue was prepared according to the method of Harden et al. (1977) with some modifications. Rats were sacrificed by decapitation, the brain rapidly removed and placed on an ice-

cold glass plate. Hippocampi were removed freehand, pooled 6 per group and sliced in two directions at right angles to each other (0.26 x 0.26 mm) with a McIlwain tissue chopper. The slices were weighed and suspended (50 mg/ml) in oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, glucose 7.8 and disodium EDTA 0.03 mM). For the in vitro experiments one of the following agents was added to the Krebs-Henseleit buffer when indicated; 10^{-6}M Metopirone, 10^{-9}M corticosterone, $5x10^{-6}$M 11-deoxycortisol, or $5x10^{-6}$M progesterone.

The suspension was incubated in an atmosphere of 95% O_2 and 5% CO_2 for 20 min at 37 ^{o}C in a shaking Dubnoff water bath. Following incubation, the samples were centrifuged at 600 x g for 20 sec. The pellet was re-suspended (50 mg/ml) in fresh Krebs-Henseleit buffer containing 2.5 μCi/ml of [8-^{3}H] adenine for an additional 40 min period. Excess radioactivity was removed by washing three times by centrifugation (600 x g, 20 sec) with pre-warmed and gassed Krebs-Henseleit buffer containing 1 mM Na ascorbate, 1 μM pargyline and 1 mM 3-isobutyl-1-methylxanthine.

Samples were divided into four aliquots and incubated in the presence of 0, 1, 10 and 100 μM noradrenaline (37 ^{o}C; 95% O_2 : 5% CO_2) for 10 min. The reaction was stopped by the addition of 50% trichloroacetic acid (107 μl/ml) and homogenized immediately. Occasionally a small portion (100 μl) was removed for protein estimation. The remaining portion was centrifuged at 18,000 x g for 10 min. The pellet was discarded and the

supernatant saved in glass tubes. Trichloroacetic acid was removed from the supernatant by extracting 3 times with 4 volumes of water-saturated ether, then placed in a 60 °C water bath for 10 min to boil off any residual ether.

Purification and Estimation of Cyclic AMP

Sequential Dowex and alumina chromatography were used to separate radioactive cyclic AMP from other labeled compounds (Salomon et al., 1974). Dowex (50 AG WX4, 200-400 mesh) was prepared by washing with 2 volumes of 1 N HCl followed by 10 volumes of water. 100 µl of 1 N HCl was added to 900 µl of trichloroacetic acid extract (final concentration of 0.1 N HCl) and the mixture was decanted onto columns (0.4 x 10 cm) containing 1 ml of prepared Dowex resin. Columns were first washed with 3.5 ml of water and a portion of this flow-through was reserved for counting (3 x 200 µl in 10 ml PCS scintillation cocktail), and the rest was discarded. Columns were then washed with an additional 4.5 ml water and a portion (3 x 200 µl) of eluate was saved for counting.

To the remaining portion (3.8 ml), 0.253 ml of 1.5 M imidazole-HCl, pH 7.2, was added. The mixture was decanted onto columns (0.4 x 10 cm) containing 0.6 g neutral alumina which had been washed with 8 ml of 0.1 M imidazole-HCl, pH 7.5. The columns were allowed to drain completely of sample and then washed with 1 ml of 0.1 M imidazole-HCl, pH 7.5. Three 200 µl portions of the eluate were saved for counting.

This final eluate contained the purified [^{3}H]-cyclic AMP

(2,000-22,000 total cpm in 4:5 ml) while the flowthrough from the Dowex column was mainly [^{3}H]-ATP (800,000-900,000 total cpm in 9.0-10.0 ml). Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP and analyzed using the paired 2-tailed t-test (except for Table 3 where an un-paired 2-tailed t-test was employed).

Protein Measurement Assay

Brain tissue protein concentrations were measured using a modification of the method of Lowry et al. (1951). To form a biuret reaction of protein with Cu_2 in alkali, 5 ml of reagent (1 ml of 1% $CuSO_4 \cdot 5 H_2O$; 1 ml of 2% K-Na tartrate; and 100 ml of 2% Na_2CO_3) were added to an incubation tube containing 0.1 ml sample and 0.4 ml water. The mixture was incubated at 45 °C for 10 mins., then cooled on ice. 0.5 ml of Folin-Ciocalteu Reagent diluted with water (1:1) was added to each tube and left at room temperature for 20 mins. This step involves the reduction of the phosphomolybdic phosphotungstic acid reagent (Folin-Ciocalteu Reagent) by the amino acids tyrosine and tryptophan present in the treated protein. The color was read on a Klett Colorimeter and measured against bovine serum albumin standards.

Corticosterone Pellet Preparation

Corticosterone pellets were prepared according to the method of Meyer et al. (1979). Corticosterone (50 mg) and cholesterol (50 mg) were melted in a stainless steel spoon and poured into shallow holes in a porcelain plate. The pellets, each weighing 100 mg, were removed after they had cooled and

solidified and were stored for future use.

Purification and Estimation of Corticosterone

Serum corticosterone levels were determined using a modification of the method of Solem and Brinck-Johnsen (1965). Serum (0.5 ml) was added to 6 ml of methylene chloride. After vortexing and centrifugation, the serum layer was removed and discarded. To remove impurities and metabolites, 1 ml of 0.1 N NaOH was added to methylene chloride, vortexed, centrifuged, and removed. An ETOH/H_2SO_4 (7:3) solution was then mixed with the remaining methylene chloride which was also discarded following centrifugation. The remaining ETOH/H_2SO_4 mixture was read on a spectrophotofluorometer at 530 emission, 470 excitation. Results are expressed as µg corticosterone/100 ml serum and analyzed using the paired 2-tailed t-test.

Materials

Sodium ascorbate (l-ascorbic acid sodium salt), pargyline, corticosterone, progesterone, 11-deoxycortisol and noradrenaline were from Sigma Chemical Company, St. Louis, Mo. whereas 3-isobutyl-1-methyl-xanthine was obtained from Aldrich Chemical Company Inc., Milwaukee, Wis. Imidazole-HCL was from Eastman Kodak Co., Rochester, N.Y. Dowex (50 AG WX4, 200-400 mesh) was obtained from Bio. Rad. Laboratories, Richmond, Calif. and neutral alumina was from Fisher Scientific Company, Fairlawn, N.J. [8-^{3}H]-Adenine and PCS scintillation cocktail were obtained from Amersham, Oakville, Ontario. [^{3}H]-cyclic AMP was purchased from New England Nuclear (Canada) Ltd.,

Lachine, Quebec, whereas Metopirone was a generous gift from Ciba-Geigy (Canada). Male Wistar rats were purchased from Charles River Canada Inc.

III. RESULTS

RESULTS

ATP stores in slices of rat hippocampi were prelabelled with [^{3}H]-adenine and incubated in the presence of various concentrations of noradrenaline (0, 1, 10, 100 μM). The results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP. Expression of the data as a percent of [^{3}H]-cyclic AMP formed from [^{3}H]-ATP stores reduces complications due to unequal tissue sizes, variations in protein levels, unmeasurable loss of sample, and normalizes the data for variations in the degree of labeling of cellular ATP. Nevertheless, protein levels were determined to ensure a relatively uniform sample preparation and were consistently between 1.7 and 2.4 mg per ml of the homogenate.

Effect of Adrenalectomy on Serum Corticosterone Levels and on Cyclic AMP Formation

Corticosterone levels, in trunk blood collected at sacrifice, were significantly lower in the adrenalectomized animals compared to the sham-operated controls [$t=8.2$, $df=5$, $p < 0.001$] (Table 1). Bilateral adrenalectomies, performed 9 days prior to sacrifice, were also found to significantly increase cyclic AMP formation at a noradrenaline concentration of 10 μM [$t=4.18$, $df=4$, $p < 0.05$] but not at the maximum concentration of 100 μM (Figure 1).

Implantation of corticosterone pellets into adrenalectomized (ADX) rats, 5 days prior to sacrifice, restored steroid levels to normal (Table 1) and prevented the adrenalectomy-induced rise in cyclic AMP production (Figure 1).

Effect of Metopirone on Serum Corticosterone Levels and on Cyclic AMP Formation

The corticosterone synthesis inhibitor, Metopirone, had the expected effect on circulating steroid levels. As seen in Table 1, corticosterone levels in animals sacrificed 2 hours following a single 50 mg/kg i.p. injection of Metopirone were significantly lower than the vehicle treated controls [$t=3.36$, $df=5$, $p < 0.05$].

An increase in cyclic AMP formation was also observed, following Metopirone treatment, which could be a consequence of the Metopirone-induced steroid reduction. As can be seen in Figure 2, treatment with Metopirone (50 mg/kg), 2 hours prior to sacrifice, resulted in an elevation of cyclic AMP levels which was statistically significant at concentrations of 10 μM [$t=2.94$, $df=5$, $p < 0.05$] and 100 μM [$t=4.75$, $df=5$, $p < 0.01$] noradrenaline when compared to the control values.

Implantation of corticosterone pellets 5 days prior to sacrifice clamped corticosterone levels within the normal range (Table 1) and prevented the Metopirone-induced increase in cyclic AMP production (Figure 2).

Noradrenaline-Induced Cyclic AMP Formation in Corticosterone Treated Animals

Steroid pellet implantation, 5 days prior to sacrifice, did not alter corticosterone values in control animals (Table 1). Similarly, steroid pellet implantation had no significant effect on the cyclic AMP generating system in these control rats (Figure 3).

Table 1. Serum Corticosterone Levels

Treatment	Control	Treated	Corticosterone Replacement
Non-treated	31.2 ±3.0	-	33.5 ±6.0
Adrenalectomized	30.8 ±7.6	4.0*** ±0.8	32.1 ±8.6
Acute Metopirone (2 hrs)	29.2 ±2.8	18.0* ±1.2	28.0 ±6.6
Chronic Metopirone (6 days)	25.4 ±3.0	22.2 ±3.8	27.4 ±4.6

Trunk blood was collected at the time of sacrifice for determination of serum corticosterone levels. Corticosterone was purified using methylene chloride, NaOH and ETOH/H_2SO_4 extractions, and measured using a spectrophotofluorometer. Results are expressed as μg corticosterone/100 ml serum. Each value represents the mean ± S.E.M. of 4-6 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

*[$t=3.36$, $df=5$, $p < 0.05$]; ***[$t=8.2$, $df=5$, $p < 0.001$] as compared to the control values.

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Effect of Adrenalectomy on Cyclic AMP Formation

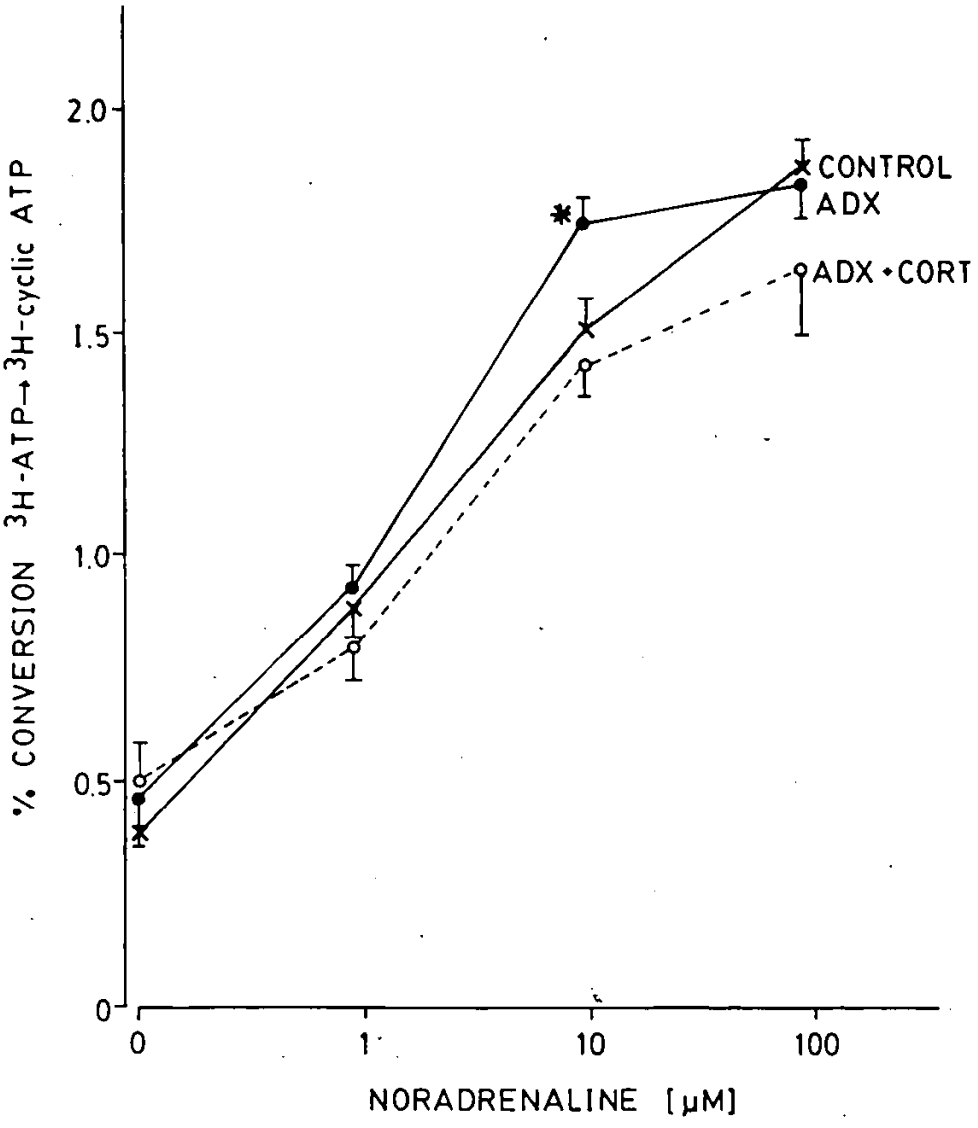
Figure 1

Bilateral adrenalectomies were performed 9 days prior to sacrifice. In sham-operated animals the adrenals were located but not removed. Rats receiving corticosterone pellets were anaesthetized with ether and pellets were implanted subcutaneously in the back of the neck 5 days prior to sacrifice.

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4-5 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

*[$t=4.18$, $df=4$, $p < 0.05$] as compared with control animals.

FIGURE 1 EFFECT OF ADRENALECTOMY ON CYCLIC AMP FORMATION



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Effect of Metopirone on Cyclic AMP Synthesis

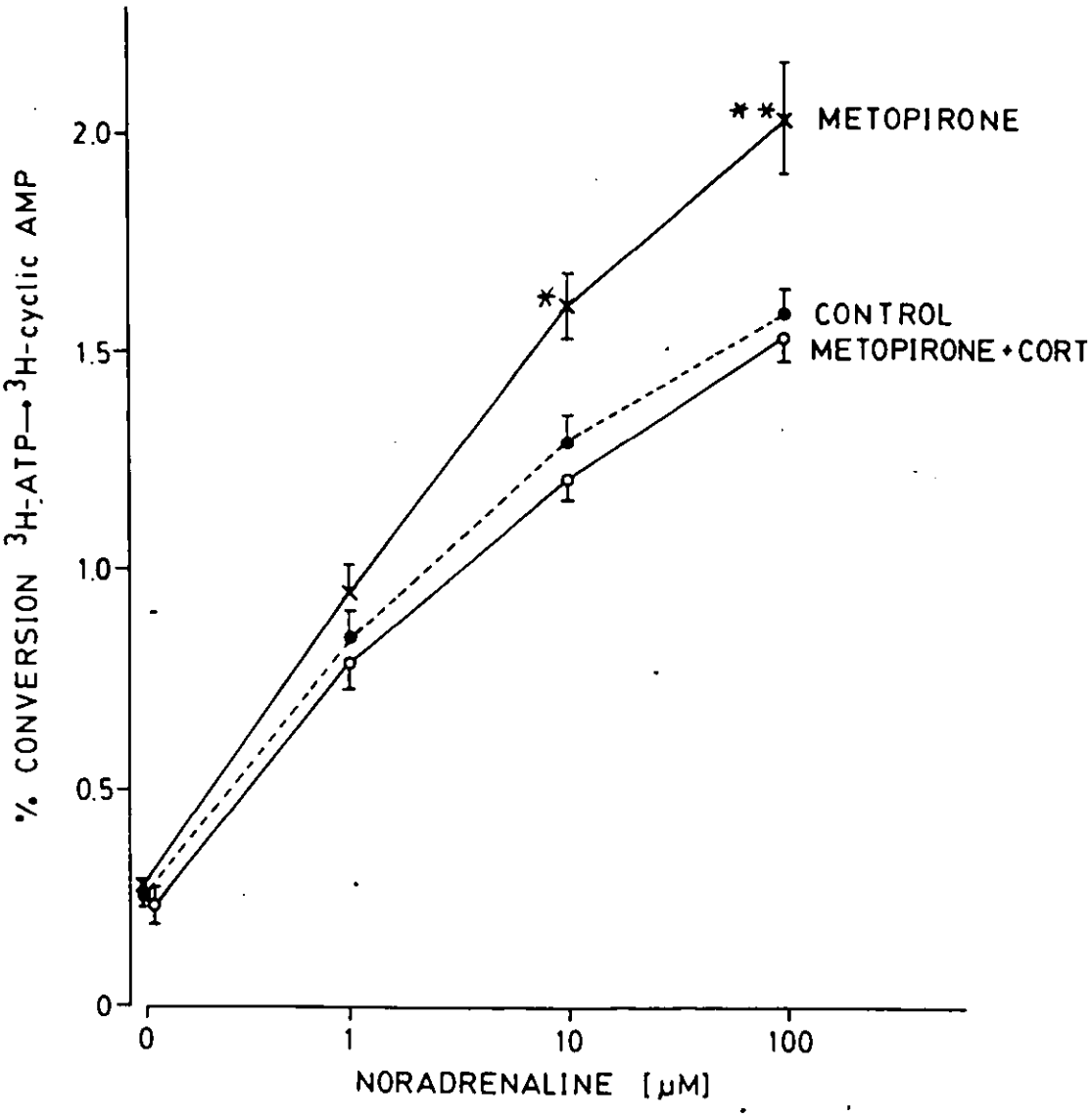
Figure 2

Metopirone treated rats received a 50 mg/kg i.p. injection 2 hours prior to sacrifice. Control animals received an equal volume of the vehicle (40% propylene glycol in water). Rats receiving corticosterone pellets were anaesthetized with ether and pellets were implanted subcutaneously in the back of the neck 5 days prior to sacrifice.

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean $\pm$ S.E.M. of 4-6 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

*[$t=2.94$, $df=5$, $p < 0.05$]; **[$t=4.75$, $df=5$, $p < 0.01$] as compared with control animals.

FIGURE 2 EFFECT OF METOPIRONE ON CYCLIC AMP SYNTHESIS



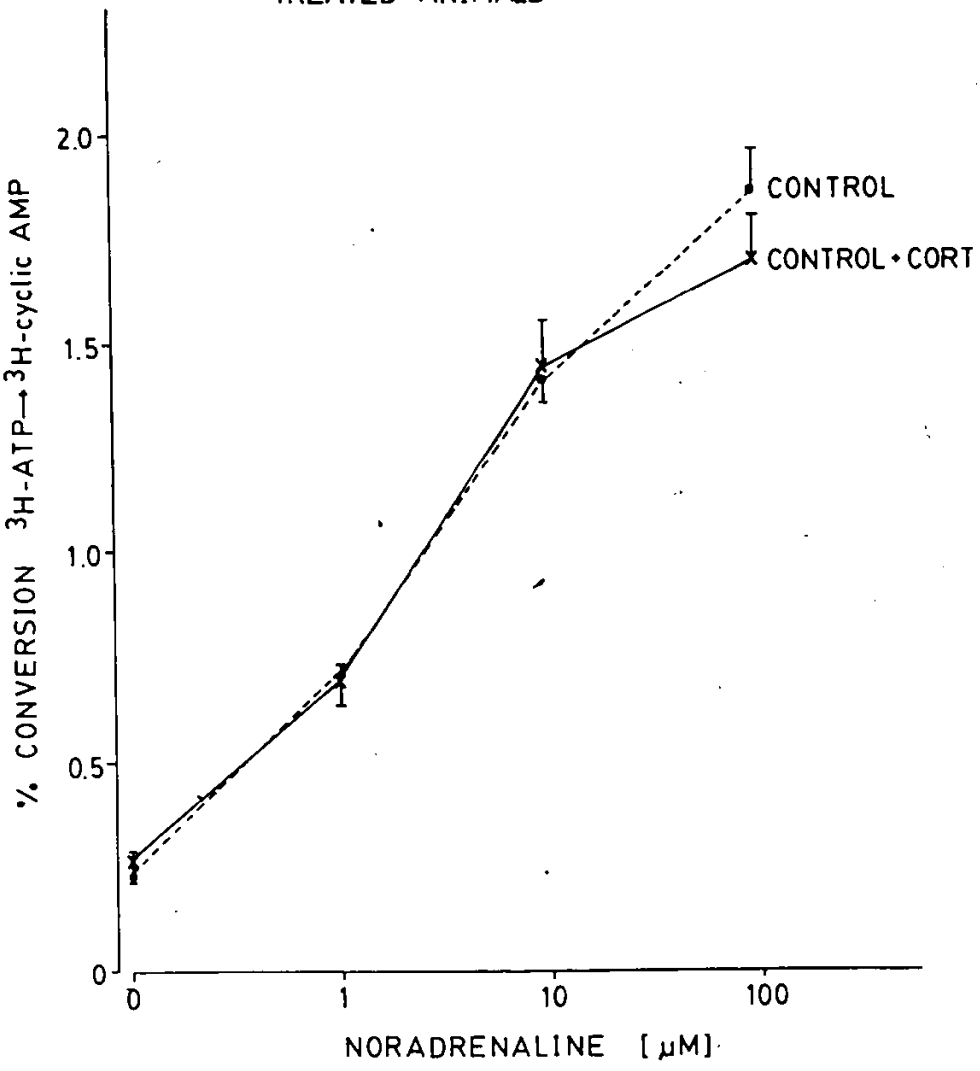
Noradrenaline Induced Cyclic AMP Formation in Control and Corticosterone Treated Animals

Figure 3

Rats receiving corticosterone pellets were anaesthetized with ether and pellets were implanted subcutaneously in the back of the neck 5 days prior to sacrifice. Control animals were anaesthetized and received an incision but no pellet.

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

FIGURE 3 NORADRENALINE INDUCED CYCLIC AMP FORMATION IN CONTROL AND CORTICOSTERONE TREATED ANIMALS



Time Course of the Metopirone Effect

ACUTE METOPIRONE TREATMENT

The effect of Metopirone treatment on serum corticosterone levels and on noradrenaline-induced cyclic AMP production was further characterized by examining these values at various times following treatment with the drug. Rats were treated approximately 3 hours into the light cycle with a 50 mg/kg i.p. injection of Metopirone or an equal volume of the vehicle (40% propylene glycol in water) and sacrificed 1, 2, or 4 hours later.

As seen in Table 2, a slight but non-significant drop was observed in serum corticosterone levels in animals sacrificed 1 hour after Metopirone treatment (50 mg/kg i.p.). This decrease reached statistical significance at 2 hours when compared to control values [$t=3.36$, $df=5$, $p < 0.05$]. Corticosterone levels in the Metopirone treated rats were also relatively low at 4 hours, but were not significantly different from the control values at this time. The relatively low values at 4 hours could possibly be due to the time of sacrifice (i.e. later in the day and longer since the i.p. injection).

These data paralleled the effect of Metopirone on noradrenaline stimulated cyclic AMP production. As can be seen in Table 3, the corticosterone synthesis inhibitor had no significant effect on noradrenaline-stimulated cyclic AMP formation 1 hour after treatment at any concentration of noradrenaline used. A statistically significant increase in

cyclic AMP formation was observed 2 hours following treatment at 10 μM [$t=2.36$, $df=18$, $p < 0.05$] and at 100 μM [$t=2.92$, $df=16$, $p < 0.01$] noradrenaline. At 4 hours there was a slight but nonsignificant elevation in noradrenaline stimulated cyclic AMP levels.

CHRONIC METOPIRONE TREATMENT

Chronically treated animals were injected i.p., once a day for 6 days, with 50 mg/kg of Metopirone and were sacrificed 2 hours following the final injection. Control animals were treated identically with an equal volume of the vehicle (40% propylene glycol in water).

Tolerance to the effect of Metopirone appeared to develop as animals treated chronically with the drug did not have reduced corticosterone levels (Table 1), and the noradrenaline-coupled cyclic AMP values were equivalent for the control and chronic Metopirone treated rats (Table 4).

Steroid pellet implantation, 6 days prior to sacrifice, did not change corticosterone levels from control values (Table 1). Similarly, no significant effect on the cyclic AMP generating system in chronically treated animals was observed following steroid replacement (Table 4).

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Table 2. Serum Corticosterone Levels in Animals Sacrificed 1, 2, or 4 Hours After Treatment with Metopirone or Vehicle

	Vehicle (Control)	Metopirone (Treated)
1 hr	29.8 ± 5.2	20.0 ± 2.2
2 hrs	29.2 ± 2.8	18.0 ± 1.2 *
4 hrs	18.6 ± 3.2	16.2 ± 3.2

Rats were treated approximately 3 hours into the light cycle with a 50 mg/kg i.p. injection of Metopirone or an equal volume of the vehicle (40% propylene glycol in water). Animals were sacrificed 1, 2, or 4 hours later at which time trunk blood was collected for determination of serum corticosterone levels. Corticosterone was purified using methylene chloride, NaOH and ETOH/H_2SO_4 extractions, and measured using a spectrophotofluorometer.

Results are expressed as μg corticosterone/100 ml serum. Each value represents the mean ± S.E.M. of 4-6 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

*[$t=3.36$, $df=5$, $p < 0.05$] as compared to the control values.

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Table 3. Noradrenaline Stimulated Cyclic AMP Production in Animals Sacrificed 1, 2, or 4 Hours After Metopirone Treatment

Time	Noradrenaline (μM) 0	1	10	100
Vehicle (control)	.260 ±.003	.849 ±.042	1.399 ±.046	1.659 ±.059
1 hr Metopirone	.287 ±.025	.762 ±.043	1.345 ±.040	1.670 ±.043
2 hr Metopirone	.274 ±.011	.950 ±.061	1.605* ±.079	2.035** ±.128
4 hr Metopirone	.278 ±.006	1.026 ±.111	1.530 ±.126	1.787 ±.173

Metopirone-treated rats received a 50 mg/kg i.p. injection 1, 2, or 4 hours prior to sacrifice. Control animals received an equal volume of the vehicle (40% propylene glycol in water). Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value in the Metopirone treated groups represents the mean ± S.E.M. of 4-6 separate determinations obtained from 12-18 animals. Cyclic AMP formation in the vehicle-treated animals from the 1, 2, and 4 hour groups was not significantly different. Data from these animals were pooled and represented as the control group (16 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate). *[$t=2.36$, $df=18$, $p < 0.05$]; **[$t=2.92$, $df=16$, $p < 0.01$] as compared to the control values.

Table 4. Chronic Metopirone Treatment

Treatment	Noradrenaline (μM) 0	1	10	100
Vehicle	.231 ±.019	.709 ±.013	1.402 ±.050	1.858 ±.096
Chronic Metopirone	.264 ±.027	.695 ±.066	1.433 ±.111	1.689 ±.109
Chronic Metopirone plus Corticosterone	.253 ±.023	.681 ±.061	1.489 ±.098	1.675 ±.150

Rats were treated chronically, once a day for 6 days, with 50 mg/kg i.p. injections of Metopirone and were sacrificed 2 hours following the final injection. Control animals were treated identically with an equal volume of the vehicle (40% propylene glycol in water).

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

Metopirone In Vitro

The effect of Metopirone was also tested in vitro (Figure 4). The presence of Metopirone in the incubation medium exerted no significant effect on the cyclic AMP generating system in hippocampal slices obtained from control animals. The lack of an effect of the drug in vitro suggests that it has an indirect action on hippocampal noradrenaline stimulated cyclic AMP formation.

Corticosterone In Vitro

As can be seen in Table 5, the addition of corticosterone to the incubation medium did not modify cyclic AMP production in hippocampal slices from Metopirone treated animals. Slices were incubated in the presence of corticosterone (10^{-9}M) for 1 hour as adenyl cyclase activity deteriorated with longer incubations. It is possible that an effect of corticosterone on cyclic AMP formation could be observed with longer incubations under different conditions. However, the possibility also exists that the observed increase in cyclic AMP formation following adrenalectomy or Metopirone treatment may not be the direct result of steroid reduction.

11-Deoxycortisol and Progesterone In Vitro

As shown in Table 6, the addition of a corticosterone receptor antagonist in vitro (11-deoxycortisol (5×10^{-6} M) or progesterone (5×10^{-6} M)) did not modify noradrenaline stimulated cyclic AMP formation in hippocampal slices from control animals. Slices were incubated in the presence of the antagonists for 1 hour as adenyl cyclase activity deteriorated

with longer incubations. It is again possible that an effect of the antagonists on cyclic AMP production could be observed with longer incubations under different conditions. However, the results also suggest that increased cyclic AMP formation, following adrenalectomy or Metopirone, may not be the direct result of reduced corticosterone action.

Effect of Hypophysectomy on Cyclic AMP Formation

The lack of an effect of corticosterone and of the corticosterone antagonists *in vitro* prompted us to investigate the role of a pituitary factor in the modification of the noradrenaline stimulated cyclic AMP generating system in rat hippocampus (Figure 5). Animals were hypophysectomized to reduce endogenous ACTH levels. Hippocampal cyclic AMP formation in these animals was significantly lower at concentrations of 10 μM [$t=6.35$, $df=8$, $p < 0.05$] and 100 μM [$t=6.36$, $df=8$, $p < 0.05$] noradrenaline compared to control values. It is interesting to note that in adrenalectomized and Metopirone treated animals, where reduced steroid levels enhance ACTH production, cyclic AMP levels are increased.

Metopirone did not significantly alter cyclic AMP formation in hypophysectomized rats (Figure 5). This would be expected as steroid levels would already be reduced in these animals due to the lack of ACTH, the corticosterone stimulating hormone.

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Metopirone In Vitro

Figure 4

The hippocampi from non-treated rats were pooled, sliced and incubated in the presence of 0, 1, 10, or 100 μM noradrenaline with or without Metopirone.

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

FIGURE 4 METOPIRONE IN VITRO

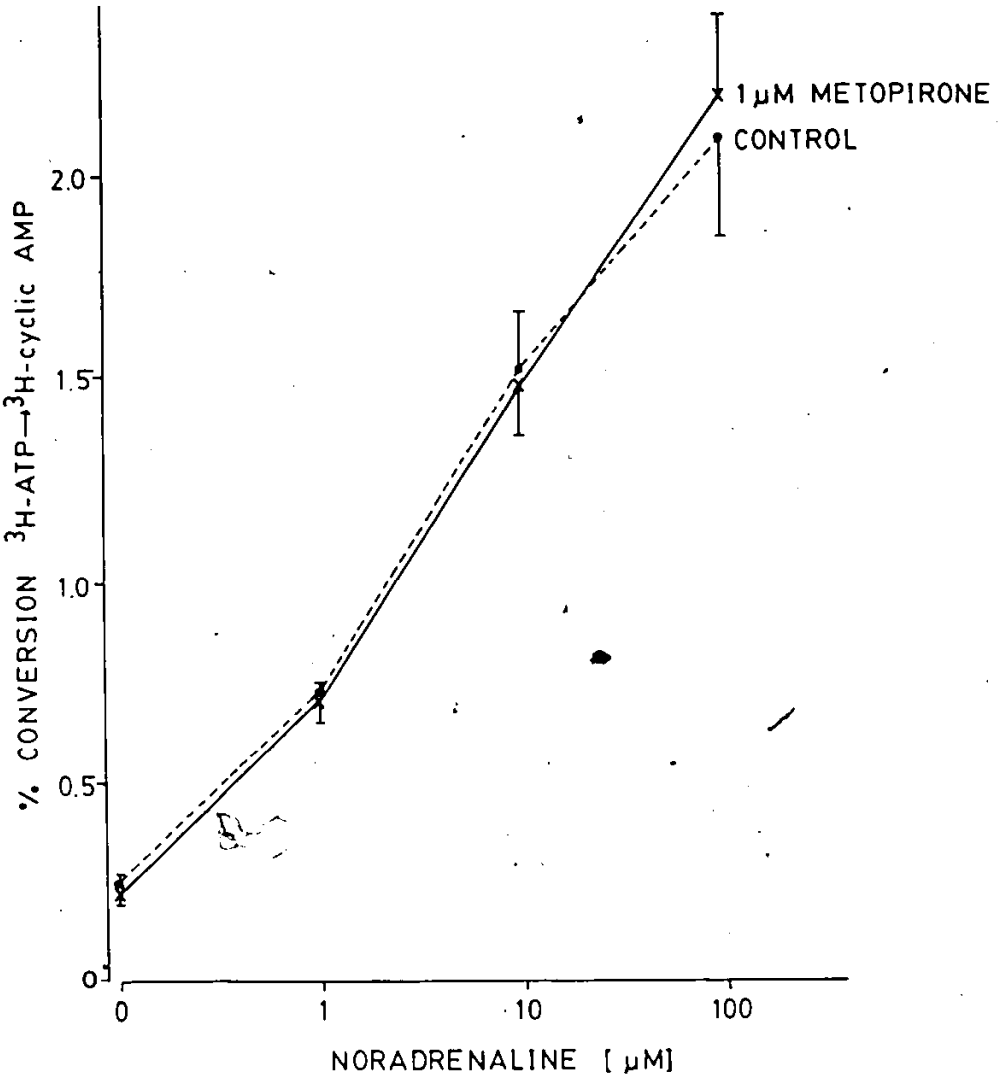

Table 5. Corticosterone In Vitro

Treatment	Noradrenaline (μM) 0	1	10	100
Control	.292 ±.004	.993 ±.039	1.559 ±.017	1.823 ±.026
Corticosterone (10-9 M)	.268 ±.017	1.077 ±.051	1.558 ±.042	1.792 ±.021

The hippocampi from animals sacrificed 2 hours after a 50 mg/kg i.p. injection of Metopirone were pooled and sliced. Slices were incubated in the presence of 0, 1, 10, or 100 μM noradrenaline with or without corticosterone (10^{-9} M). Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

Table 6. 11-Deoxycortisol and Progesterone In Vitro

Treatment	Noradrenaline (μM) 0	1	10	100
Control	.195 ±.003	.725 ±.056	1.160 ±.056	1.305 ±.066
Progesterone (5 x 10-6 M)	.208 ±.008	.794 ±.020	1.145 ±.073	1.430 ±.075
Control	.217 ±.022	.906 ±.129	1.251 ±.172	1.620 ±.173
11-Deoxycortisol (5 x 10-6 M)	.244 ±.019	.963 ±.084	1.319 ±.140	1.766 ±.142

The hippocampi from non-treated animals were pooled, sliced, and incubated in the presence of 0, 1, 10, or 100 μM noradrenaline with or without a corticosterone antagonist (11-deoxycorticol (5×10^{-6} M) or progesterone (5×10^{-6} M)). Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 4 separate assays. Within each assay, 3 animals were and points were assessed in triplicate.

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Effect of Hypophysectomy on Cyclic AMP Synthesis

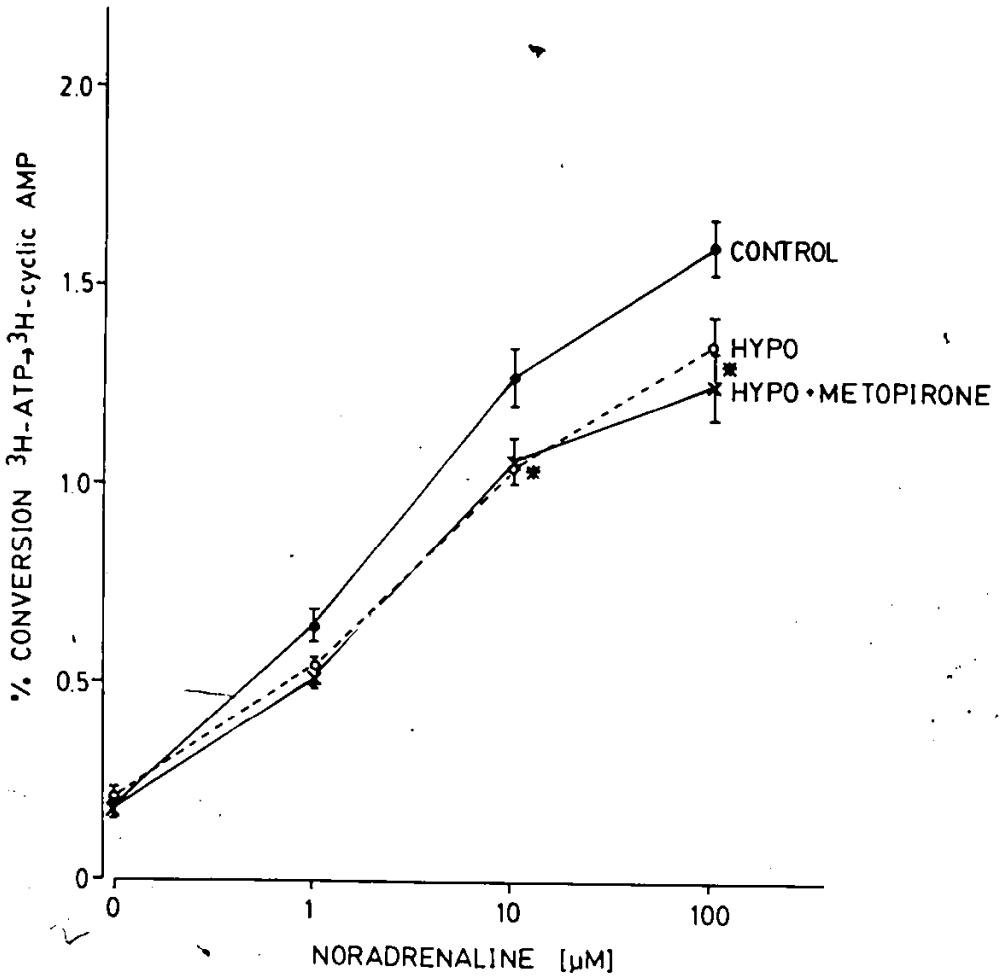
Figure 5

Hypophysectomized rats were operated on approximately 5 days before sacrifice. Animals treated with Metopirone received a 50 mg/kg i.p. injection 2 hours prior to sacrifice.

Results are expressed as percent conversion of [^{3}H]-ATP to [^{3}H]-cyclic AMP as a function of noradrenaline concentration. Each value represents the mean ± S.E.M. of 3 separate assays. Within each assay, 3 animals were used and points were assessed in triplicate.

*[$t=6.35$, $df=8$, $p < 0.05$] for 10 μM noradrenaline and *[$t=6.36$, $df=8$, $p < 0.05$] for 100 μM noradrenaline as compared to control animals.

FIGURE 5 EFFECT OF HYPOPHYSECTOMY ON CYCLIC AMP SYNTHESIS



IV. DISCUSSION

DISCUSSION

Introduction

Adrenergic receptor stimulation is known to produce a number of biochemical responses such as gluconeogenesis in liver and lipolysis in adipose tissue. These effects are mediated via adenyl cyclase stimulation and require the "permissive" effect of corticosterone. Recent evidence suggests that corticosterone modulates noradrenergic mechanisms in the brain as well. Mobley and Sulser (1980 a,b) reported an increase in noradrenaline stimulated cyclic AMP production in rat frontal cortex 2 weeks following bilateral adrenalectomy. This adrenalectomy induced increase in cyclic AMP formation was prevented by daily injections of corticosterone. The present study examined the interaction between adrenal corticoids and the noradrenaline-coupled cyclic AMP generating system in another brain region, the hippocampus. This structure specifically retains more corticosterone than any other part of the brain (McEwen et al., 1969).

In the present experiments adrenalectomy as well as Metopirone, which inhibits the synthesis of corticosterone, were used to reduce endogenous steroid levels. Adrenalectomy causes changes in electrolyte balance, carbohydrate metabolism (Exton et al., 1972) as well as in ACTH and endorphine secretion (Guillemin et al, 1977). Alterations in noradrenergic mechanisms following such an operation might be secondary to these effects, or due to some compensatory

response. Therefore, convergent evidence for the role of corticosterone in the modulation of central noradrenergic mechanisms was sought using an acute pharmacological manipulator of adrenal steroids, Metopirone. This drug has been shown to inhibit the 11-beta-hydroxylating enzyme responsible for the synthesis of corticosterone (Liddle et al., 1958).

Main Results of the Experiments

1. Noradrenaline stimulated cyclic AMP synthesis was measured in rat hippocampus 9 days following bilateral adrenalectomy. We found a significant increase in cyclic AMP levels which was less pronounced than that reported by Mobley and Sulser (1980a,b). This smaller increase could perhaps be due to the different brain region examined (i.e. hippocampus vs. frontal cortex and limbic forebrain), the shorter period following adrenalectomy (9 days vs. 2 weeks), or to difference(s) in assay procedure. Corticosterone replacement prevented the adrenalectomy induced increase in cyclic AMP synthesis in both the present experiment as well as in the reports by Mobley and Sulser.

2. Metopirone had no significant effect on noradrenaline stimulated cyclic AMP formation 1 hour after treatment. A statistically significant increase in cyclic AMP formation was observed at 10 μM and 100 μM noradrenaline 2 hours following Metopirone treatment. A slight but non-significant elevation in noradrenaline stimulated cyclic AMP levels remained at 4 hours.

3. The above data paralleled the corticosterone synthesis inhibiting effect of Metopirone. One hour after treatment, a slight but non-significant drop was observed in serum corticosterone levels. This decrease reached statistical significance at 2 hours. Corticosterone levels in Metopirone treated animals remained relatively low at 4 hours but were not significantly different from the vehicle treated animals. The lower corticosterone levels in the 4 hour Metopirone treated and control animals could be due to the time of sacrifice (i.e. later in the day and longer since the i.p. injection).

Thus Metopirone significantly lowers corticosteroid levels within 2 hours of treatment. The increase in hippocampal noradrenaline stimulated cyclic AMP formation, at this time, may be a consequence of the Metopirone reduced steroid levels.

4. Consistent with this possibility, corticosterone pellet implantation prevented the increase in cyclic AMP synthesis following Metopirone. These pellets effectively maintained plasma corticosterone levels within the normal range. This would presumably inhibit the de novo synthesis of corticosterone in adrenal glands and thus render the Metopirone inhibition of corticosterone synthesis inconsequential.

5. Tolerance to the corticosterone inhibiting effect of Metopirone seems to develop as animals treated for 6 days with the drug did not have reduced corticosterone levels. Similarly, the noradrenaline-coupled cyclic AMP generating system appeared to adapt to chronic Metopirone treatment,

possibly as a consequence of the normal steroid values. Alternatively in the case of adrenalectomy, where steroid levels remain low, cyclic AMP production fails to return to control values.

6. In vitro Metopirone had no effect on hippocampal cyclic AMP formation. This again supports the suggestion that Metopirone has an indirect action on noradrenaline stimulated cyclic AMP production, mediated by its inhibitory effect on corticosterone synthesis.

7. The addition of corticosterone to the incubation medium did not modify cyclic AMP production in hippocampal slices from Metopirone treated animals. Futhermore, while 11-deoxycortisol and progesterone have been shown to act as corticosterone antagonists in vitro (Makman et al., 1966, 1967, Turnell et al., 1974, and Cutler et al., 1979) this action did not, in the present study, alter cyclic AMP production in hippocampal slices from control animals. It is possible that, with longer incubations, the observed changes in noradrenaline stimulated cyclic AMP formation following adrenalectomy or Metopirone would be mimicked by corticosterone and corticosterone blockers in vitro. However, the lack of an effect of corticosterone and of the corticosterone antagonists in vitro prompted us to investigate the role of a pituitary hormone in the modification of noradrenaline stimulated cyclic AMP production.

8. Cyclic AMP formation was significantly lower at 10 μM and 100 μM noradrenaline in hypophysectomized animals compared to the controls. Since hypophysectomy eliminates pituitary

hormones, including ACTH which stimulates the synthesis and release of adrenal glucocorticoids, this experiment suggests that reducing circulating levels of ACTH, and hence corticosteroids, decreases noradrenaline stimulated cyclic AMP formation in rat hippocampus. Interestingly, noradrenaline stimulated cyclic AMP levels are increased in adrenalectomized and Metopirone treated animals in whom reduced steroid levels enhance ACTH production through feedback mechanisms.

9. Metopirone treatment did not increase cyclic AMP synthesis in hypophysectomized animals. The corticosterone synthesis inhibitor would be expected to have a very limited effect in these animals as adrenal corticoid synthesis and release would already be reduced as a result of decreased ACTH levels. This result is a further indication that the effect of Metopirone on hippocampal cyclic AMP synthesis is due to this drug's inhibition of adrenal corticosteroid synthesis or to its stimulation of ACTH release through endogenous feedback mechanisms.

In conclusion, the present study suggests that treatment with a corticosterone synthesis inhibitor, Metopirone, increases noradrenaline stimulated cyclic AMP formation in rat hippocampus in as little as 2 hours. This may be the direct result of altered steroid levels or this modulation could be due to the reflexive release of a pituitary factor, possibly ACTH, when corticosterone synthesis is impaired.

General Discussion

At present, we can only speculate as to the nature of this interaction. The biochemical sequelae of noradrenaline receptor stimulation in the brain are not yet clearly understood. However, there are growing parallels between the well studied peripheral reactions and those found in the central nervous system. In liver, adrenergic stimulation is known to produce glycogen breakdown through an adenyl cyclase mediated phosphorylation of glycogen phosphorylase. This reaction requires the "permissive" effect of corticosterone (Exton et al., 1972). Adrenalectomy causes blockade of glycogen hydrolysis and, presumably as a compensatory response, also causes an up regulation of noradrenaline stimulated adenyl cyclase (Wolfe et al., 1976).

It is possible that a similar mechanism is operative in brain as well. The enzymes responsible for glycogen metabolism, glycogen phosphorylase and phosphorylase kinase, are present in brain and the activation of these enzymes has been shown to be dependent upon cyclic AMP (Wilkening and Makman, 1977). Noradrenaline stimulates glycogenolysis in brain cortical slices, and this action is mediated via beta-adrenergic receptors and cyclic AMP formation (Quach et al., 1978; Magistretti et al., 1981). If the parallels between central and peripheral mechanisms hold this would suggest that glycogenolysis in brain is dependent on adrenal glucocorticoids and that steroid reduction is compensated by an up regulation of noradrenaline stimulated cyclic AMP formation to maintain

cellular responses.

Alterations in noradrenaline stimulated cyclic AMP formation as well as steroid imbalances are associated with behavioral disorders. There has been much research indicating that antidepressant treatment decreases the sensitivity of the noradrenaline receptor-coupled adenyl cyclase system in brain. It has been hypothesized that depression occurs as a result of an increase in the sensitivity of the cyclic AMP generating system and that antidepressant therapy alters this supersensitivity (see Sulser, 1982). There is also clinical evidence suggesting that adrenocortical steroids may have a role in affective disorders as steroid imbalances and steroid therapy are often accompanied by mood disorders (Carpenter and Gruen, 1982). If noradrenaline stimulated cyclic AMP production and adrenal corticosteroids are critically linked to brain energy balance, then it is possible that mood disorders are a consequence of an imbalance in brain oxidative metabolism. Just as low blood sugar levels can induce fatigue and depression, endogenous depression may also be due to an abnormal glucose metabolism in the brain.

A variety of animal experiments provide evidence for an interaction between brain noradrenergic mechanisms and adrenal steroids in learning and memory. A severe impairment of avoidance performance in rats has been reported following bilateral adrenalectomy in combination with 6-OHDA lesions of the dorsal noradrenergic bundle. This behavioral impairment

does not occur after either treatment alone and is prevented by corticosterone replacement (Ogren and Fuxe, 1974, 1977; Roberts and Fibiger, 1977). It appears that central noradrenergic mechanisms and adrenal steroids interact in such a way that either system is able to compensate for the loss of the other.

Recently, Bialik et al. (1984) reported learning deficits in rats following treatments to alter levels of ACTH (i.e. dexamethasone and Metopirone). It was suggested that learning impairments in central noradrenaline depleted rats occur as a result of their inability to adapt to chronically elevated ACTH levels following steroid reduction.

Changes in circulating corticosterone levels and brain noradrenaline content have also been shown to occur in response to stress. Psychological and physical stressors activate the sympathetic nervous system to release noradrenaline, and the adrenal medulla to release adrenaline and noradrenaline (see Mason, 1968 a, b). The adaptive value of this response was first recognized by Cannon in the early 1900's. He suggested that these physiological responses enabled the organism to cope with the stress. Indeed, adrenaline stimulates glycogen breakdown in the liver and lipolysis in adipose tissue thus increasing the supply of glucose and free fatty acids for energy utilization. In addition, ACTH which is released during stress, stimulates the adrenal cortex to release glucocorticoids which are also involved in stimulating glycogenolysis and lipolysis (see Dunn and Kramarcy, 1984).

In common with the peripheral response, noradrenergic

systems are activated in the brain as well. Synthesis and utilization of noradrenaline are increased by stress (Anisman, 1978; Stone, 1975). In addition, decreases in either or both noradrenaline stimulated cyclic AMP formation and beta-adrenergic receptor density have been observed following stress (see Stone, 1983). These decreases may be a consequence of the increased availability of brain noradrenaline (i.e. subsensitivity) or, as the present paper suggests, these responses could also be influenced by corticosteroids.

In conclusion, I would like to suggest that the observed changes in circulating corticosterone levels and in brain noradrenergic mechanisms in response to stress could be adaptive alterations to maintain cyclic AMP linked cellular responses such as brain energy metabolism. In addition pituitary, adrenal, and noradrenergic insufficiency may cause an impairment in cellular responses such as brain energy metabolism. Such intracellular deficits could explain many of the diverse effects of altered steroid, ACTH, and central noradrenergic mechanisms such as mood disorders as well as learning and memory impairments.

V. SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

In conclusion, the present study has shown that noradrenaline stimulated cyclic AMP formation in rat hippocampus is significantly increased 2 hours following treatment with a corticosterone synthesis inhibitor, Metopirone. Implantation of corticosterone pellets 5 days prior to sacrifice prevented this increase. Metopirone had no significant effect on cyclic AMP production after 1 hour, while a slight but non-significant elevation remained at 4 hours. These observations paralleled the corticosterone synthesis inhibiting effect of Metopirone. One hour after treatment, a slight but non-significant drop was observed in serum corticosterone levels. This decrease reached statistical significance at 2 hours. Corticosterone levels in Metopirone treated animals remained relatively low at 4 hours but were not significantly different from the vehicle treated animals. These findings suggest that Metopirone significantly increases noradrenaline stimulated cyclic AMP formation within 2 hours of treatment. This increase may be a consequence of the Metopirone reduced steroid levels.

Metopirone had no significant effect on the cyclic AMP generating system *in vitro*. This suggests an indirect action of the drug which we presume is its inhibitory effect on corticosterone synthesis. However, corticosterone and two *in vitro* corticosterone antagonists (progesterone and 11-deoxycortisol) did not alter noradrenaline stimulated cyclic AMP formation *in vitro*. It is possible that, with longer

incubations, the observed changes in noradrenaline stimulated cyclic AMP formation following adrenalectomy or Metopirone would be mimicked by corticosterone and corticosterone blockers *in vitro*. However, the lack of an effect of corticosterone and of the corticosterone antagonists *in vitro* present the possibility that the observed increase in cyclic AMP formation following steroid reduction may be due to secondary changes such as in ACTH levels. This is supported by the finding that noradrenaline stimulated cyclic AMP formation was significantly lower in the hippocampi of hypophysectomized animals compared to the controls. These results demonstrate that the modulation of noradrenaline stimulated cyclic AMP production in rat hippocampus may be the direct result of altered steroid levels or due to the reflexive release of a pituitary factor, possibly ACTH, when corticosterone synthesis is impaired.

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RESUME

Evidence from a variety of sources has shown that alterations in central noradrenergic mechanisms and changes in circulating steroid levels are associated with various abnormalities such as mood disorders, learning and memory impairments as well as with the condition of stress. Antidepressant treatment decreases the sensitivity of the noradrenaline receptor-coupled adenyl cyclase system in brain and there is clinical evidence that steroid imbalances and steroid therapy are associated with mood disorders as well. Behavioral impairments in rats have been reported following bilateral adrenalectomy in combination with 6-OHDA lesions of the dorsal noradrenergic bundle. The impairments do not occur after either treatment alone and are prevented by corticosterone replacement. These results suggest that circulating adrenal corticosteroids and noradrenergic mechanisms in the brain interact in such a way that either system is able to compensate for the loss of the other. Increases in plasma corticosterone levels and in brain noradrenaline synthesis and utilization have been shown to occur in response to stress. Decreases in either or both noradrenaline stimulated cyclic AMP formation and beta-adrenergic receptor density have also been observed during stress. The present study examines the interactions between corticosterone and noradrenaline stimulated cyclic AMP formation in the brain for the purpose of finding an answer to the cause and effect of central abnormalities.

Adrenalectomy as well as Metopirone, which inhibits the synthesis of corticosterone, were used to reduce endogenous steroid levels. Noradrenaline stimulated cyclic AMP formation was measured in rat hippocampus 9 days following bilateral adrenalectomy or at various time points after Metopirone treatment (50 mg/kg i.p.). The effect of Metopirone, corticosterone, and corticosterone antagonists on the cyclic AMP generating system were also examined in vitro (i.e. in slices of rat hippocampus). Hypophysectomized animals were used to determine if changes in the levels of circulating ACTH could also affect central cyclic AMP production.

Bilateral adrenalectomies, performed 9 days prior to sacrifice, significantly lowered serum corticosterone levels and presumably as a compensatory response significantly increased noradrenaline stimulated cyclic AMP formation in rat hippocampus. Implantation of corticosterone pellets into adrenalectomized rats, 5 days prior to sacrifice, restored steroid levels to normal and prevented the adrenalectomy-induced rise in cyclic AMP production.

Treatment with the corticosterone synthesis inhibitor, Metopirone, significantly lowered serum corticosterone levels after 2 hours. A slight but non-significant drop in serum corticosterone levels was observed 1 hour and 4 hours following treatment with the drug. These data paralleled the effect of Metopirone on noradrenaline stimulated cyclic AMP production. A statistically significant increase in cyclic AMP formation

was observed 2 hours following Metopirone treatment at 10 μM and at 100 μM noradrenaline. The corticosterone synthesis inhibitor had no significant effect on noradrenaline stimulated cyclic AMP formation 1 hour and 4 hours after treatment at any concentration of Metopirone used. Steroid pellet implantation, 5 days prior to sacrifice, maintained serum corticosterone levels within the normal range during all of these treatment schedules and prevented the rise in noradrenaline stimulated cyclic AMP formation as well.

These data suggest that Metopirone significantly lowers corticosteroid levels within 2 hours of treatment. The increase in hippocampal noradrenaline stimulated cyclic AMP formation, at this time, may be a consequence of the Metopirone reduced steroid levels.

Tolerance to the corticosterone inhibiting effect of Metopirone seems to develop as animals treated for 6 days with the drug did not have reduced corticosterone levels. Similarily, the noradrenaline-coupled cyclic AMP generating system appeared to adapt to chronic Metopirone treatment, possibly as a consequence of the normal steroid values. Alternatively in the case of adrenalectomy, where steroid levels remain low, cyclic AMP production fails to return to control values.

In vitro Metopirone had no effect on hippocampal cyclic AMP formation. This again supports the suggestion that Metopirone has an indirect action on noradrenaline stimulated cyclic AMP production, mediated by its inhibitory effect on corticosterone

synthesis.

The addition of corticosterone to the incubation medium did not modify cyclic AMP production in hippocampal slices from Metopirone treated animals. Futhermore, while 11-deoxycortisol and progesterone have been shown to act as corticosterone antagonists in vitro, in the present study, this action did not alter cyclic AMP production in hippocampal slices from control animals. It is possible that, with longer incubations, the observed changes in noradrenaline stimulated cyclic AMP formation following adrenalectomy or Metopirone would be mimicked by corticosterone and corticosterone blockers in vitro. However, the lack of an effect of corticosterone and of the corticosterone antagonists in vitro prompted us to investigate the role of a pituitary hormone in the modification of noradrenaline stimulated cyclic AMP production.

Cyclic AMP formation was significantly lower at 10 μM and 100 μM noradrenaline in hypophysectomized animals compared to the controls. Since hypophysectomy eliminates pituitary hormones, including ACTH which stimulates the synthesis and release of adrenal glucocorticoids, this experiment suggests that reducing circulating levels of ACTH, and hence corticosteroids, decreases noradrenaline stimulated cyclic AMP formation in rat hippocampus. Interestingly, noradrenaline stimulated cyclic AMP levels are increased in adrenalectomized and Metopirone treated animals in whom reduced steroid levels enhance ACTH production through feedback mechanisms.

Metopirone treatment did not increase cyclic AMP synthesis in hypophysectomized animals. The corticosterone synthesis inhibitor would be expected to have a very limited effect in these animals as adrenal corticoid synthesis and release would already be reduced as a result of decreased ACTH levels. This result is a further indication that the effect of Metopirone on hippocampal cyclic AMP synthesis is due to this drug's inhibition of adrenal corticosteroid synthesis or to its stimulation of ACTH release through endogenous feedback mechanisms.

In conclusion, the present study suggests that a reduction in circulating corticosterone levels causes a compensatory increase in noradrenaline stimulated cyclic AMP formation in rat hippocampus. This may be the direct result of altered steroid levels or this modulation could be due to the reflexive release of a pituitary factor, possibly ACTH, when corticosterone synthesis is impaired. Stress increases circulating corticosterone levels and alters central noradrenergic mechanisms. It is possible that such changes could be adaptive responses to maintain steroid and cyclic AMP linked cellular systems such as brain energy metabolism. Many of the diverse effects of altered steroid, ACTH, and central noradrenergic mechanisms, such as mood disorders and learning and memory impairments, could be due to an insufficiency in the maintenance of cellular systems.