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OF ACTH SECRETION.

The Ohio State University, Ph.D., 1970
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RECEPTOR MECHANISMS INVOLVED IN THE
REGULATION OF ACTH SECRETION

DISSER TATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

Madeline Molnar Hall, B.S.

* * * * * *

The Ohio State University
1970

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INTRODUCTION

General Historical Review

There appear to be three distinct relationships of nerve cells to endocrine secretory processes. These are exemplified by the neurohypophysis, the adrenal medulla and the anterior pituitary. The fiber tract of the neurohypophysis originates in the supraoptic and paraventricular nuclei of the hypothalamus, forms the supraoptico-hypophyseal tract, and eventually expands into the pars nervosa. This unique tract contains (within its fibers) secretion granules containing the polypeptides, oxytocin and vasopressin. In this system, nerve activity directly causes the liberation of these hormones from the granules located within this neural tract (Douglas et al., 1967; Douglas, 1968). It is characteristic of this system that neurotransmitter substances are not known to play a part in the process by which the polypeptides are released; the only known impulses being the propagated action potential in the nerve, as demonstrated by the use of electrical or chemical (i.e., excess K) stimuli or the application of calcium ions.

In the adrenal medulla, on the other hand, nerve terminals synapse on chromaffin cells. Stimulation of splanchnic nerves results in the release of acetylcholine from their nerve endings, which then diffuses across the synaptic cleft causing depolarization of the chromaffin cell, with the resultant secretion of epinephrine
and norepinephrine (Douglas et al., 1967). This is a typical relationship between nerves and effector cells, and the chromaffin cells respond not only to nerve stimuli but to a variety of nicotinic agents. Neither of these two endocrine structural prototypes however resemble the relationship that exists between nerve cells and the cells of the anterior pituitary. The distribution of nerve fibers or terminals within the adenohypophysis has been shown to be very scanty (Harris, 1955). A fiber tract originating in the hypothalamus and terminating in the anterior pituitary does not exist. Neither are there hormone-containing nerve fibers as seen in the neurohypophysis nor termination of nerve endings on anterior pituitary cells as seen in the adrenal medulla.

The nature of the relationship between the neural centers in the hypothalamus and the pituitary became more clearly defined by transplantation experiments (Harris, 1955). The present view of pituitary regulation hinges on these experiments, which involved transplantation of the adenohypophysis to the anterior chamber of the eye or to the subarachnoid space. These experiments demonstrated the following:

Revascularization of the anterior pituitary does not restore normal function to the gland. There is a loss of weight and function of the target organs, indicating a decrease in trophic hormone secretion. Further, basophils become highly dedifferentiated during transplantation to a heterotrophic site (Szentagothai, 1962). Transplantation of the neurohypophysis or the adrenal medulla also resulted in a loss of normal function and eventual atrophy. However, there is a greater dependency of these two glands on neural input. Due to the chronic nature of these experiments, neural degeneration could occur within the time course necessary for revascularization to take place. As stated above, neural input
to the anterior pituitary is quite limited, and was thought at this time not to be the cause of the decreased function, as is the case for the neurohypophysis and the adrenal medulla. The marked decrease of normal secretory activity did indicate that the adeno-hypophysis was neither autonomous nor was it controlled by humoral substances within the systemic circulation. Thus, the anterior pituitary was unlike its endocrine target organs such as the ovary, testis, thyroid or adrenal cortex, which can maintain a normal functional level after transplantation and are regulated by humoral agents within the systemic circulation.

Lending support to the conclusions inherent in Harris' experiments, Popa and Fielding (1930, 1933) described the blood supply of the anterior pituitary to consist of hypothalamo-hypophyseseal portal blood vessels. These vessels arose as arterial twigs from the internal carotid and posterior communicating arteries; they supply the capillary plexus of the pars tuberalis and the median eminence, referred to as the primary plexus of the hypophyseseal portal vessels. These vessels drain into the pituitary via the relatively few portal venous trunks of the pituitary stalk, there breaking up into smaller vessels and distributing the blood supply to the sinusoids of the pars distalis.

Again utilizing pituitary homografts, Harris could demonstrate a specific humoral transmission role for these portal vessels. After placing pituitary grafts into the hypothalamic area of hypophyssectomized rats, the functional state of trophic hormone secretion was re-established. Thus the work of Harris defined the role of the hypothalamus as a primary factor in pituitary regulation:
"It may be argued from the results of these experiments that the anterior pituitary tissue is plastic in nature and that its pattern of secretory activity is not due to any intrinsic property of the tissue itself but to some outside 'drive' or stimulus, derived probably from the central nervous system."

Harris (1955)

The drive or stimulus to which Harris alludes came to be identified as chemical substances contained within the hypothalamus and secreted into the hypophyseal portal vessels. The exact chemical identity, the localization, and the specificity of these humoral substances are not known, but the term "releasing factor" has been used to describe them since they activate pituitary hormone secretion. Recently the chemical nature of TRF has been identified. Utilizing ovine or porcine hypothalami, TRF has been shown to be tripeptide in nature, containing the amino acids, proline, histidine and glutamic acid (Schally et al., 1969; Vale et al., 1970). Releasing factors appear to be specific for each of the pituitary hormones; I have chosen to concentrate my attention on the regulation of adrenocorticotropic hormone (ACTH) by means of its hypothalamic regulatory substance, corticotropin-releasing factor (CRF).

Although CRF has not been characterized as a homogeneous chemical substance, it is thought to be a short-chained polypeptide. Relatively purified CRF has been isolated and purified from sheep hypothalami (Guillemin, 1967). However, it appears that hypothalamic CRF is quite distinct from the neurohypophyseal polypeptide, vasopressin (McCann, 1966). For some time there existed a controversy in the literature regarding vasopressin as the possible physiological releasing factor for ACTH (Ganong, 1963; Fortier, 1966; McCann, 1966). Its action of stimulating ACTH
release had been found in both intact rats as well as in animals with hypothalamic lesions (McCann, 1966). ACTH-secreting tumors could be stimulated following an injection of vasopressin (Grindel-and and Andersen, 1962). It was also found that both vasopressin and ACTH may be released concurrently (Martini, 1966). However, other experiments disassociated the causal interdependence of ACTH and vasopressin secretion. In animals with insulin-induced hypoglycemia, there was the release of ACTH, as measured by plasma corticosterone, without a concomitant increase in vasopressin secretion (McDonald et al., 1957). Furthermore, the experiments of Smelik et al. (1962) demonstrated that not all stress responses require the mediation of vasopressin.

Using posterior-lobectomized rats, Smelik found the response to so-called neurogenic stimuli, such as sound or pain, was either prevented or reduced; however, responses to other systemic stimuli, such as hemorrhage, ether or histamine were unaffected. It was the work of Saffran and Schally (1955) which clarified the chemical nature of hypothalamic regulation of ACTH release. Incubating isolated pituitary halves, they demonstrated an active chemical substance within the hypothalamus which caused the secretion of ACTH. The activity of this crude stalk median eminence extract could not be accounted for by the quantity of vasopressin present (McCann, 1966). Thus the role of vasopressin remains unanswered; although involved in the secretion of ACTH, it does not appear to be the final common mediator of its release.

Although CRF is not yet defined in structure, it is very likely that the chemical information which the hypothalamus liberates in order to control pituitary ACTH secretion is contained within this substance. Among the compelling reasons for believing
this is that lesions of the CRF-containing portion of the hypothalamus block ACTH secretion (Brodish, 1963, 1968). Injection into the arterial supply of the pituitary of a hypothalamic extract produces ACTH secretion with a time course resembling closely that of an acute stress. Furthermore, there is evidence that alteration of ACTH secretion is mediated by changes in CRF content or secretion (Vernikos-Danellis, 1964; Vernikos-Danellis and Marks, 1969). The ACTH hypersecretion induced by reserpine has been thought to be mediated by the secretion of CRF, ACTH and corticosterone in both adrenal and plasma (Bhattacharya and Marks, 1969). The limits of our present knowledge of this system, therefore, may be stated in the question--What regulates the secretion of CRF?

The Influence of Neurohumoral Transmitters on CRF Release

In this work, the basic assumption has been made that CRF secretion is under neuronal control. Neuronal activity has only been associated with the release of CRF and ACTH in a general way, namely by electrical stimulation studies (Endrocz et al., 1959, 1960, 1962, 1963). These methods attempt to implicate definitive centers responsible for the activation and inhibition of ACTH release. Various components of the limbic system, the amygdaloid nucleus and the hippocampus, are shown to regulate ACTH release. Electrical stimulation of the amygdala results in activation of the ACTH response, whereas impulses generated in the hippocampus result in inhibition of secretion. Depression of the ACTH response occurred after stimulation of the basal septum, anterolateral and lateral hypothalamus and similarly in the area of the dorsal tegmentum at the level of the superior colliculus. Activation of the pituitary-adrenal system resulted from electrical stimulation of pre-mammillary region, the mesencephalic reticular
formation and the ventral tegmentum. Although this evidence at best is only indirect, it does imply neural regulation of CRF release. Yet these studies are held in abeyance due to the diffuse nature of the electrical stimuli and the complexity of neuronal networks within these areas. That neurogenic impulses provide the stimulus for secretion appears likely on general grounds only. The best understood expression of neural information is in the form of chemical substances known as neurotransmitters. That neurochemical influences affect the response of glands, muscles and organ systems has been well-established in the peripheral nervous system. Neurotransmitter actions have not been well characterized within the central nervous system with respect to the functional level of pituitary secretions. Investigation of the specific transmitter substances involved within the hypothalamic nerve centers and these subsequent effects on the regulation of ACTH secretion and CRF is discussed in the following section.

The Use of Adrenergic and Cholinergic Agonists and Antagonists: At one time the regulation of ACTH release was believed to be under the control of the systemic circulation. Epinephrine secreted from the adrenal medulla in response to stress was an early candidate as the potential "releasing factor" for ACTH (Fortier, 1966). Although many of the stimuli capable of activating ACTH secretion also evoked an increase in the secretion of epinephrine (Fortier, 1966), it was necessary to distinguish the various components of the endocrine apparatus which might be activated by epinephrine and subsequently act on stimulating ACTH release. Following slow intravenous infusion, epinephrine could activate ACTH release independently from its pressor effect (Vogt, 1944). Though not entirely conclusive, this evidence showed that activation of peripheral vasomotor receptors by epinephrine was not
responsible for its action on ACTH. Perfusion of isolated adrenal glands did not show increased corticoid secretion with the addition of epinephrine (Vogt, 1952). Secondly, depletion of adrenal cholesterol and ascorbic acid with epinephrine administration did not occur in hypophysectomized animals (Long and Frey, 1955), which further demonstrates a lack of direct adrenal effect.

To show that systemic epinephrine was not required for ACTH release, concomitant measurements of both ACTH secretion as well as the plasma concentration of epinephrine were made. The plasma of adrenal demedulated rats showed epinephrine concentrations of less than 0.5 μg/ml, whereas the ACTH response to emotional stress, though depressed, was still present (Vogt, 1952). Furthermore, intramuscular injection of epinephrine in doses insufficient to produce a fall in adrenal ascorbic acid elevated the plasma epinephrine to levels of 1.0 and 4.5 μg/ml. Substantiating these findings, Gray and Munson (1951) found that histamine was capable of evoking an ACTH response which did not involve the participation of epinephrine.

Although epinephrine is no longer believed to be the systemic factor which acts on the pituitary to cause secretion of ACTH, it is one of the earliest adrenergic agents studied. More recent experiments, using direct measurements of ACTH itself substantiate these earlier findings. With a dose of 0.2 mg, Kitay (1959) demonstrated stimulation following epinephrine by measuring the fall in pituitary ACTH content and the concomitant depletion of adrenal ascorbic acid.

The exact mechanism by which epinephrine finally acts on ACTH release is not known. In vitro studies of epinephrine's
action on pituitary ACTH have not been extensively made. Incubation of norepinephrine with pituitary halves and median eminence homogenates did not potentiate the action of CRF on ACTH release (Bhattacharya and Marks, 1969; Saffran and Schally, 1955; Guillemin, 1957). Direct injection of epinephrine into the grafted pituitaries in the anterior chamber of the eye show no stimulation, thus precluding any direct pituitary effect (Casentini, et al., 1959). High concentrations of epinephrine are not found in either the peripheral or central nervous system. Thus it has not been shown to be a local endogenous neurotransmitter, as is the case for norepinephrine and acetylcholine. The major source of endogenous epinephrine is the amount released from the adrenal medulla into the systemic circulation. Access of epinephrine to the brain from the general circulation is certainly limited by the existence of the blood-brain barrier. However, the immediate area of pituitary regulation, the median eminence of the hypothalamus will concentrate epinephrine in nerve endings, appearing not to possess a blood-brain barrier (Lichensteiger and Langemann, 1966; Samorajski and Marks, 1962). Although not entirely conclusive, the action of catecholamines on ACTH release may well be via the neural units involved in the secretion of CRF.

Other adrenergic agents have been studied in relation to activation of pituitary ACTH secretion. Attempting to define a common adrenergic neural path for ACTH, Vernikos-Danellis (1967) utilized the pharmacological principles of alpha and beta receptor actions of various catecholamines. Following intraperitoneal administration, the relative potencies of these catecholamines demonstrated the following: norepinephrine, primarily an alpha agonist, is more potent in stimulating ACTH secretion than is
epinephrine, which possesses both alpha and beta agonist activity. Isoproterenol, principally a beta agonist, had little effect on ACTH secretion and is the least potent of the catecholamines. Attempting to assess the alpha and beta effects of these sympathomimetic agents, selective blockade with either phentolamine, an alpha antagonist, and MJ 1999, a potent beta blocker, was studied. Blockade with phentolamine, eliminated the stimulatory effects of both norepinephrine and epinephrine. Stimulation of ACTH after vasopressin and histamine, as well as the effects of ether stress, were unaffected by alpha blockade. Therefore, it seems probable that not all stress responses are mediated through the common mechanism of alpha receptor activation. Following MJ 1999, there was a marked reduction in the response to epinephrine and ether, as well as a slight decrease in the response to lysine vasopressin. Since the effects of neither histamine nor of norepinephrine are eliminated by beta blockade, it appears that there is no common beta receptor involved in ACTH release.

The administration of dopamine has been shown to cause stimulation of ACTH secretion (King and Thomas, 1968). Measuring the fall in adrenal ascorbic acid one hour after subcutaneous administration of various concentrations of dopamine, a significant depletion was observed with doses as low as 0.1 mg/kg. However, measurements of ACTH directly (Vernikos-Danellis, 1967) did not show any increase in plasma content ten minutes after a single dose of dopamine administered i.p. It is possible that the time course and the route of administration might account for the discrepancies in these experiments. Stimulation of ACTH secretion after dopamine was not blocked by phenoxybenzamine (King and Thomas, 1968). However, the effect of dopamine was abolished
in animals pretreated with pentobarbital-morphine, thus impli-
cating some central neural involvement in the mechanism of dop-
amine's action and eliminating any possible direct effect on the
adrenal.

Attempting to disassociate central and peripheral neuro-
transmitter effects on ACTH secretion, Naumenko (1967) utili-
zed various drug prototypes of adrenergic and cholinergic trans-
mitters. Pipradrol, a centrally acting sympathomimetic, caused
only a slight elevation of plasma steroids. The greatest stimu-
latory effect in intact guinea pigs was seen with amphetamine,
classed as having both central and peripheral actions as well as
naphtyzine, mainly peripherally acting. Midbrain transection
abolished all stimulatory effects of these adrenergic prototypes.
In studying cholinergic mechanisms, Naumenko utilized the anti-
cholinesterases, galanthamin, which was classified as possessing
central activity and neostigmine, which primarily acts in the peri-
phery. The stimulation of ACTH which resulted was again abolish-
ed by midbrain transection. Although the blood supply to the hypo-
thalamus might have been depressed by surgical intervention, there
was a considerable decrease in acetylcholinesterase levels in the
area above the section with both agents. These experiments imply
a neural regulation; their mechanism appears to be primarily be-
low the section and mainly peripheral in origin.

In order to totally bypass the possibility of peripheral acti-
vation following systemic administration of drugs, Naumenko
(1968) locally injected carbachol, norepinephrine and serotonin
(5-HT) into various hypothalamic areas of the guinea pig. All
neurotransmitter agonists demonstrated marked stimulation of ACTH
secretion. After midbrain transection, eliminating any reverberat-
ing neural circuitry from the brain stem, stimulation was obtained
only with 5-HT. Since the stimulation produced by carbachol and norepinephrine was abolished, it was proposed that their actions were below the region of the transection, while serotonin acted within the hypothalamic area, implying that the regulation by 5-HT is by a more direct neural path for CRF release.

Similarly, Endroczí, Schrieberg and Lissak (1963) utilized the method of direct local injection into various hypothalamic regions to study adrenergic and cholinergic effects on ACTH secretion. Control samples of adrenal venous blood showed elevated corticoid secretion. However, further stimulation by norepinephrine, epinephrine and ephedrine was prominent in the posterior hypothalamus and the ventral tegmental area. Cholinergic activation occurred in the tuber cinereum and also the posterior hypothalamus. When either eserine or carbaminoylcholine was injected into the anterolateral hypothalamic area, there was a reduction in ACTH secretion. Reversal of cholinergic inhibition on forebrain structures was abolished by repeated injection of norepinephrine into basal septum, the posterior hypothalamus and the ventral tegmentum.

Although a cholinergic inhibitory effect has been observed after local administration, peripherally administered acetylcholine demonstrates stimulation. Casentini et al (1957, 1959) could abolish cholinergic stimulation by hypophysectomy, which rules out a direct effect on the adrenal; in animals bearing pituitary grafts in the anterior chamber of the eye, no cholinergic activation was observed, which further eliminates any direct pituitary effects. Guillemin (1955) elicited adrenal ascorbic acid depletion after methacholine administration. The cholinesterase inhibitor, eserine, also showed a fall in adrenal ascorbic acid; chronic treatment with eserine resulted in hypertrophy of the adrenal, suggesting an activation of ACTH secretion (Guillemin, 1955).
The muscarinic antagonist, atropine, blocked the stimulatory effects of systemic methacholine. However, this could only be obtained after repeated daily injections of this antagonist (Guillemin, 1955). Crystalline atropine, implanted within the anterior hypothalamus, antagonized the stimulation seen after lysine vasopressin and ether stress (Smelik and Hedge, 1968). Implants in either the septum or the posterior hypothalamus were ineffective against these stress responses. Thus the regional specificity of atropine may represent the area of cholinergic activation for CRF release. The dose of atropine, however, was extremely high (200-250 µg). Control animals had only an implantation cannula containing no atropine.

It appears feasible that a physiologically or osmotically active salt could have been used as the control since the use of high salt concentrations implanted in various brain regions might cause local necrosis (Kreiger and Kreiger, 1965).

The Influence on Pituitary ACTH Secretion of Drugs which Affect Neural Components Indirectly: In attempting to decipher the neural components involved in CRF regulation, studies, utilizing drugs which influence the content, release, synthesis or storage of biogenic amines, have been investigated with regard to endocrine function.

Reserpine, an agent which depletes endogenous amine stores, specifically those of norepinephrine, dopamine and 5-hydroxytryptamine, has been shown in a number of studies to cause ACTH hypersecretion. Within two hours after reserpine administration, there was a fall in pituitary ACTH content along with a concomitant decrease in adrenal ascorbic acid (Kitay et al., 1959). Both of these parameters imply activation of CRF secretion. In order to more fully study
reserpine's effects on all components of the endocrine apparatus, Bhattacharya and Marks (1969) investigated changes in both CRF and ACTH at various times after a single reserpine dose. Using an in vitro assay of pooled median eminence extracts, they noted a dramatic decrease in CRF content after reserpine administration. Reduction in the CRF content was greatest at two hours and continued to be decreased for the following forty-eight hours. Concomitant measurement of pituitary ACTH content along with adrenal and plasma corticosterone levels all demonstrated the pattern of endocrine responsiveness for the period of time studied.

The depleting action of reserpine has implied that the catecholamines might possess an inhibitory function with regard to the endocrine response. Examining the effects of systemic reserpine (2 mg/kg) on both brain catecholamines as well as changes in the endocrine response, Hirsch and Moore (1968) found that, as corticosterone levels approached maximal concentrations in the plasma, there was the simultaneous decrease in whole brain catecholamines. However these two systems did not recover synchronously. Although plasma corticosterone levels returned to normal within 24 hours, the whole brain catecholamine content is not an adequate reflection of local amine changes and that these local amine changes could in turn account for the termination of the ACTH response.

Attempting to localize reserpine's action to central catecholamine-containing neuronal systems, Smelik (1967) implanted reserpine into the area of the ventral medial hypothalamus, thereby depleting the tubero-infundibular system. Histochemical studies showed an absence of both catecholamine and serotonin fluorescent neurons within this area. No increase in ACTH secretion could be seen subsequent to the reserpine implant; after the systemic administration of
reserpine, in implanted animals, an elevated plasma corticosterone level was observed. The action of reserpine to initiate ACTH secretion would therefore not appear to be blocked by depletion of the catecholamines and serotonin in the tuberal region.

These studies imply that catecholamines and possibly serotonin might function as inhibitors but not as activators of the ACTH response. In order to further characterize the actions of these two systems, selective removal of either catecholamines or 5-HT by the use of the biosynthetic inhibitors, alpha methyl tyrosine or para-Cl-phenylalanine (PCPA) was performed. Interruption of monoamine synthesis has not conclusively shown a single participation of either catecholamines or serotonin in ACTH secretion. Animals, so treated, respond normally to acute or chronic stress as well as to the effects of reserpine administration (Bhattacharya and Marks, 1969). However, further stimulation of ACTH activity, twenty-four hours after reserpine could be shown with the highest dose of alpha methyl tyrosine, (Carr and Moore, 1968). At this time, whole brain norepinephrine and dopamine remained depressed and no further decrease in monoamine content could be seen with alpha methyl tyrosine.

Blockade of the stress response following alpha methyl tyrosine has been obtained in rats twenty-four hours after adrenalectomy (Vernikos-Danellis, 1967). Intact controls responded normally to stress following alpha methyl tyrosine, whereas adrenalectomized rats demonstrated a graded decrease in the ACTH response to ether stress with varying doses of this drug. The highest dose of this agent blocked the ACTH release after ether stress as well as ether/sham unilateral adrenalectomy when compared with tyrosine injected controls. These results indicate that catecholamines may be required for initiation of ACTH secretion in-
adrenalectomized rats by providing a stimulatory path through which the stress response expresses itself.

Despite the marked decrease in brain serotonin after either PCPA (Preziosa et al., 1968; de Schaepdryver et al., 1969) or 4-chloro amphetamine (Dixit and Buckley, 1969) no activation in basal ACTH secretion occurred. The plasma corticosterone response to stress remains unchanged in animals depleted of endogenous serotonin by treatment with 4-Cl-amphetamine (Dixit and Buckley, 1969). Although the effects of amine depletion with PCPA (de Schaepdryver et al., 1969) resulted in a decrease in brain serotonin levels along with a fall in brain norepinephrine, only a slight rise in plasma corticosterone was observed. Preliminary reports by Stolk et al. (1969) show a reduction in both dopamine and norepinephrine levels following single or multiple doses of PCPA. It is therefore apparent that there exists an interrelationship between neuronal systems. The selective depletion of a single system could result in the activation or depression of a second neuronally dependent one. Control of brain serotonin and norepinephrine by specific neural systems following lesioning has been recently described by Heller and Moore (1968). Chemical removal of neurotransmitters could simulate the effects of lesioning, thereby causing similar changes in non-depleted neural systems. Thus it must be considered that depletion of a biogenic amine in one brain region may influence other neural mechanisms both locally as well as remotely.

The results summarized above have failed to dispel the confusion surrounding concepts of neurochemical regulation of CRF secretion, due, in most cases, to the indirect relationship between drug administration or amine depletion and the observed endocrine
response. It was therefore determined to examine the effects of biogenic amines following intraventricular administration, thus attempting to localize the distribution of these agents to central structures, and to limit effects of these drugs on blood pressure, epinephrine secretion from the adrenal medulla or other neural receptors outside the CNS which reflexly could activate CRF release.
MATERIALS AND METHODS

Animals

Female Wistar rats weighing 180 - 230 grams were used throughout the experiment. Animals were housed in communal cages kept in a controlled environmental room at 23°C with 12-hour light-dark cycles (7:00 a.m. to 7:00 p.m. light cycle). Animals were kept in the animal room for at least 3 days prior to experimentation with food (Purina rat chow) and water provided ad lib in order to accustom them to the novel environment. One day prior to the experiment animals were weighed and placed in individual cages.

Experimental and Surgical Procedure

General Format: All experiments were performed in the morning, sacrificing the animals between 11:30 a.m. and 1:00 p.m. to avoid any possible change due to the diurnal variation in ACTH release. Nembutal (35 mg/kg, i.p.) was administered 30 minutes prior to surgery. At the time of surgery, an incision was made across the skull and an opening was bored 1.5 mm lateral and 0.5 mm caudal to the bregma, according to the method of Noble et al. (1967). One hour later, intraventricular injections were performed with a Hamilton microliter syringe. The syringe was fitted with an 18 gauge needle sleeve, cut to allow the needle to penetrate to a depth of 4 mm, thereby placing the tip within the
right lateral ventricle of the rat. Merlis Solution, an artificial cerebrospinal fluid (Merlis, 1940; Glowinski et al., 1965), was used both for control injections and as a vehicle for all drugs (Table 1); the total volume equalled 25 microliters (μl). Animals were sacrificed by decapitation 30 or 60 minutes after injection. The adrenal glands were removed, cleaned and weighed; after homogenization in 13 % ethanol saline, the adrenals were stored and frozen for later corticosterone assay according to the method of Bhattacharya and Marks (1969). Plasma samples were also collected and stored frozen for later assay.

Lesioned Animals

Partial hypothalamic isolation was performed with a Halasz knife (Halasz et al., 1965). The blade was housed in a stainless steel barrel (19 gauge needle shank) extending 1.5 mm lateral to the barrel and 3.0 mm from the tip of the blade to the end of the barrel. Animals were lightly anesthetized with Nembutal (20 mg/kg, i.p.). Thirty minutes later they were mounted on a Stoelting stereotaxic apparatus for surgery. Anesthesia was maintained with ether. Ear bars were elevated with steel shims or wedges in order to maintain a 13° angle between the upper jaw and the tooth bar, thereby allowing the hypothalamus to lie in a straight horizontal plane (Szentagothai et al., 1962; Halasz et al., 1962; Halasz et al., 1965; Rogers, 1968). An opening for the knife was made in the skull with a small electrical hand drill and dental burr (No 2), extending 1.0 mm on both sides of the saggital suture. The opening, medially placed, was 3.5 mm in length, lying 1.5 mm caudal to the bregma and 1.0 mm rostral to the lambdoidal suture.
### Table 1

**Merlis Solution**

<table>
<thead>
<tr>
<th></th>
<th>gm/liter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>moles/liter&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.98</td>
<td>Na 0.141</td>
</tr>
<tr>
<td>KCl</td>
<td>0.25</td>
<td>K 0.0033</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.14</td>
<td>Ca 0.00125</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.11</td>
<td>Mg 0.0012</td>
</tr>
<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.07</td>
<td>Cl 0.152</td>
</tr>
<tr>
<td>Urea</td>
<td>0.13</td>
<td>HPO&lt;sub&gt;4&lt;/sub&gt; 0.00048</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.16</td>
<td>HCO&lt;sub&gt;3&lt;/sub&gt; 0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea 0.0022</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose 0.0034</td>
</tr>
</tbody>
</table>

<sup>a</sup>Glowinski et al. (1965)

<sup>b</sup>Merlis (1940)
The knife, located in the stereotaxic holder, was positioned 5.0 mm caudal to the bregma, then lowered through the sagittal sinus to a depth of 9.5 mm. The tip of the blade was located just rostral to the posterior portion of the Circle of Willis and above the sella diphragmatica of the hypophysis. The blade was rotated 90°, then moved 1.0 mm rostrally and caudally. It was then rotated 180° and again moved 1.0 mm rostrally and caudally. Rotation of the blade 90° returned the tip to its original placement and allowed for removal. Gelfoam pads were used to cover the skull opening and to prevent excess bleeding. The opening for intraventricular injection was made at this time and covered with bone wax. Wound clips were used to close the incision. After surgery, the animals were given Bicillin (0.25 cc) intramuscularly, along with 5% glucose/saline (5 cc) intraperitoneally. They were then kept in a heated, oxygen-filled chamber until recovery from the anesthesia and acute effects of the brain lesion.

This process resulted in partial isolation of the posterior part of the medial basal hypothalamus. Lesioned animals were used four days after surgery, following the experimental format mentioned above. Both adrenals and plasma were collected for corticosterone assay. The completeness of the lesion and the vascular integrity of the pituitary gland were grossly examined at the end of all experiments. Data were rejected if the lesion transected the Circle of Willis, or if the lesion was off center as determined by gross anatomical observations or as indicated by hemorrhage.

**Reserpine Pretreatment**

Normal and lesioned rats were pretreated with reserpine (2.5 mg/kg, i.p.) sixteen hours prior to intraventricular injection.
In these animals the dose of Nembutal anesthesia was reduced to 20 mg/kg, i.p. because the anesthetic effect was potentiated by reserpine.

**Blood Pressure Measurements**

In order to determine if there were changes in blood pressure due to the intraventricular injection of biogenic amines, animals were anesthetized with Nembutal; an opening for intraventricular injection was made in the skull and PE 10 polyethylene cannula was inserted in the right femoral artery and the left femoral vein. Recordings were made on a Grass polygraph using a Statham pressure transducer. Blood pressure was observed for 15-30 minutes after injection into the ventricles in both normal and reserpine pretreated rats.

**Chemical Determinations**

The fluorometric assay of corticosterone was done according to the method of Bhattacharya and Marks (1969). Aliquot samples of 1 ml were taken from the adrenal extract after centrifugation and made up to a volume of 2 ml with 13 % absolute ethanol saline. Corticosterone standards (0.1 to 0.4 µg) along with blank determinations were run with unknowns throughout all assays. The unknowns and standards were washed with iso-octane; after aspiration of the iso-octane layer, the steroid was extracted into methylene chloride, shaken, centrifuged and finally extracted into the fluorescence reagent (70 % absolute ethanol and sulfuric acid). Corticosterone fluorescence was read at an activation 470 nm, emission 525nm on an Aminco fluorometer one hour later.

Plasma samples were assayed similarly. A plasma aliquot of 0.2 ml was brought up to 2 ml volume with 13 % ethanol-saline.
After the iso-octane wash and subsequent extraction into methylene chloride, a 4 ml aliquot of the methylene chloride was transferred to a clean centrifuge tube prior to addition of the fluorescence reagent. Standards of 0.02 ml to 0.1 μg corticosterone along with blank determinations were run with each assay.

Drugs

All drugs used for intraventricular injection are expressed as the concentration of the free base. Drugs used: 1-norepinephrine, dopamine hydrochloride, carbamylcholine chloride (carbachol), and serotonin creatininsulfate were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Reserpine (Serpasil-Ciba) was obtained from the Ohio State University Hospital Pharmacy.

Chemicals

RESULTS

Adrenal Corticosterone Concentration at Different Time Periods after Control Procedures

The adrenal corticosterone concentration in normal, unanesthetized animals, kept in a controlled environment, is shown by the hatched area in figure 1. Control values for experimental animals were determined by a sequential time study. Non-injected controls sacrificed at ninety minutes, one hour after surgery, demonstrate a reduction below normal values, presumably due to the anesthetic. At this time there was a recovery from any anesthetic or surgical stress. Therefore, this time period was chosen for intraventricular administration of either drug or vehicle.

Fifteen minutes after injection of Merlis solution (105 minutes in figure 1) the adrenal corticosterone values were markedly elevated. The stimulation seen at this time period may indicate a non-specific stress response possibly due to an increase in cerebrospinal fluid pressure. At both thirty and sixty minutes following the injection, the adrenal values for the Merlis-injected animals had returned to normal levels. On the basis of these controls, the response of drug-injected animals was examined at the last two time periods.

The Time-Response Relationship for Norepinephrine, Dopamine and Carbachol

Adrenal corticosterone values obtained at both thirty and sixty minutes after intraventricular amine injection are shown in

24
Figure 1

Control Adrenal Responses after Intraventricular Injections of Merlis Solution

Adrenal corticosterone values obtained at various time intervals after surgery and intraventricular injection of control vehicle, Merlis solution (25 µl). The shaded area represents adrenal values for normal, non-anesthetized rats.
CONTROL ADRENAL RESPONSES AFTER INTRAVENTRICULAR INJECTIONS OF MERLIS' SOLUTION

ADRENAL CORTICOSTERONE (μg/100 mg)

TIME (min)

Nembutal  Surgery
Table 2. Stimulatory doses of norepinephrine, dopamine and carbachol all showed a maximal effect on adrenal corticoid secretion at thirty minutes (except for the highest dose (40 µg) of norepinephrine). Doses of these amines, which produced maximal elevation at this earlier time, did not cause further activation at sixty minutes. There was a reduction in the responses to both norepinephrine and carbachol one hour after injection to most doses studied except for the highest dose of norepinephrine. Further, the dopamine-induced activation had completely subsided at this later time.

The Dose-Response Curves of Adrenal Corticosterone, after Intraventricular Amine Administration

The corticosterone response to intraventricularly administered monoamines thirty minutes after injection, can be seen in figure 2. Carbachol at 0.1 and 0.25 µg was no different from controls, whereas, doses of 5 µg and higher produced maximal stimulation. Dopamine doses from 0.1 to 10.0 µg were similar to controls, while a higher dose of 25 µg was required to produce stimulation. Neither of the catecholamine curves showed any graded response 30 minutes after intraventricular injection. However, it should be noted that the dopamine curve is shifted to the right, the potency relative to norepinephrine being approximately twenty percent. One hour after monoamine injection (figure 3), low doses of norepinephrine (0.1 and 1.0 µg) were no different from control. An intermediate elevation was observed at 5, 10 and 20 µg; these doses at thirty minutes had given maximal stimulation. At the highest dose tested (40 µg) an equivalent stimulation was obtained at both time intervals. Again the dopamine response is reduced. Low doses of carbachol did not show stimulation at the sixty minute time period; the higher
Table 2

Adrenal Corticosterone Values at Various Time Intervals after Intraventricular Amine Administration

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Adrenal Corticosterone (µg/100mg)±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 minutes</td>
</tr>
<tr>
<td>Control</td>
<td>Merles solution</td>
<td>0.69 ± 0.11 (23)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>0.1 µg</td>
<td>0.35 ± 0.16 (6)</td>
</tr>
<tr>
<td></td>
<td>0.25 µg</td>
<td>0.66 ± 0.57 (7)</td>
</tr>
<tr>
<td></td>
<td>1.0 µg</td>
<td>2.05 ± 1.20 (9)</td>
</tr>
<tr>
<td></td>
<td>2.5 µg</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td></td>
<td>5.0 µg</td>
<td>5.24 ± 1.80 (5)*</td>
</tr>
<tr>
<td></td>
<td>25.0 µg</td>
<td>4.63 ± 0.34 (4)*</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.1 µg</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td></td>
<td>1.0 µg</td>
<td>0.34 ± 0.11 (3)</td>
</tr>
<tr>
<td></td>
<td>2.0 µg</td>
<td>0.76 ± 0.59 (3)</td>
</tr>
<tr>
<td></td>
<td>5.0 µg</td>
<td>5.71 ± 0.77 (3)*</td>
</tr>
<tr>
<td></td>
<td>10.0 µg</td>
<td>5.23 ± 0.61 (3)**</td>
</tr>
<tr>
<td></td>
<td>20.0 µg</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td></td>
<td>40.0 µg</td>
<td>4.85 ± 0.62 (8)**</td>
</tr>
<tr>
<td>Dopamine</td>
<td>0.1 µg</td>
<td>- - - - - - - -</td>
</tr>
<tr>
<td></td>
<td>1.0 µg</td>
<td>0.97 ± 0.64 (7)</td>
</tr>
<tr>
<td></td>
<td>5.0 µg</td>
<td>0.63 ± 0.42 (5)</td>
</tr>
<tr>
<td></td>
<td>10.0 µg</td>
<td>1.03 ± 0.23 (5)</td>
</tr>
<tr>
<td></td>
<td>25.0 µg</td>
<td>5.76 ± 0.49 (4)**</td>
</tr>
</tbody>
</table>

(N) = number of animals
* Significant at P = 0.05 level
** Significant at P = 0.01 level
Figure 2

Dose-Response Curves Obtained 30 Minutes Post-Injection

The adrenal steroid response obtained at thirty minutes after intraventricular administration of monoamines. Control values are shown in the shaded area for this time period as indicated in the Materials and Methods.
ADRENAL CORTICOSTERONE (µg/100 mg)

CARBACHOL

NE

DA

DRUG DOSE

0.18

1.08

108
Figure 3

Dose-Response Curves Obtained 60 Minutes Post-Injection

The dose-response curve obtained at sixty minutes after monoamine injection. Shaded area represents the control values.
ADRENAL CORTICOSTERONE (μg / 100 mg)

CARBACHOL

NE

DRUG DOSE

ADRENAL CORTICOSTERONE (μg / 100 mg)
doses showed only an intermediate effect, when compared with the stimulatory responses at thirty minutes (Table 2). Plasma corticosterone values were assessed under different experimental conditions and were found to correspond with adrenal steroid values.

The Effect of Biogenic Amines Following Pretreatment with Reserpine

Reserpine has been shown to cause depletion of central catecholamine and serotonin stores, along with an activation of the release of both CRF and ACTH. In order to examine the possibility that replacement of one or more of these amines might reverse the stimulatory response to reserpine, the action of these agents after reserpine pretreatment was examined.

In studies of Bhattacharya and Marks (1968) on reserpine effects, CRF release was maximal from two to 24 hours subsequent to reserpine administration. Therefore, in the present experiments, reserpine was administered 16 hours prior to intraventricular administration of biogenic amines. Maickel et al. (1961) have shown that after an injection of radiolabeled corticosterone, values returned to control levels within one hour. Since circulating corticosterone levels were elevated at the time of amine administration following reserpine, it appeared necessary to wait for one hour after intraventricular administration in order for reversal of the reserpine effect to be measurable.

Varying doses of reserpine were administered 16 hours prior to rat surgical preparation for experiment: one hour following control intraventricular injection of Merlis solution, maximal increase of adrenal corticosterone was observed with both 2.5 mg and 5.0 mg/kg. The 2.5 mg/kg dose was chosen for these experiments, since maximal depletion of endogenous catecholamine stores at this dose and time was observed by Hirsch and Moore (1968). Following Nembutal (20 mg/kg) and surgery, intraventricular
doses of biogenic amines were given at 90 minutes; both adrenal
and plasma corticosterone activity were examined one hour later.
The effects of norepinephrine, carbachol and dopamine are shown
in figures 4, 5, and 6. In the first panel of figure 4, the effects
of 0.1 and 0.25 µg of carbachol are shown not to stimulate ACTH
secretion in normal animals. The effect of reserpine pretreatment
resulted in activation of ACTH release as seen in the first column
of the second panel. Neither dose of carbachol inhibited reserpine
effects. The 0.25 µg dose showed significant potentiation.

The changes observed after catecholamine administration are
seen in the next two figures (figures 5, 6), showing the responses
of both normal and reserpine treated animals. Both doses of nor-
epinephrine caused significant depression of the reserpine re-
ponse as can be seen in the second panel of figure 5. In normal
animals, 1.0 µg of norepinephrine was without effect, whereas the
10 µg dose caused maximal stimulation. Dopamine, at both 1.0
and 10.0 µg doses had no effect in normal rats, but caused signifi-
cant suppression of the reserpine stimulation.

Pituitary Responses in Normal and Reserpine Treated Rats Follow-
ing Intraventricular Serotonin

The responses of the pituitary-adrenal system to intra-
ventricular serotonin are shown in Table 3. In normal animals
only a slight elevation of adrenal corticosterone was observed,
thirty minutes after the amine. At all doses used, the responses of
corticosterone to serotonin (5-HT) were similar and not graded
although there was a twenty-five fold range of amine doses used.
The highest dose tested was significant from the control state but
was not equivalent to the maximal stimulation observed after either
catecholamine or carbachol. Hypersecretion of ACTH observed
Carbachol Effects in Normal and Reserpine Pretreated Animals

The adrenal responses in reserpine (2.5 mg/kg) pretreated rats observed with various intraventricular doses of carbachol. The first panel represents the response to carbachol of rats not given reserpine; second panel demonstrates the effects of carbachol in reserpine pretreated animals. The open bars represent the control injection of Merlis solution (25 μl). The shaded and darkened histograms indicate different drug concentration.
Figure 5

Norepinephrine Effects in Normal and Reserpine Pretreated Animals

The adrenal responses in reserpine (2.5 mg/kg) pretreated rats observed with various intraventricular doses of norepinephrine. The first panel represents the response to norepinephrine of rats not given reserpine; second panel demonstrates the effects of norepinephrine in reserpine pretreated animals. Open bars represent the control injection of Merlis solution (25 μl). The shaded and darkened histograms indicate different drug concentration.
Figure 6
Dopamine Effects in Normal and Reserpine Pretreated Animals

The adrenal responses in reserpine (2.5 mg/kg) pre-treated rats observed with various intraventricular doses of dopamine. The first panel represents the response to dopamine of rats not given reserpine; second panel demonstrates the effects of dopamine in reserpine pretreated animals. The open bars represent the control injection of Merlis solution (25 μl). The shaded and darkened histograms indicate different drug concentrations.
ADRENAL CORICOSTERONE (mg/100 mg)

NO PRETREATMENT

RESERPINE PRETREATMENT

ADRENAL CORICOSTERONE (mg/100 mg)

MERLIS CONTROL  D.A 1.0 µg  D.A 10.0 µg

(14)  (7)  (7)

MERLIS CONTROL  D.A 1.0 µg  D.A 10.0 µg

(8)  (5)  (5)
Table 3  
Adrenal Corticosterone Levels in Normal and Reserpine Pretreated Animals Following Intraventricular 5-HT

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Adrenal Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Merlis Solution (25 μl)</td>
<td>0.69±0.11 (23)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>1 μg</td>
<td>1.38±0.66 (7)</td>
</tr>
<tr>
<td></td>
<td>10 μg</td>
<td>1.07±0.45 (8)</td>
</tr>
<tr>
<td></td>
<td>25 μg</td>
<td>2.05±0.65 (6)*</td>
</tr>
<tr>
<td>Reserpine</td>
<td>Merlis Solution (25 μl)</td>
<td>4.21±0.43 (8)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>1 μg</td>
<td>5.70±0.41 (4)*</td>
</tr>
<tr>
<td></td>
<td>10 μg</td>
<td>3.79±0.55 (4)</td>
</tr>
</tbody>
</table>

(N) = number of animals used in each group
* Significant at P = 0.05 level
** Significant at P = 0.01 level
after reserpine has been related to the depletion of monoamines, norepinephrine, dopamine and 5-hydroxytryptamine. In attempting to relate monoamine depletion to a single neurotransmitter, it appears that selective replacement of depleted amines would demonstrate an inhibitory effect. However, following reserpine, 5-hydroxytryptamine did not cause a dramatic decrease in ACTH hypersecretion sixty minutes after intraventricular amine administration. Although a slight increase was observed at the lower amine concentration, 1 μg (significant at P = 0.05), the higher amine concentration of 10 μg did not cause a significant reduction in ACTH hypersecretion.

The Effect of Intraventricularly Administered Biogenic Amines in Animals Bearing Lesions in the Posterior Hypothalamus

Animals with lesions resulting in posterior hypothalamic deafferentation were examined following intraventricular administration of carbachol and norepinephrine. Naumenko (1969) has shown that these two agents were without effect in midbrain transected guinea pigs indicating that their action was not within the area of the hypothalamus. In order to eliminate any activation which might arise from the afferent pathways entering the hypothalamus from the brain stem, in the present studies surgical cuts were placed in the posterior hypothalamus.

Biogenic amines were administered 96 hours after the brain lesioning, in order to allow recovery from any effect of this surgical stress. Control values for adrenal corticosterone in posterior de-afferented animals are shown in figure 7 along with adrenal values 30 minutes after varying doses of carbachol. In normal intact rats, carbachol at 1.0 μg gave an intermediate response; however, after posterior de-afferentation this response was reduced to
Carbachol Effects in Intact and Posterior Deafferented Animals

The response of intact rats (first panel) and posterior deafferented animals (second panel) to varying concentrations of carbachol are represented. Open bars indicate control injection of Merlis solution (25 μl). The shaded and darkened areas represent various amine doses.
IN TA C T ANIM ALS

MERLIS CONTROL

CARBACHOL 1 μg 5 μg

POSTERIOR DEAFFERENTED ANIMALS

MERLIS CONTROL 1 μg 2.5 μg 5 μg

CARBACHOL

ADRENAL CORticosterone (μg/100 mg)
control levels (Table 4). Although maximal stimulation was seen at 5 μg, in both normal and lesioned rats, an intermediate response was obtained at 2.5 μg in lesioned animals. This indicates that the higher dose was still capable of eliciting hypothalamic activation, whereas with the lower doses of 1.0 μg, the response was reduced following de-afferentation.

The effect of norepinephrine was potentiated after lesioning. At 0.1 μg both normal intact and lesioned rats did not show any stimulation as can be seen in figure 8. A maximal stimulation was obtained in intact rats with doses of 5 μg and higher; whereas, de-afferentation caused a dose of 1.0 μg to be markedly effective (Table 4) significant at P < 0.01 level, resulting in a 5-fold increase of potency of norepinephrine for the biological activity which results in stimulation of ACTH release.

The Adrenal Responses to Biogenic Amines in Reserpine Pretreated Rats After Posterior De-Afferentation

In order to test the effect of amine depletion within the area of hypothalamus after posterior de-afferentation, reserpine (2.5 mg/kg, i.p.) was administered 16 hours prior to intraventricular amine injection. As can be seen in figure 9, reserpine controls were reduced in lesioned animals when compared with the adrenal steroid responses in normal intact rats. In the first panel the effect of carbachol in intact rats shows that at the 0.1 μg dose, there is no difference from control, however, there is a marked potentiation of adrenal response to reserpine after 0.25 μg of carbachol. In lesioned animals both doses of carbachol, 0.25 and 1.0 μg, as can be seen in the second panel of figure 9, did not bring about a significant change in the response.
Table 4
Comparison of Monoamine Effects on Adrenal Corticosterone Content in Lesioned and Intact Animals Before and After Reserpine

<table>
<thead>
<tr>
<th>Intraventricular Injection Drug</th>
<th>Adrenal Corticosterone (µg/100 mg)</th>
<th>Intact Animals</th>
<th>Posterior deafferented</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Merlis Solution</td>
<td>0.69 ± 0.11 (24)</td>
<td>0.86 ± 0.51 (9)</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 µg</td>
<td>0.34 ± 0.11 (3)</td>
<td>5.33 ± 0.08 (4)</td>
<td></td>
</tr>
<tr>
<td>5.0 µg</td>
<td>5.71 ± 0.77 (3)*</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 µg</td>
<td>2.05 ± 1.20 (9)</td>
<td>0.29 ± 0.04 (4)</td>
<td></td>
</tr>
<tr>
<td>2.5 µg</td>
<td>-</td>
<td>2.08 ± 0.68 (4)</td>
<td></td>
</tr>
<tr>
<td>5.0 µg</td>
<td>5.24 ± 1.80 (5)</td>
<td>4.98 ± 0.67 (7) **</td>
<td></td>
</tr>
<tr>
<td>Reserpine Pretreated (2.5 mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls Merlis Solution</td>
<td>4.21 ± 0.43 (8)</td>
<td>2.89 ± 0.91 (9)</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1.58 ± 0.62 (3)*</td>
<td>2.14 ± 0.64 (5)</td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>4.63 ± 1.27 (4)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.25 µg</td>
<td>5.49 ± 0.27 (5)*</td>
<td>2.75 ± 0.70 (5)</td>
<td></td>
</tr>
<tr>
<td>1.0 µg</td>
<td>-</td>
<td>2.36 ± 0.66 (4)</td>
<td></td>
</tr>
</tbody>
</table>

(N) = number of animals used in each group
* Significant at P = 0.05 level
** Significant at P = 0.01 level
Figure 8

Norepinephrine Effects in Intact and Posterior Deafferented Animals

The response of intact rats (first panel) and posterior deafferented animals (second panel) to varying concentrations of norepinephrine are represented. Open bars indicate control injection of Merlis solution (25 µL). The shaded and darkened areas represent various amine doses.
Figure 9
Carbachol Effects in Reserpine Pretreated Intact and Posterior Deafferented Animals

The response obtained after reserpine (2.5 mg/kg) pretreatment and intraventricular carbachol in both intact (first panel) and posterior deafferented animals (second panel). The open bars indicate control Merlis injection. The shaded and darkened areas represent the drug concentrations.
<table>
<thead>
<tr>
<th>Reserpine Pretreatment</th>
<th>Adrenal Corticosterone (μg/100 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact Animals</strong></td>
<td></td>
</tr>
<tr>
<td>Merlis Control</td>
<td>4.0 (6)</td>
</tr>
<tr>
<td>Carbachol 0.1 μg</td>
<td>5.0 (5)</td>
</tr>
<tr>
<td>Carbachol 0.25 μg</td>
<td>6.0 (4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reserpine Pretreatment</th>
<th>Posterior Deafferented Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merlis Control</td>
<td>4.0 (17)</td>
</tr>
<tr>
<td>Carbachol 0.25 μg</td>
<td>3.0 (5)</td>
</tr>
<tr>
<td>Carbachol 1.0 μg</td>
<td>2.0 (4)</td>
</tr>
</tbody>
</table>
Norepinephrine showed a marked inhibitory action at both 1.0 and 10.0 µg following reserpine in normal, intact rats (figure 5). As can be seen in figure 10, reserpine treatment followed by intraventricular norepinephrine (1.0 µg) was not different from control values in de-afferented animals (Table 4).

**Blood Pressure Effects Observed after Administration of Biogenic Amines**

Systemic arterial blood pressure responses were monitored as a measurement of possible peripheral drug distribution. Both normal and reserpine pretreated animals were examined following intraventricular administration of various doses of norepinephrine and carbachol. The effects of drug administration were evident within the first minute. In untreated animals (figures 11, 12), norepinephrine, at both 1.0 and 10.0 µg, produced a slight rise (approximately 25 mm Hg) in blood pressure after intraventricular administration. This elevation in pressure lasted for approximately five minutes and returned to control level within seven minutes following the injection. In order to determine to some degree the quantity of norepinephrine which might exchange with the systemic circulation, a comparison was made between the magnitude of the response seen after intraventricular administration with those effects produced by systemic injection. Norepinephrine was administered intravenously at doses of 0.1, 0.2 and 0.4 µg (figure 13). The pressor responses produced after the intravenous norepinephrine administration demonstrated increases of 40, 65, and 85 mm Hg, respectively. Thus it appears that the pressor responses produced by 0.1 µg of intravenous catecholamine was greater in magnitude than the effect of either 1.0 or 10 µg norepinephrine administered intraventricularly. The responses of blood pressure to higher doses of intravenous
Figure 10

Norepinephrine Effects in Reserpine Pretreated Intact and Posterior Deafferented Animals

The response obtained after reserpine (2.5 mg/kg) pretreatment and intraventricular norepinephrine in both intact (first panel) and posterior deafferented animals (second panel). The open bars indicate control Merlis injection. The shaded and darkened areas represent the drug concentrations.
Figure 11

Blood Pressure Responses Following Intraventricular Norepinephrine Administration
Figure 12

Blood Pressure Responses to Intraventricular Norepinephrine Administration
Control - Norepinephrine 0.1 μg (3 minutes)

Control - Norepinephrine 0.2 μg (3 minutes)

Control - Norepinephrine 0.4 μg (3 minutes)
norepinephrine showed a much more marked effect within the first few minutes than any of these doses studied after intraventricular administration.

Intraventricular carbachol administration of 0.25 µg produced a pressor response similar to the effects seen after intraventricular administration of norepinephrine (figure 14), a rise of about 40 mm Hg. The response terminated within eight minutes. At higher doses of 2.5 and 10.0 µg of carbachol (figures 15, 16), an initial fall of blood pressure could be observed. With the intermediate dose of 2.5 µg, the depressor response (a fall of approximately 25 mm Hg) returned to normal by six minutes and was not followed by recurrent pressor activity at either 10 or 15 minutes post injection. Following 10 µg of intraventricular carbachol there was a fall in blood pressure (50 mm Hg) lasting for eight minutes. The characteristic hypotensive response of cholinergic stimulation could be seen after 0.1 µg of carbachol administered intravenously, the fall in blood pressure was around 50 mm Hg occurring within the first few minutes following injection. Thus it is possible that the higher doses of carbachol do exchange with the systemic circulation but that the effects of peripheral cholinergic stimulation are terminated within the first ten minutes.

Reserpine pretreated animals, injected intraventricularly, showed no marked change in blood pressure following the control administration of Merlis solution (figure 17). Norepinephrine at a dose of 1.0 µg (figure 17), demonstrated a slight fall in blood pressure within the first two minutes. This was followed by an increase in blood pressure (25 mm Hg) which returned to control level by ten minutes. At a higher dose of 10.0 µg norepinephrine (figure 18) the slight decrease in blood pressure was initially
Figure 14

Blood Pressure Response Following Intraventricular Carbachol Administration
Figure 15
Blood Pressure Responses Following Intraventricular Carbachol Administration
Figure 16

Blood Pressure Responses Obtained After Intraventricular Carbachol Administration
Control - Merlis Solution

arrows indicate injection time

Time Scale 1 min.

Control - Norepinephrine 1.0 μg (2 minutes)

Norepinephrine Response (5 minutes)
Norepinephrine Response (8 minutes)

Time Scale 1 min.

Norepinephrine Response (10 minutes)

Figure 17

Blood Pressure Responses in Reserpine Pretreated Rats
Following Intraventricular Merlis and Norepinephrine Injection
Figure 18

Blood Pressure Responses in Reserpine Pretreated Rats Following Intraventricular Norepinephrine Administration
observed, followed by a sustained elevation, an increase of 50 mm Hg, which terminated within twenty minutes.

A pressor response was elicited after intraventricular carbachol in reserpine pretreated animals. The 0.25 µg dose of carbachol (figure 19) produced a rather sustained elevation in blood pressure. Only at the highest tested dose, 25 µg (figure 20) was a marked depressor response observed, again indicating possible peripheral cholinergic activation.

It requires approximately 10 times as much norepinephrine injected intraventricularly as systemically to produce an equivalent pressor response. Although the magnitude of these responses are similar, the duration and pattern of this pressor activity is quite different; the time course for intraventricular amine injection is longer in duration and develops slowly. An equivalent fall in pressor activity following intraventricular carbachol was obtained at approximately 100X the systemic dose.
Figure 19

Blood Pressure Responses in Reserpine Pretreated Rats Following Intraventricular Carbachol Administration
Figure 20

Blood Pressure Responses in Reserpine Pretreated Rats Following Intraventricular Carbachol Administration
DISCUSSION

The Presence of Neurotransmitter Substances Within the Hypophysiotrophic Area and Central Nervous System

From studies of pituitary transplantation in specific hypothalamic regions, the area of the medial basal hypothalamus, designated as the hypophysiotropic area (HPA), is found to be the only site capable of maintaining the structure and function of the pituitary (Halasz et al., 1962; Flament-Durant, 1965). This region extends from the optic chiasm to the mammillary body including the arcuate, supraoptic and ventromedial nuclei of the hypothalamus. From these studies as well as from the studies of the localization of CRF (Brodish, 1968; Saffran and Schally, 1955) this area represents the site of CRF storage (figure 21).

In this region as well as other brain areas, a rather static picture of neurotransmitter function within the central nervous system (CNS) emerges from measurements of monoamine content and distribution. However, this distribution pattern provides a reference framework for interrelating the possible monoamine receptors involved in neuroendocrine regulation.

From the measurements of fluorescent histochemistry (Dahlstrom and Fuxe, 1964, 1965; Dahlstrom et al., 1964; Fuxe et al., 1965, 1968a, 1968b) the topography and location of neuronal distribution for both catecholamines and serotonin within cell bodies, axons and terminals has been established in the
### Explanation of Abbreviations used in Figures 21 and 22

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHA</td>
<td>anterior hypothalamus</td>
</tr>
<tr>
<td>AL, APL, ACO</td>
<td>amygdaloid complex</td>
</tr>
<tr>
<td>ARH</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>CA</td>
<td>anterior commissura</td>
</tr>
<tr>
<td>CC</td>
<td>corpus colossum</td>
</tr>
<tr>
<td>CO</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>CP</td>
<td>posterior commissure</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsal medial hypothalamic nucleus</td>
</tr>
<tr>
<td>Fx</td>
<td>fornix</td>
</tr>
<tr>
<td>HPC</td>
<td>hippocampus</td>
</tr>
<tr>
<td>LAHY</td>
<td>anterior pituitary</td>
</tr>
<tr>
<td>LHA</td>
<td>lateral hypothalamic area</td>
</tr>
<tr>
<td>LPHY</td>
<td>posterior pituitary</td>
</tr>
<tr>
<td>LT, MD, VP, VE</td>
<td>thalamic nucleus</td>
</tr>
<tr>
<td>M</td>
<td>mammillary region</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
</tr>
<tr>
<td>MM</td>
<td>medial mammillary nucleus</td>
</tr>
<tr>
<td>MP</td>
<td>posterior mammillary nucleus</td>
</tr>
<tr>
<td>MT</td>
<td>mammillothalamic tract</td>
</tr>
<tr>
<td>PH</td>
<td>posterior hypothalamic nucleus</td>
</tr>
<tr>
<td>PMD</td>
<td>posterior medial dorsal nucleus</td>
</tr>
<tr>
<td>PV</td>
<td>periventricular nucleus of thalamus</td>
</tr>
<tr>
<td>PVH</td>
<td>paraventricular hypothalamus</td>
</tr>
<tr>
<td>RE</td>
<td>reuniting nucleus of the thalamus</td>
</tr>
<tr>
<td>SC</td>
<td>supra chiasmatic area</td>
</tr>
<tr>
<td>ST</td>
<td>striatum</td>
</tr>
<tr>
<td>SUM</td>
<td>submammillary nucleus</td>
</tr>
<tr>
<td>VM</td>
<td>ventromedial nucleus</td>
</tr>
<tr>
<td>VMH</td>
<td>ventral medial hypothalamic nuclei</td>
</tr>
</tbody>
</table>
Figure 21

Sagittal Midline Section

A sagittal midline section through the rat brain indicating the areas of pituitary grafts as well as the extent of the lesion in posterior deafferented animals.
Figure 22

Coronal Section

A coronal section through the posterior hypothalamic area indicating the lateral extent of the lesion in posterior de-afferented animals.
hypothalamus. There exists ascending serotonin and catechol-
amine-containing fiber tracts within the medial forebrain
bundle as well as the specialized adrenergic neurons of the arcuate
nucleus--the tubero-infundibular tract (Fuxe, 1964).

Studies on the ultrastructure of the anterior hypothalamus
(de Iraldi et al., 1963) demonstrated the presence of granulated
vesicles indicative of catecholamine granules. Biochemical de-
termination of this region as well as whole brain analysis shows
the presence of norepinephrine within the vesicular fraction
(de Robertis, 1965). Neither the adrenergic or serotonergic
neuronal system have been shown to be directly involved with the
regulation of CRF synthesis or secretion. Their proximity to
hypothalamic-hypophyseal structures, however, suggest such a
regulatory control by the release of the neurotransmitter sub-
stances within the area of the hypothalamus.

Microiontophoresis of chemical neurotransmitters within
various brain regions suggest that these substances act to cause
either stimulation or inhibition of neuronal activity. Both acetyl-
choline and norepinephrine have been shown to possess both ex-
citatory as well as depressant effects in the brain stem and hypot-
thalamus (Curtis and Crawford, 1969). Although these studies
attempt to relate these neurotransmitter and neuronal activities,
it is difficult to project from this type of experiment to the neural
influence on endocrine function.

The distribution of cholinergic pathways within the CNS
has been shown through measurements of acetylcholinesterase
(Krnjevic, 1969). Various cholinergic projections ascending
from the brain stem to the forebrain are related to the limbic
system as well as the striatum. However, these findings as well as the measurements of choline acetylase provide only indirect evidence for the functional role of cholinergic substances as a neurotransmitter.

Again, arguing from the position of anatomical proximity, it appears that a cholinergically active system at the site of CRF storage might regulate its secretory activity.

Although endocrine function has been examined after systemic administration of chemical prototypes of these neurotransmitter systems, the results from these findings are difficult to interpret. There are the inherent problems of exclusion from the CNS due to the blood-brain barrier, dilution of the drug concentration by whole body distribution and finally the possibility of stimulating peripheral receptors which in turn activate the secretion of ACTH. Therefore, it appeared desirable to study drug effects after a rather localized injection into the cerebrospinal fluid.

The method of intraventricular administration has been used for the estimation of drug effects on general behavioral and motor activity as well as changes in temperature responses (Feldberg and Fleischhauer, 1965).

Recently, monoamine distribution within the CNS has been examined after intraventricular administration of labeled neurotransmitter substances. Radioautography combined with electron microscopy illustrates the distribution of $^3$H-norepinephrine (0.8 μg) and $^3$H-5-HT within central neuronal stores (Aghajanian and Blood, 1966; 1967; Bloom and Giarman, 1968). After intraventricular administration, $^3$H-norepinephrine (0.21 μg) and $^3$H-dopamine (0.24 μg) localize within the adrenergic neurons and mix with the endogenous pools (Glowinski and Axelrod, 1965; Glowinski and Iverson, 1966; Glowinski et al., 1966a, 1966b). Within the first hour
following intraventricular administration, 43% of the drug is found as unmetabolized amine in the brain. Later the unmetabolized concentration falls exponentially followed by a concomitant rise in de-aminated and o-methylated metabolites. In view of these observations, it appears reasonable to expect that receptor sites for these substances could be activated after intraventricular administration; and subsequently bring about changes in endocrine function.

Assessment of Drug Distribution in the Systemic Circulation Following Intraventricular Administration

Drugs introduced into the cerebrospinal fluid are able to exchange with the systemic circulation thus stimulating peripheral receptors. In the present experiments, the quantity of drug accessible to peripheral nervous structures, has been estimated by measurements of blood pressure following intraventricular administration. In normal animals, norepinephrine, 1.0 and 10 μg, caused a small pressor response of relatively short duration. In reserpine pretreated animals the pressor response of the lower dose of norepinephrine is similar to the effects observed in normal animals; whereas the pressor response to the higher dose (10 μg) is longer in duration and gives a more pronounced effect. Since hypertension occurred after intraventricular administration of norepinephrine, it is implied that there is involvement of peripheral sympathetic receptors due to exchange with the systemic circulation. However, the responses obtained were not as great as those observed after direct systemic administration.

Following intraventricular carbachol administration, normal animals showed a slight increase of blood pressure at low doses, while reserpine-pretreated animals showed larger and more
prolonged pressor responses. Higher concentrations of carbachol elicited a fall in blood pressure both in normal and reserpine pre-treated animals, that is characteristic of the peripheral action of cholinergic agonists. Thus, cholinergic stimulation of peripheral parasympathetic receptors was observed only at high amine concentrations. The pressor effect seen after low doses of carbachol could be the result of either the activation of central vasomotor receptors as well as the stimulation of the secretion of adrenal medullary hormones, epinephrine and norepinephrine. However, the neural pathway for the production of pressor responses following intraventricular carbachol has not been established at this time.

Finally, it should be asked whether the observed cardiovascular responses account for the subsequent changes in ACTH secretion. Marked hypotensive responses elicited secretion of ACTH release (Ganong, 1963; Ganong et al., 1965; Redgate, 1968), whereas, inhibition of ACTH secretion has been related to the hypertensive effects following alpha ethyltryptamine and other pressor amines (Lorenzen and Ganong, 1967). The reduction in ACTH secretion was observed after a very marked fall (70 mm Hg systolic and 25 mm Hg diastolic) and prolonged pressor response (approximately 40 minutes or longer in dogs. The duration and magnitude of these pressor effects are much greater than those observed in the present experiments after intraventricular amine administration. Furthermore, at doses which produce pressor effects after injection into the CSF, the responses of the endocrine system were variable.

A slight pressor effect was observed after intraventricular norepinephrine in both normal and reserpine pretreated animals. However, stimulation of ACTH secretion was observed in normal animals and inhibition of secretion was seen in reserpine pretreated rats. The pressor responses produced by low doses of carbachol
(0.25 µg) was not associated with an elevation of ACTH release. In reserpine pretreated animals, this same dose of carbachol caused a marked pressor effect but did not decrease the reserpine induced secretion of ACTH. It did however bring about a significant potentiation. Due to the lack of association between vascular effects and endocrine responses, it appears that these two events are not interdependent.

It is difficult to assess at this time the possible role of the cardiovascular system on ACTH release from these or other experiments. Presently it is thought that the small increments of vascular changes observed after intraventricular administration are at best only partially responsible for the changes observed in ACTH release. The activation of neural receptors within the CNS following monoamine administration intraventricularly is probably the primary cause of stimulation of ACTH release.

The ACTH Response to Monoamines in Normal and Reserpine Pretreated Animals

Following intraventricular administration of varying doses of carbachol, norepinephrine and dopamine, stimulation of ACTH could be elicited. Carbachol produced a characteristic dose-response curve at the concentrations studied. Responses to norepinephrine and dopamine on the other hand demonstrate a threshold phenomenon. Adrenal steroid outflow showed stimulation at both thirty and sixty minutes after administration. At sixty minutes the response to carbachol and norepinephrine were diminished. Dopamine activation at this time was absent. It is difficult to explain the duration of effects seen after norepinephrine and carbachol, in terms of pure receptor mechanisms alone. The maximal stimulation seen at thirty minutes with these amines and the subsequent decline
at later time intervals may represent the turnover of adrenal corticosterone and plasma ACTH (Ganong, 1963; Maickel et al., 1961). The decline in steroid output to high doses of ACTH required approximately one hour, (Ganong, 1963). The time period necessary for labeled corticosterone to decline in the plasma was one hour (Maickel et al., 1961). Thus, in the present experiments, the decrease in adrenal steroid content could be representative of a fall in blood ACTH. Sustained stimulation of ACTH secretion at sixty minutes was not seen except at the highest dose of norepinephrine. A prolonged effect at this time would represent either activation of CRF sites or a possible involvement of other neural pathways stimulating ACTH release. On the other hand, the diminished response could represent the diffusion away from the receptor site or the action of catabolic enzymes involved in neurotransmitter metabolism.

In comparing the durational effects of norepinephrine and dopamine on ACTH secretion, it should be noted that the maximal responses observed after these amines fall off at a different rate. In other words norepinephrine's effect on stimulation, although reduced, are still stimulatory at sixty minutes; whereas, the ACTH response to dopamine has completely subsided. Dopamine could be exerting an initial stimulation followed by inhibition. However, without having made measurements of ACTH directly, it is difficult to assess these monoamine effects.

High amine doses did cause activation of the ACTH response. In general, the concentrations of stimulatory doses were greater than the endogenous monoamine content (Carr and Moore, 1968; Glowinski and Iversen, 1966). Lower amine doses, more within the level of endogenous concentrations, did not cause stimulation. The lack of potency of these doses could be explained by distribution to nonspecific
sites, so that the concentration at the receptor was insufficient to elicit any stimulatory response. However, the effect of small doses of amines might be responsible for another phenomenon, one of inhibition, which would not have been apparent under resting conditions.

In order to study an inhibitory effect on ACTH secretion, it was necessary to bring about an elevated steady state level of ACTH release. Reserpine has been shown to activate CRF release (Bhattacharya and Marks, 1969), with the concomitant depletion of central monoamines serotonin, dopamine and norepinephrine (Carr and Moore, 1968). It appears that supplying the missing inhibitory factor would terminate the reserpine-induced hypersecretion. If inhibition were to occur after the intraventricular injection of any of these amines, it would be necessary to allow sufficient time for pre-existing corticosterone levels to decline. Although decreases in adrenal steroid content and changes in plasma ACTH content have not been investigated, the fall in plasma $^3$H-corticosterone from an elevated state required forty minutes for several half-lives to elapse. Thus a longer time period after injection was required to allow these changes in corticoid production to occur (Maickel et al., 1961).

Carbachol at the doses studied did not reverse the ACTH hypersecretion induced by reserpine. Augmentation of the ACTH response was significant with 0.25 μg carbachol. Both dopamine and norepinephrine inhibited ACTH hypersecretion. This effect was observed at low amine concentrations which did not affect the basal secretion in normal animals. Dopamine reduced ACTH secretion to a lesser extent that equivalent doses of norepinephrine. It should be realized that these minimally effective doses of amines had little effect of ACTH secretion in normal animals. Catecholamines act as inhibitors of
ACTH secretion. Administration of the adrenergic precursor 1-DOPA has recently been shown to decrease the elevated levels of ACTH-induced hypotension after both systemic and intraventricular administration of the amine (Van Loon et al., 1969). Since 1-DOPA crosses the blood-brain barrier, direct central receptor activation is implied in these studies.

The neuronal inhibitory role of catecholamines has been demonstrated in studies on superior cervical ganglia. Following maximal or submaximal pre-ganglionic stimulation, the response of the nictitating membrane was reduced after injection of epinephrine into the arterial supply of the ganglion (Weir and McLennan, 1963). The depressant action of epinephrine was blocked by dibenamine and dibenzyline. Responses observed after injected acetylcholine were reduced in the presence of epinephrine. Similar responses were observed in isolated ganglia (Christ and Nishi, 1969). By recording from intracellular micropipettes inserted in the post-ganglionic cell body, epinephrine produced a rapid blockade of orthodromic action potentials; this blockade could be inhibited by phenoxybenzamine but not by propranolol. These results lend support to the possible inhibitory nature of alpha adrenergic receptor function on neuronal activity.

In summary it appears that carbachol caused activation of ACTH release both in normal and reserpine-pretreated animals, whereas, a dual effect can be attributed to the actions of the catecholamines, that is, both inhibitory (reserpine pretreated animals) as well as stimulatory (normal animals).

Serotonin has been postulated as a final common stimulatory path for CRF release after localized hypothalamic infusion (Nau menko, 1968). The stimulation of corticoid production was observed in guinea pigs bearing midbrain transection. Localized infusion
of either carbachol or norepinephrine were without effect in transected animals. In the present experiments, as well as others, the action of serotonin remains ambiguous. A slight rise in ACTH release was observed following intraventricular administration in normal rats. The stimulation of ACTH following intraventricular administration occurred only at drug concentrations (25 µg) five times greater than the dose (5 µg) utilized by Naumenko (1968). Further, if reserpine caused hypersecretion of ACTH due to its amine depleting effects replacement of the serotonergic component should have resulted in inhibition. However, following 5-HT administration, no inhibition of elevated ACTH occurred in reserpine pretreated animals but a significant augmentation of ACTH release was apparent at the 1 µg dose. Further, selective depletion of serotonin with PCPA or chloroamphetamine (Preziosi et al., 1968; de Schaepdryver et al., 1969; Dixit and Buckley, 1969; Bhattacharya and Marks, 1970) did not cause an elevation of basal secretion nor inhibition of stress-induced ACTH secretion. It is not possible to assess the role of serotonin on ACTH secretion by the methods presently utilized or to discuss species variations as a source of these discrepant observations.

Relationship of the Hypothalamus to Other Brain Regions: Effects of Monoamines in Posterior Deafferented Animals

It would be delightful to postulate that the neuronal mechanisms involved in ACTH release receive their information from receptor sites originating in the hypothalamus after intraventricular monoamine administration. However, the medial basal hypothalamus represents a region to which all neuronal connections which affect CRF secretions from various brain regions eventually send terminals (Fortier, 1966), thereby affecting the secretion of both CRF and ACTH.
No final neuronal system or tract has been anatomically isolated and shown to be exclusively involved in ACTH release. That is, no neural tract leaves the central nervous system and terminates within this anterior pituitary lobe. The position of the hypothalamus in respect to the rest of the CNS represents an anatomical network of interconnecting systems (Nauta, 1963). No sensory afferents arising from the spinal or bulbar lemnisci send fiber tracts directly to the hypothalamus; thus, this region appears to be outside the direct distribution of a secondary sensory pathways. The extrinsic afferent connections to the hypothalamus arise in the brain stem within the mammillary peduncle and the dorsal longitudinal fasciculus (Nauta and Haymaker, 1969). Both of these tracts traverse the ventral tegmental region. The peduncular tract terminates within the medial mammillary body; whereas the fascicular fibers can either end in the posterior hypothalamus or extend to the dorsal and lateral hypothalamic region. Afferent input which connects the hypothalamus with the limbic forebrain structures, are represented by the fornix system, the hippocampal formation, the amgdaloid complex, and the olfactory cortex as well as the gyrus fornicatus and the piriform lobe. The medial forebrain bundle which passes laterally through the hypothalamus is accessible to impulses arising in virtually all limbic structures.

Efferent pathways arising within the nuclei of the hypothalamus, are carried by the medial forebrain bundle rostally to the septal region and caudally to the medial mesencephalic region. The dorsal longitudinal fasciculus contains efferents arising in the ventromedial and dorsomedial nuclei as well as the posterior hypothalamus to the dorsal tegmental nucleus. Thus the hypothalamus appears as a nodal way station of both the descending and ascending limbs of the limbic system-midbrain circuit. It is important to note that the distribution of catecholamines and serotonin within the CNS
has been specifically shown to exist within these structures of the limbic-midbrain system. The histochemical picture demonstrates high concentrations of the monoamines within these areas (Fuxe, 1969). Further, radioautography of whole brain slices following intraventricular $^{14}$C-norepinephrine shows a gross brain distribution which parallels the anatomical and histochemical localization of these neural tracts. Amine concentration is primarily along the midline regions and areas lateral to the ventricular system. These regions represent the structures associated with the limbic system. Also these neural tracts contain the greatest concentrations of catecholamines and serotonin (Reivich and Glowinski, 1967).

Thus following intraventricular monoamine administration receptor activation at these sites could possibly occur. It appeared feasible that posterior deafferentation of the hypothalamus would eliminate peripheral influences arising from the brain stem afferent regions. That is, it might no longer be possible for influences resulting from either the involvement of peripheral amine receptors, or the direct stimulation of monoamine receptors within the medullary and pontine regions to exert their effects, if the medial forebrain bundle and other ascending tracts could be cut.

Posterior deafferentation did not result in an elevation of basal ACTH secretions. This is in agreement with the findings of Halasz et al. (1967). Thus tonic inhibition of ACTH secretion was still present, originating from structures rostral to the lesion. Activation of ACTH release did occur following injection of both norepinephrine and carbachol. There was slight potentiation of the response observed after norepinephrine administration, which could represent a type of denervation supersensitivity. Comparing these
observations with studies of peripheral sympathomimetic responses, chronic ganglionic denervation results in a pronounced increase in the adrenergic receptor activity following exogenous norepinephrine (Trendelenburg, 1963). The supersensitivity observed in peripheral adrenergic neurons after denervation has been correlated to a decrease in the reuptake mechanisms of these terminals.

Denervation of the iris does demonstrate a loss of amine fluorescence following bilateral cervical sympathectomy as well as a decreased accumulation of exogenous amine (Malmfors and Sachs, 1965). Within twenty-eight hours after denervation, fluorescence disappeared from the main post-ganglionic fibers, as well as the terminal and pre-terminal axons. Accumulation of exogenous norepinephrine in animals treated with nialamide could not be obtained after denervation times of 24 hours or longer. Thus it appears that, following denervation, adrenergic neurons might be characterized by a decrease in content and synthesis as well as a loss of neuronal re-uptake. Evidence for a similar mechanism within the CNS is only suggested by these experiments. Although it has been demonstrated that there is a decrease in the synthetic ability of both adrenergic and serotonergic neurons following lesions placed within the medial forebrain bundle (Heller and Moore, 1968), any changes existing in the re-uptake mechanism after interruption of this neural tract have not been evaluated.

However, the potentiation of the ACTH response observed after norepinephrine in these experiments could be attributed to an increased concentration at adrenergic receptors due to a decreased re-uptake of the exogenous amine.

Although the activity of carbachol is decreased at the lower doses, it can still elicit an equivalent stimulation at higher dose levels (5 μg). The stimulatory responses are similar to those obtained in intact animals. The diminished responses observed in
deafferented animals might indicate that at least part of carbachol's stimulation is exerted posterior to the lesion. However, other neural factors due to transection might account for the reduction in carbachol's effect. In the peripheral nervous system sectioning of the cholinergic supply to the superior cervical ganglion results in a loss of acetylcholine content (MacIntosh, 1938). Conduction through the ganglia in response to electrical stimulation is absent within 72 hours after decentralization. This is due to a greatly decreased release of acetylcholine from the denervated pre-ganglionic neuron. Carbachol has been proposed to act in part by release of the cholinergic transmitter in the sympathetic ganglia (Volle and Koelle, 1961). Thus, following lesioning, any loss of endogenous amines within the central nervous system could reduce the responsiveness of the endocrine apparatus to carbachol's stimulation.

Removal of part of the peripheral neural input by posterior deafferentation did not bring about an elevation of the basal state. Therefore the inhibitory mechanisms involved in tonic maintenance of basal ACTH were still operative. Anterior deafferentation as well as a complete hypothalamic island preparation does result in prolonged hypersecretion of ACTH (Halasz et al., 1967), suggesting that inhibitory pathways originate in higher cortical centers or structures anterior to the lesion and send fiber tracts into the anterior hypothalamic area controlling ACTH secretion. It was of interest to determine if reserpine would affect the neurochemical nature of these inhibitory structures by mechanisms similar to those observed in animals with an intact neural supply. That is, are the inhibitory tracts involved in maintainence of basal ACTH secretion either serotonin or catecholamine-containing systems which are depleted by reserpine? Following reserpine, posterior deafferented
animals demonstrated a moderate elevation of ACTH secretion. Assuming that there is depletion of endogenous monoamines from adrenergic and serotonergic neurones rostral to the lesion, these experiments indicate that removal of inhibitory neurotransmitter influences possibly from the arcuate nucleus within the hypothalamus or the neural stores within the medial forebrain bundle, contributed to this elevated secretion of ACTH seen after reserpine. The arcuate nucleus is not transected by this surgical procedure and thus would be intact; whereas the medial forebrain bundle is only partially interrupted by posterior deafferentation at this level (de Groot Atlas) (figure 22). Although it is difficult to attribute the ACTH responses seen after reserpine depletion to one specific anatomical site, the partial elevation observed after reserpine in lesioned animals is subject to several possible interpretations. The lesion may transect neural tracts originating posterior to the lesion which carry inhibitory information, but are not in themselves depleted by reserpine. Or deafferentation could transect only part of the neural inhibitory tracts which are depleted by reserpine. Thus the lesion may transect inhibitory information which originates posterior to the lesion or else effect the ability of the lesioned area to response to reserpine. Since the deafferented animals were observed at only a single time period and at a single dose of reserpine, it is possible that depletion occurs at a slower rate and requires a dosage level quite different than the one used in intact animals.

At this point it is important to discuss the population of neurons present within the area anterior to the lesion. It can be assumed that any cholinergic or adrenergic nerve terminals which have been transected have lost their amine content (MacIntosh, 1938; Malmfors and Sacha, 1965; Heller and Moore, 1967). The
neurones which are depleted by reserpine or acted on by exogenous amines can therefore be considered to be decentralized, that is, without presynaptic control.

Drawing again from our knowledge of the peripheral nervous system and making certain analogous assumptions regarding neural function, it seems reasonable to assume that neurotransmitter activity is affected after either surgical or chemical removal of presynaptic effects. Decentralization of superior cervical ganglia results in a moderate increase in the sensitivity of the nictitating membrane to exogenously administered norepinephrine; but this increase is not as great as the supersensitivity observed after denervation (Trendelenburg, 1963). Thus decentralization does result in a decrease in the functional state of the post-ganglionic neuron, possibly due to a decreased re-uptake phenomenon. Endogenous amine loss, which characterized the denervated neural unit is not observed in the decentralized ganglia (Kirpekar et al., 1962). Further spontaneous release of $^3$H-norepinephrine is slowed following either surgical or "chemical" decentralization (Hertting et al., 1962). Chemical decentralization obtained with the use of long lasting ganglionic blocking drugs, pempidine and chloroisodamine, gave effects similar to those observed after surgical decentralization. It therefore appears that the spontaneous amine release as well as the endogenous amine content is affected by the quantity of transmitted synaptic information.

In studying the rate of synthesis in normal as well as decentralized submaxillary glands of the rat, the conversion of $^{14}$C-tyrosine to labelled norepinephrine was not totally impaired but was reduced to 50% when compared with intact controls (Sedval, 1968). No reduction of tyrosine hydroxylase activity was observed in the adrenal medulla following sectioning of the splanchnic nerve supply.
(Thoenen et al., 1969). Three days after surgery both the endogenous catecholamine content and the level of tyrosine hydroxylase was the same as intact controls. Although there is some functional reduction in the decentralized neurons, this has not been correlated to a change in the synthetic mechanisms of the neuron. However, measurements of tyrosine uptake have not been made which could explain a limitation of synthetic rate due to a decreased availability of substrate. Although the re-uptake of norepinephrine has not been studied in decentralized neurons, the uptake of indirect acting amines amphetamine, ephedrine and meta-tyrosine did not appear to be altered as indicated by the response of the nictitating membrane (Trendelenburg et al., 1963). Thus, it can be concluded that amine release and synthesis are decreased but there is no loss of tyrosine hydroxylase activity nor impairment of the re-uptake mechanisms.

The action of reserpine on decentralized neurons has only been studied in a limited number of cases. A slight reduction in the depleting activity along with a decreased rate of recovery in amine content following reserpine was observed in the superior cervical ganglion (Kirpekar et al., 1962). In intact adrenal medulla, reserpine activates tyrosine hydroxylase activity along with a concomitant depletion of catecholamines. Following splanchnic nerve section, this elevation of the hydroxylase enzyme does not occur after reserpine (Thoenen et al., 1969). In the innervated gland, reserpine reduced catecholamine content by 85%, whereas, following splanchnic nerve sectioning, reserpine caused only a 50% reduction in amine content. This possibly implies that the action of reserpine is dependent on the neural input. Thus decentralization might limit either the uptake of reserpine into neurons or possibly change the responsiveness of the catecholamine stores to the depleting action of this agent due to a decreased rate of neuronal discharge.
Applying these limited observations to the CNS, it appears that the action of reserpine might not occur to the same degree in the deafferented preparation. Also the rate of amine depletion could be reduced. Since a moderate increase in ACTH secretion occurs, this implies that removal of adrenergic or serotonergic inhibitory influences by reserpine has not been totally impaired. Further the inhibitory influences which arise anterior to this lesion might well be represented by these neurotransmitter substances. Neither carbachol nor norepinephrine augmented or depressed the ACTH secretion induced by reserpine in deafferented animals. Low doses (0.25 μg) of carbachol which produced a potentiation of reserpine's effect in intact animals did not increase the response in lesioned animals. The higher dose (1.0 μg) was similar in effect to the lower carbachol concentration. Carbachol did not exhibit any inhibitory effects under the conditions studied in these experiments.

Catecholamine inhibition of ACTH hypersecretion induced by reserpine did not occur following deafferentation. This dose of norepinephrine did show effective inhibition of the reserpine effect in intact animals. The absence of catecholamine inhibition in these animals could be interpreted to originate posterior to the lesioned area. It is difficult to interpret these results in this manner only since reserpine did cause a moderate elevation in adrenal response. In the light of these observations the only feasible approach is to question primarily the reserpine effect. It is possible that since maximal adrenal activation did not occur in the lesioned animals after reserpine it would not be possible to see the inhibitory influence after catecholamine administration. The adrenal corticoid level in lesioned animals is the same as the response observed following either doses of norepinephrine.
This threshold for inhibition may be related to the dual action of norepinephrine. However, it would be necessary to test this hypothesis with reserpine and norepinephrine in posterior de-afferented animals at other doses and time periods.
SUMMARY AND CONCLUSIONS

In general it appears that the cholinergic responses are stimulatory. This is supported by the observation made in intact and lesioned animals as well as in reserpine pretreated groups. This concept is supported by the experiments investigating atropine implants in various hypothalamic regions (Smelik and Hedge, 1968). Animals bearing crystalline atropine implanted in the anterior hypothalamus had reduced steroid levels after ether stress as well as after lysine vasopressin. Carbachol was stimulatory in intact guinea pigs after localized injection into specific hypothalamic regions, namely the mammillary area and ventral tegmentum (Naumenko, 1968). However, carbachol has not always exhibited a stimulatory response. Inhibition was observed after carbachol administration into areas anterior to the hypothalamus, such as Broca's diagonal band and the pre-optic area (Endroczi et al., 1963). Stimulation occurred with both carbachol and norepinephrine in the mammillary body, posterior hypothalamus and ventral tegmental area. In the region of greatest inhibition by carbachol no other amine was tested. If one accepts the Burn and Rand hypothesis (Koelle, 1965; Burn and Rand, 1963) that cholinergic agonists may release norepinephrine from adrenergic nerves, it is possible to interpret these results in another light. Both carbachol and norepinephrine cause stimulation in the same areas, it is possible that this is due to cholinergic affects on adrenergic terminals. However the areas of greatest cholinergic inhibition were not tested.
with norepinephrine; thus the inhibitory aspects cannot be evaluated in these experiments but can be questioned on the basis of the Burn and Rand theory.

In attempting to localize the origin of cholinergic stimulation, carbachol's effects were observed after lesioning. The effects of carbachol were still stimulatory although reduced at the lower dose levels. Midbrain transection however abolished the stimulation to carbachol in guinea pigs (Naumenko, 1968). It is difficult to explain these results in the light of any neuronal mechanisms which are presently known. However, it is possible that carbachol administered intraventricularly distributes to other regional sites thereby causing stimulation of an afferent supply from areas anterior to the lesion. The nature of the stimulation in these experiments may not be due only to cholinergic activation but also to release of other transmitter substances. It does appear from the observations in midbrain transected animals that cholinergic stimuli arising from the afferent hypothalamic supply, that is, within the posterior region, do not originate in this region, although both species differences and the technical methodology have to be explored.

Catecholamines have been shown to be stimulatory in the intact as well as the lesioned preparation. These results are supported by the localized infusion of adrenergic substances in specific brain regions (Endroczi et al., 1963; Naumenko, 1968) in intact animals. However, stimulation by norepinephrine was abolished after midbrain transection (Naumenko, 1968). Again the stimulatory areas were limited to the posterior regions of the hypothalamus. It is possible that in the posterior deafferented animals stimulation arose from either lateral or anterior regions of the hypothalamus after intraventricular administration.
It must be noted that Naumenko observed stimulation with serotonin in both normal as well as midbrain transected animals and describes this transmitter as the final common path for ACTH release. However, serotonin in the present experiments did not cause a maximal stimulation of ACTH response in intact animals. Potentiation, however was observed after reserpine pretreatment. Although these observations might suggest stimulation, it would be necessary to assess the role of serotonin in lesioned, i.e., posterior deafferented animals, which were not done in the present experiment. Catecholamines have further been shown to demonstrate inhibition in reserpine pretreated animals. 1-DOPA has also been shown to exert an inhibitory effect on ACTH release induced by hypotension (Van Loon et al., 1969).

Reserpine implanted into the medial basal hypothalamus, specifically the median eminence were shown not to block the stimulatory responses of ACTH elicited by various stress stimuli, nor was an elevation of basal secretion noted (Smelik, 1967). From these implant experiments, catecholamines and possibly serotonin are not responsible for transmission of neural information evoked by the stress responses nor do they function as inhibitors which maintain tonic inhibition of basal ACTH release. By histochemical analysis, the neurons within the median eminence have been shown to be depleted by the reserpine implants; these same neural tracts are primarily dopaminergic (Fuxe et al., 1968a, 1968b). Furthermore they do not represent the entire adrenergic population of the hypothalamus (Fuxe et al., 1968a). Certainly, the adrenergic neural tracts of the medial forebrain bundle are accessible to catecholamines after intraventricular administration and could operate in the regulation of CRF.
It is not possible at this time to state the exact mechanisms by which the brain monoamines exert their influence on CRF and ACTH. However from these experiments it appears feasible to derive the following conclusions as illustrated in the drawing (figure 23). Carbachol under the conditions studied appears to possess a stimulatory effect on CRF and ACTH release. In intact, reserpine pretreated and lesioned animals the principal effect was one of stimulation. The monoamines dopamine, and especially, norepinephrine show dual actions producing either stimulation or inhibition of CRF secretion, depending on the state of the system, the dose, and the time.

These effects may not all be related to central neural receptor activation. These effects may not involve exclusively the neural network within the hypothalamic area. It may be possible, however, to propose a simple functional model of monoamine regulation of CRF secretion. The primary stimulatory path for CRF release is postulated to be a cholinergic system. A cholinergic muscarinic receptor mechanism is supported by the atropine implant experiments (Smelik and Hedge, 1968). Thus, it must be presumed that this mechanism stimulates release of CRF from the terminals of the ventral hypothalamic neurons at the neurovascular junction indicated in the illustration.

Stimulation observed after norepinephrine could be obtained in intact as well as lesioned preparations. The potentiation observed in posterior deafferented animals could represent a type of denervation supersensitivity. Due to the degeneration of adrenergic fibers arising from the medial forebrain bundle, and thereby decreasing the available sites for reuptake of norepinephrine, there may be a change in distribution of the injected monoamine increasing the effective concentration available at the receptor site.
The administration of catecholamines to reserpine-pretreated rats demonstrates marked inhibition to CRF secretion. Although the neural mechanism by which these neurotransmitters operate is not known, evidence for inhibitory actions of catecholamines on central neurons is given from microelectrophoresis data (Ruf and Steiner, 1967; Steiner et al., 1968; 1969). Inhibition of neuronal firing by dexamethasone was localized to neurons within the hypothalamus as well as the mesencephalon. Acetylcholine activated about half of these steroid sensitive neurons. In contrast, norepinephrine as well as dopamine caused primarily inhibition. Iontophoresis of ACTH however resulted in stimulation of the dexamethasone sensitive neurons. Thus it appears that a relationship exists between monoamines, steroid and ACTH sensitive neurons. Yet, the question should be asked as to whether these neurons represent the final common path for CRF release. Further, if the action of norepinephrine on neuronal activity is one of inhibition, the resulting stimulation could be explained on the basis of dual inhibition. Thus inhibition of an inhibitory neuron would result in facilitation.

However, it is not at present possible to draw a model that would demonstrate the functional states that have been observed at the isolated neuronal level and correlate these changes with the function of the physiological system. In all studies, attempting to integrate neural factors with the endocrine system, the major problem is limiting the origin of the stimulus, first to the central nervous system and secondly to specific brain areas. It therefore appears feasible to study in the future the interrelationships of neural systems with endocrine function in an isolated state. It would be necessary to show:

1. that electrical and chemical stimulation caused release of CRF.
2. that a neurotransmitter function is in a manner similar to electrical and chemical stimulation.

3. that neurotransmitter action can be blocked by pharmacological antagonists.

4. that the final pathway, if cholinergic, would be blocked by atropine and that stimulation arising from either electrical or chemical activation could be decreased by this blockade.

Figure 23

Hypothetical Model for Central Monoamine Regulation of ACTH Release
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