BRAIN CATECHOLAMINES AND THE PITUITARY-ADRENAL RESPONSE TO STRESS

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ABSTRACT

BRAIN CATECHOLAMINES AND THE
PITUITARY-ADRENAL RESPONSE TO STRESS

By Laurence A. Carr

The purpose of this investigation was to determine if norepinephrine and dopamine, the catecholamines present in the brain, play a role in the response of the pituitary-adrenal system to a stressful stimulus. Neurons containing norepinephrine and dopamine have been shown to converge in the hypothalamus. These neurons may be involved with the perception of stressful stimuli or with the secretion of corticotropin releasing factors.

The potential roles of these amines were studied with the use of two pharmacological tools, reserpine and α-methyltyrosine. When reserpine, which disrupts catecholamine storage granules, and α-methyltyrosine, which inhibits catecholamine synthesis, are administered sequentially, they deplete the brain of norepinephrine and dopamine. Male rats pretreated with these drugs were subjected to a variety of stressful procedures which normally stimulate the pituitary-adrenal system. The ability of this system to respond to the various stimuli was determined by measuring plasma corticosterone levels.

A single injection of reserpine depleted brain catecholamines within 3 hours and the levels remained low for up to 7 days.

Except for a transient increase at 3 hours after its administration, reserpine had no effect on plasma levels of corticosterone. α-Methyltyrosine caused a moderate reduction in the brain content of catecholamines for up to 8 hours but had no effect upon plasma corticosterone levels.

When administered to nonstressed rats, reserpine (for 24 hours) and α-methyltyrosine (for 4 hours) markedly reduced the brain content of catecholamines but did not alter the plasma levels of corticosterone. At this time, vehicle (control) and drug-pretreated animals were subjected to a variety of stressful procedures (administration of ether, histamine or formalin; restraint or altered environment). Formalin and restraint stresses significantly reduced the brain content of norepinephrine; the dopamine content was not altered by any stress. Neither reserpine nor the stressful procedures altered the plasma levels of α-methyltyrosine. All stresses increased plasma corticosterone levels; there was no difference between control and drug-pretreated animals.

The concentration or corticosterone increased significantly in the plasma of animals which were placed in motor activity cages. Reserpine and α-methyltyrosine pretreatment (alone and in combination) depressed motor activity concurrently with the

depletion of brain catecholamines; plasma corticosterone levels were unaffected by the drug pretreatments.

Pretreatment with centrally acting cholinergic blocking drugs, in combination with reserpine and α-methyltyrosine, did not prevent the rise in plasma corticosterone in response to the altered environment stress.

Since the normal functioning of the pituitary-adrenal system was not impaired by depleting brain stores of norepinephrine and dopamine, it was concluded that these amines do not play an essential role in the activation of the pituitary-adrenal system by stress.

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By

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INTRODUCTION

It has long been known that vertebrates and many lower forms of animal life possess two organ systems specializing in communication and the integration of all body processes. These two regulatory systems, the nervous and endocrine systems, were once thought to be discrete entities. However, in the late 1940's it was suggested that these systems might be linked together functionally and anatomically and that environmental stimuli exert their effects on endocrine glands via the central nervous system (Green and Harris, 1949).

Several lines of evidence indicate that the central nervous system exerts a controlling influence over the anterior lobe of the pituitary. Anterior pituitary function is disrupted when the pituitary stalk is sectioned and the portal vessels are not permitted to regenerate (Jacobsohn, 1954; Fortier, et al., 1957) or when the pituitary gland is transplanted to ectopic sites (Kendall et al., 1966). These experiments suggest that the brain controls endocrine functions by transmitting signals through neurons converging in the hypothalamus (Mangili et al., 1966) and by translating these signals into chemical factors which are carried by the hypothalamic-hypophyseal portal system to the pituitary (Duffy and Menefee, 1965).

One of the functions of the anterior pituitary is the secretion of adrenocorticotropic hormone (ACTH) which regulates the rates of synthesis and release of glucocorticoids in the adrenal cortex.

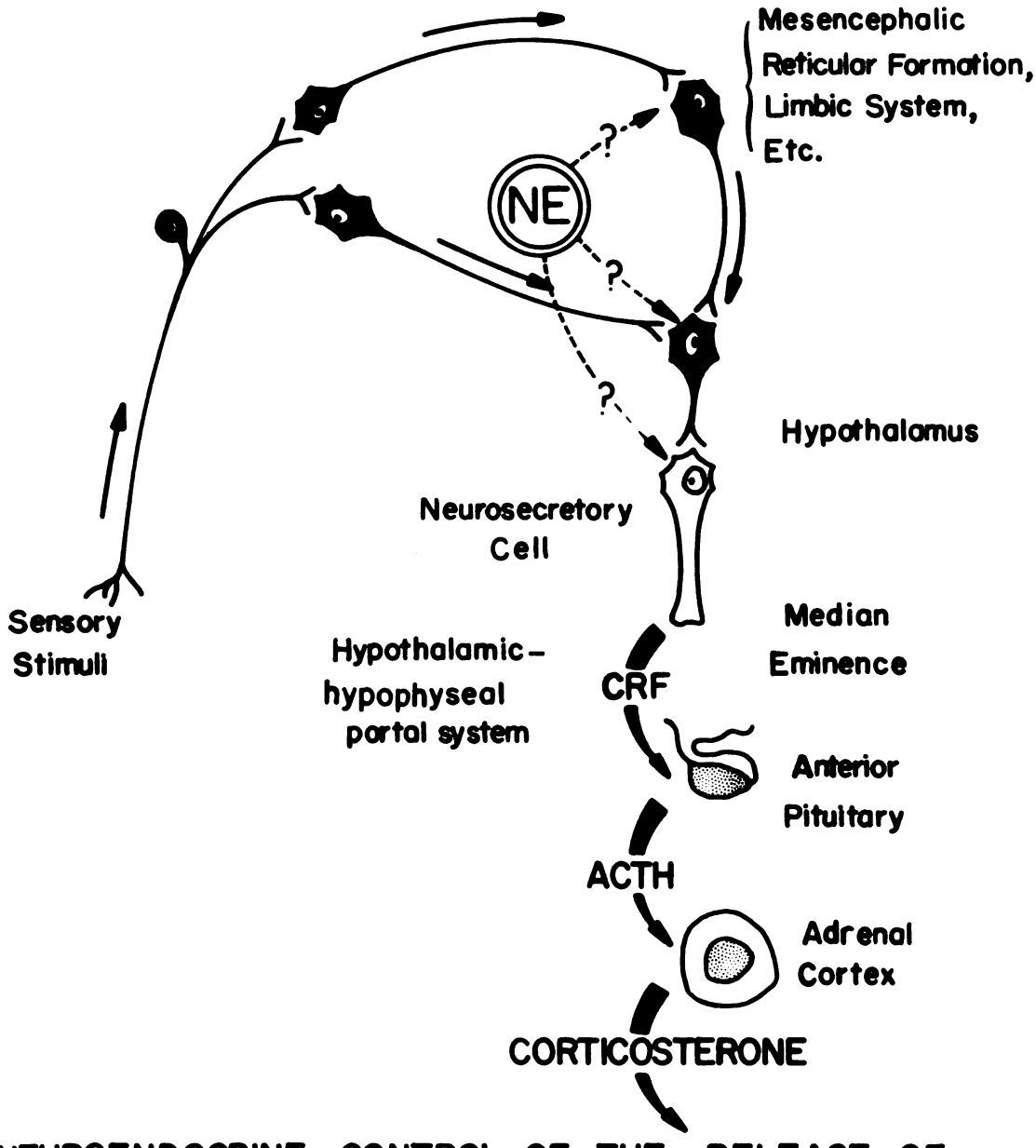
Since these two glands function as a unit in response to stressful stimuli, they are referred to as the pituitary-adrenal system. Laqueur et al. (1955) and Brodish (1963) have demonstrated by means of lesions placed in various areas of the hypothalamus that the central nervous system triggers the release of ACTH from the anterior pituitary. However, these studies indicate that it is very difficult to restrict hypothalamic control of ACTH secretion to a specific, circumscribed locus within the brain. Rather, this regulatory function is diffusely interrelated with all the other endocrine control systems in the central nervous system (Knigge, 1967). Although a large number of stimulatory and inhibitory neural circuits enter the hypothalamus to ultimately influence ACTH secretion, electrical stimulation studies have revealed that these neurons converge in the median eminence to form a final common pathway to the hypothalamic-hypophyseal portal system (Anand and Dua, 1955; D'Angelo, et al., 1964).

A number of chemicals have been postulated to be released into the portal system from the hypothalamus which cause the secretion of ACTH. Since the exact chemical structure of most of these substances is unknown, they are generally referred to as corticotropin releasing factors (CRFs).

Antidiuretic hormone (ADH) has been implicated as the chemical involved because of the close association between the antidiuretic and adrenocorticotropic responses to noxious stimuli. In addition, the intracarotid injection of synthetic ADH elevates blood corticosterone levels (Gavazzi, et al., 1964). However, ADH is presently considered to be an unlikely mediator of ACTH release. The release of ACTH by ADH may be explained by its similarity in chemical structure to another CRF, by a nonspecific permeability action or by portal vascular constriction (Schally et al., 1964). It may also release ACTH in response to specific stimuli whose afferent route involves the supraoptic-paraventricular system (Knigge, 1967).

Recent studies have indicated that the CRFs are probably polypeptides which have virtually no pressor activity but do possess corticotropin releasing activity considerably greater than that of ADH (Schally et al., 1964). The most active material purified from hypothalamic extracts appears to be different from ADH, oxytocin, ACTH, histamine, acetylcholine, norepinephrine, epinephrine and serotonin (Guillemin et al., 1957; Porter, et al., 1967). Significant amounts of this substance have been observed in the peripheral blood of animals under conditions of physiological stress (Anderson, 1966).

The present concept of the control mechanisms for the release of adrenal glucocorticoids is outlined in Figure 1.



NEUROENDOCRINE CONTROL OF THE RELEASE OF CORTICOSTERONE FROM THE ADRENAL CORTEX

Figure 1.

Environmental stimuli influence the peripheral and central nervous systems, giving rise to neural signals which ultimately converge on the neurosecretory cell in the median eminence of the hypothalamus. This cell secretes a CRF which enters the hypothalamic-hypophyseal portal system and finally acts on the anterior lobe of the pituitary to release ACTH. ACTH enters the peripheral circulation and, when it reaches the adrenal gland, induces the synthesis and release of glucocorticoids from the adrenal cortex.

Since norepinephrine and dopamine were initially identified in the brain (Vogt, 1954; Carlsson, 1959), their specific locations and functions have been actively investigated. It has been suggested (Carlsson, 1964) that these catecholamines may function as transmitter substances in certain neuronal pathways within the central nervous system.. That is, upon appropriate stimulation, norepinephrine and/or dopamine may be released from nerve terminals to influence the activity of adjacent neurons. This investigation deals with the role of catecholamines as possible chemical mediators in neurons which converge on the hypothalamus to influence the pituitary-adrenal system. Chemical analysis of gross anatomical areas of the central nervous system indicates that norepinephrine is most highly concentrated in the hypothalamus (Carlsson, 1959). The recent development of fluorescent histochemical techniques

(Dahlstrom and Fuxe, 1964) have permitted a more discrete localization of norepinephrine and dopamine within the hypothalamus. Catecholamine-containing nerve terminals have been detected in the dorsomedial hypothalamic nucleus, the periventricular nucleus, in the region between the ventromedial nucleus and the lateral hypothalamic area, in the supraoptic and paraventricular nuclei and in the external layer of the median eminence (Carlsson et al., 1962; Fuxe, 1965). These are the same areas which were found by electrical stimulation and lesion studies to influence the functional activity of the anterior pituitary.

Other studies lend support to a role for catecholamines in brain-pituitary regulating mechanisms. For example, some of the neurons which terminate in the median eminence are rich in monoamines (Falck et al., 1962). These neurons have been postulated to play an essential role in the control of adenohypophyseal functions by monitoring the discharge of hypothalamic releasing factors (Scharrer, 1967). Other synapses where norepinephrine or dopamine may act as transmitter substances in this scheme are indicated in Figure 1. Thus, catecholamine-containing neurons in the hypothalamus, brainstem reticular formation and other areas may be involved in the perception of stressful stimuli and/or may function in the activation of corticotropin releasing factors.(Saffran and Schally, 1955; Long, 1956; Schally et al., 1964).

If, indeed, norepinephrine or dopamine play any role in these functions, then depleting these amines from the central nervous system should impair the functional activities in which they participate. The primary aim of this study was to deplete brain catecholamines with the use of pharmacological techniques and observe the resulting changes, if any, in pituitary-adrenal function following stress. The use of drugs to study this possible role of brain catecholamines is necessary because of anatomical complexities and the poor accessibility of exogenous catecholamines to the brain (Samorajski and Marks, 1962).

Reserpine has been used to deplete the brain of norepinephrine and dopamine because of its effect of disrupting catecholamine storage granules in adrenergic nerve terminals (Glowinski, et al., 1966). However, because of its many and varied actions, both in the central nervous system and peripheral tissues, one cannot attribute the effects of this drug solely to depletion of catecholamines (Zaimis, 1961).

There have been conflicting reports on the action of reserpine on the pituitary-adrenal axis. For example, reserpine has been observed to block the decrease in adrenal ascorbic acid following a stress (Mahfouz and Ezz, 1958; Kitay, et al., 1959). The mechanism of this blockade has not been clearly established. It has been suggested that reserpine initially decreases the content of ACTH in the pituitary to such an extent

that the animals cannot respond to an additional pituitary stimulus (Maickel et al., 1961). On the other hand, reserpine stimulates the acute release of ACTH (Maickel et al., 1961). This stimulation has been attributed to depression of inhibitory pathways in the hypothalamus which control the secretion of a humoral factor influencing ACTH discharge (Maickel et al., 1961) or to a nonspecific stress reaction (Halkerston et al., 1964; Rosecrans and DeFeo, 1965). In spite of this stimulatory effect, reserpine was employed in this study because of its ability to deplete tissues of catecholamines. However, efforts to correlate reserpine's action with a depletion of brain catecholamines are complicated by the fact that the drug does not block the biosynthesis of these amines (Glowinski, et al., 1966).

Most of the norepinephrine in neurons is bound within vesicles which are distributed along the axon and at nerve endings (Kopin, 1966). Reserpine causes these vesicles to release norepinephrine intracellularly where most of it is destroyed by monoamine oxidase. While reserpine can deplete most of the norepinephrine in tissues, there appear to be two small sites which are resistant to the action of this drug. One is an extraneuronal pool (Fischer et al., 1965) and the other is an intracellular pool from which nerve stimulation can release norepinephrine. This latter pool, which represents a "functionally active store," is maintained by the reserpine-

sensitive reserve stores and by synthesis. Therefore, following a single dose of reserpine, this small functional store of norepinephrine and/or dopamine may maintain the functions of neurons containing this mediator. In support of this view, it has been observed that following the administration of reserpine the rate of synthesis of catecholamines increases (Sedvall and Kopin, 1967) and the animals recover from the behavioral depression long before the brain levels of norepinephrine and/or dopamine are restored (Bertler, 1961; Pirch et al., 1966). Thus, depleting the major store of catecholamines from neurons with reserpine does not necessarily disrupt adrenergic transmission since the functionally active pool can be maintained by newly synthesized amines.

α-Methyltyrosine can reduce tissue concentrations of catecholamines by blocking their synthesis. It does this by inhibiting tyrosine hydroxylase (Nagatsu et al., 1964). This enzyme controls the rate-limiting step in the synthesis of catecholamines (Levitt et al., 1965). Because of the large reserve store of catecholamines in vesicles, there is a prolonged latent period before α-methyltyrosine can deplete tissues of norepinephrine or dopamine. However, when α-methyltyrosine is administered to animals which have been pretreated with reserpine, a prompt disruption of noradrenergic or dopaminergic neurons is obtained.

These concepts may best be summarized by means of the schematic diagram depicted in Figure 2. Newly synthesized catecholamine enters the small functional pool of mediator. Norepinephrine or dopamine can exchange between this small pool and a larger reserve pool in which the catecholamine is bound more tightly. Norepinephrine which is released from the functional pool to excite the postsynaptic receptor is replenished in large measure by reuptake in the nerve terminal. The balance is supplied by synthesis. Reserpine impairs the ability of binding sites in the reserve pool to hold the amines and they are released into the neuron. There they are destroyed by monoamine oxidase and lost from the neuron as deaminated products. α-Methyltyrosine inhibits tyrosine hydroxylase and thus stops the supply of newly synthesized mediator. When the two drugs are administered sequentially, both sources of amine are eliminated and functional activity of the neuron should be disrupted.

The role of brain catecholamines in regulating the pituitary-adrenal system was examined in the light of these recent discoveries on the dynamic aspects of noradrenergic and dopaminergic neurons. Changes in the function of the pituitary-adrenal response to stress were examined after the disruption of noradrenergic and dopaminergic nerve function by the use of reserpine and α-methyltyrosine.

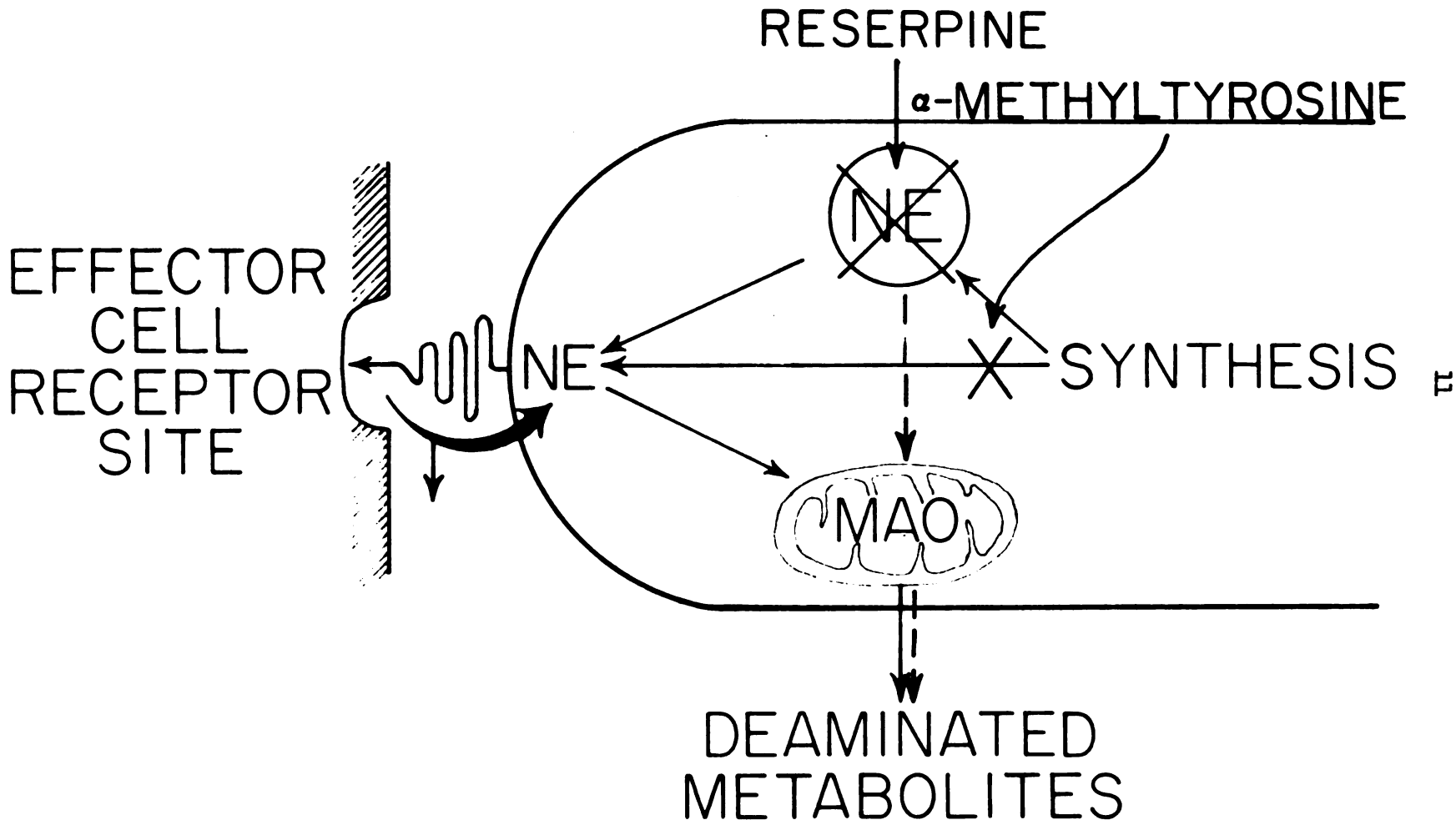


Figure 2. Diagramatic representation of a noradrenergic or dopaminergic nerve terminal showing locus of action of α-methyltyrosine and reserpine.

MATERIALS AND METHODS

A. Materials

1. Animals

Male albino rats (CD_1, Charles River Breeding Labs) weighing 200-250 g were used throughout the investigation. They were housed in groups of 4 or 5 in 18" x 10" x 5" opaque plastic cages with wire mesh tops and they received water and food (Wayne Lab Blox) ad libitum. The animal room temperature was maintained at 70-74°F. The lighting was automatically controlled so that the room was illuminated from 7 a.m. to 7 p.m. To minimize variation due to circadian rhythmicity, all animals were sacrificed between 10 a.m. and 1 p.m. All animals were conditioned to the animal room for at least 5 days prior to their use.

2. Drugs

All drugs were administered by the intraperitoneal route. The reserpine stock solution (5 mg/ml) was made by dissolving crystalline reserpine (S.B. Penick & Co.) in an aqueous vehicle composed of 4.5% acetic acid and 10% polyethylene glycol 400. This solution was diluted 1:10 with distilled water for injection (2 mg/kg).

l-α-Methyltyrosine was administered as an aqueous suspension (25 mg/ml).

Animals serving as controls for the reserpine treatment received the reserpine vehicle, those for the α-methyltyrosine treatment received saline.

The animals were divided into the following groups for drug pretreatments:

1. Vehicle + saline (control)
2. Vehicle + α-methyltyrosine (α-MT)
3. Reserpine + saline (reserpine)
4. Reserpine + α-methyltyrosine (reserpine + α-MT)

B. Methods

Brain catecholamines and plasma corticosterone were measured in the same animals in order to correlate amine concentration with steroid levels. In addition, plasma levels of α-methyltyrosine were measured in those animals which received this drug.

At appropriate times after various treatments, rats were sacrificed by decapitation within 3-7 seconds after removal from cages. Blood was collected from the trunks in beakers containing heparin. Plasma was separated by centrifugation and stored at -20°C until assayed.

1. Catecholamine assays

Brain norepinephrine and dopamine were determined by modification of methods reported by Moore and Rech (1967).

Whole brains were quickly removed from the skull, weighed and homogenized in 6 ml of cold 0.4 N perchloric acid. The homogenate was allowed to stand for 30 minutes and then centrifuged at 10,000 x g for 5 minutes. The supernatant was collected, the pellet rehomogenized in 6 ml of 0.4 N perchloric acid and the resulting homogenate centrifuged as before. The supernatants were combined and adjusted to pH 4 with 10 N potassium hydroxide. The resulting precipitate of potassium perchlorate was separated from the clear supernatant by centrifugation. All extraction steps were carried out at 0-4°C.

The tissue extracts were transferred to 50 ml capacity glass stoppered centifuge tubes containing approximately 400 mg of aluminum oxide (Woelm) and 0.5 ml of 0.2 M disodium ethylenediaminetetraacetate. The alumina was prepared by washing repeatedly with 2N hydrochloric acid and then with distilled water to remove the finer particles. The alumina-tissue extract mixture was adjusted to pH 8.6-8.7 with 5 M and 0.2 M potassium carbonate and the tubes were shaken for 5 minutes. The supernatant was removed by aspiration and the alumina washed first with 5 ml of 0.2 M sodium acetate and then with 10 ml of distilled water. The amines were eluted from the alumina with 8 ml of 0.2 N acetic acid. Five minutes of shaking was used in all wash steps and 10 minutes in the elution step. Seven

to nine tubes, one of which contained catecholamine standards (0.8 μg norepinephrine and 2.0 μg dopamine), were shaken concurrently. The eluate from each tube was divided into 4 ml aliquots, one of which was used for the norepinephrine assay and the other for the dopamine assay.

Norepinephrine was determined in the following manner. To each 4 ml sample 0.8 ml of 0.1 M phosphate buffer (pH 6.5) was added and the pH adjusted to 6.5-6.8 with 5 M potassium carbonate. Each sample was then divided into two 2.4 ml aliquots. To one aliquot, in which norepinephrine was determined, 0.05 ml of 0.25% potassium ferricyanide was added. The other aliquot served as the blank. At 2 minutes after adding the ferricyanide, 0.25 ml of freshly prepared alkaline ascorbate was added (1 ml of 2% ascorbic acid + 9 ml of 5 N sodium hydroxide). Alkaline ascorbate was also added to the blank. Fluorescence was determined 5 minutes later in an Aminco-Bowman spectrophotofluorometer at activation-fluorescence wave lengths of 391-510 mμ (uncorrected). Recovery of norepinephrine standards was 84 ± 1% (mean ± S.E.) and the norepinephrine content of brain was calculated from these standards.

Dopamine was determined in the following manner. To each 4 ml sample 0.2 ml of 95% ethanol and 2.0 ml of 0.5 M

phosphate buffer (pH 7.0) were added. Each sample was then divided into two 3.1 ml aliquots. To one aliquot, in which dopamine was determined, 0.2 ml of freshly prepared 0.5% solution of sodium periodate was added. The other aliquot served as the blank. At 1 minute after adding the periodate 1.0 ml of alkaline sulfite (2.65 g of sodium sulfite + 10 ml of water + 90 ml of 5 N sodium hydroxide), 2.8 ml of distilled water, 1.0 ml of 0.5 M citrate buffer (pH 4.0) and 1.7 ml of 3 M phosphoric acid were added. Fluorescence was determined in 5 minutes in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wave lengths of 325-385 mμ (uncorrected). The blank was treated exactly as the standards except that 0.2 ml of water was substituted for the periodate. The recovery of dopamine standards was 70 ± 2% (mean ± S.E.) and the dopamine content of the brain was calculated from these standards. No adjustment was made for the percent recovery of norepinephrine or dopamine.

2. α-Methyltyrosine assay

α-Methyltyrosine was assayed fluorometrically by a modification of the method of Porter et al. (1966). To plastic centrifuge tubes containing 3.0 ml of 6% trichloroacetic acid 0.5 ml of plasma was added. The samples were

centrifuged for 5 minutes at 10,000 x g. The supernatant was added to 15 ml capacity glass stoppered centrifuge tubes containing 1.5 ml of 1% ninhydrin and 0.3 ml of pyridine. The tubes were shaken briefly and placed in a boiling water bath for 10 minutes. The samples were immediately cooled in ice and 0.35 ml of concentrated hydrochloric acid was added to each tube. After thoroughly mixing, each tube was washed in 5 ml of ethyl acetate. The supernatant was removed by aspiration and 2 ml of the remaining solution was transferred to another 15 ml capacity glass stoppered centrifuge tube. To this were added 1 ml of nitric acid reagent (1 ml of 2.5% sodium nitrite + 49 ml of 1:5 nitric acid) and 1 ml of nitroso-naphthal reagent (100 mg of 1-nitroso-2-naphthal in 100 ml of 95% ethanol). The tubes were shaken and heated for 30 minutes at 55°C. They were then cooled and 5 ml of ethylene dichloride was added to each tube. The tubes were shaken for 5 minutes and centrifuged. The supernatant was removed and its α-methyltyrosine content was measured in an Aminco-Bowman spectrophotofluorometer at activating-fluorescent wave lengths of 456-560 mμ (uncorrected). α-Methyltyrosine standards of 5, 10, and 15 μg were run through the entire assay and the α-methyltyrosine content of the plasma was calculated from these standards.

3. Corticosterone assay

The pituitary adrenal response to stress has been determined by measuring the plasma concentration of corticosterone, the chief corticosteroid secreted by the rat adrenal cortex. Although this concentration represents a balance between secretion and metabolism, changes in the rate of the latter were assumed to be of minor importance in the acute experiments carried out in this investigation.

Plasma corticosterone was measured by a modification of the method of Guillemin et al. (1958). Aliquots of 0.5 ml and 0.1 ml of plasma were placed in 15 ml-capacity glass stoppered centrifuge tubes. The total volume in each tube was brought to 2.0 ml with 13% ethanol. Two ml of petroleum ether were added to each tube which was shaken for 1 minute and centrifuged. The supernatant was removed by aspiration and 5 ml of chloroform was added to each tube. After shaking for 1 minute and centrifuging, the aqueous phase was removed by aspiration. One ml of cold 0.1 N sodium hydroxide was added to each tube which was shaken and centrifuged. A 4 ml aliquot of the chloroform extract was transferred to another 15 ml capacity glass stoppered centrifuge tube containing 1.5 ml of 30 N sulfuric acid. After shaking for 1 minute and centrifuging, the acid was removed and its corticosterone content was measured in an

Aminco-Bowman spectrophotofluorometer at activation-fluorescent wave lengths of 460-525 mμ (uncorrected). The measurement was made 60 minutes after the 4 ml aliquot was shaken with sulfuric acid. Blanks were calculated by plotting the fluorometer readings versus the volume of plasma (0.5 and 0.1 ml). The line connecting these points was extended to the y axis and the y intercept was taken as the blank value. Corticosterone standards of 0.05, 0.10 and 0.20 μg were run through the entire assay. A standard curve plotted from their fluorescence readings was used to calculate the plasma corticosterone content.

4. Stresses

The following stressful procedures were utilized in order to stimulate the pituitary-adrenal system:

a. Strange environment: The animal was removed from its home cage in the animal room and placed individually in a small holding cage (9" diameter x 7" high). This cage was placed in the main laboratory for 15 minutes where the animal was exposed to the usual laboratory noise and activity. At the end of this time, the animal was quickly sacrificed.

b. Histamine: Histamine phosphate was dissolved in distilled water and injected intraperitoneally, 2 mg/100 g. The animal was sacrificed after 30 minutes.

c. Formalin: A 10% solution of formalin was injected subcutaneously, 0.3 ml/100 g. The animal was sacrificed after 30 minutes.

d. Ether: The animal was placed for 1 minute in a glass jar (6" diameter x 9" high) which was saturated with ether vapor. The animal was then removed and placed in its own cage for 30 minutes before sacrificing.

e. Restraint: The animal was placed in a small (4" x 2" x 2-1/2") enclosed chamber whose sides and floor were constructed of wood and the top of transparent plastic. Although unable to move in this space, the animal received adequate ventilation through several 1/4" holes in the top and ends.

f. Motor activity cage: The animal was placed in a doughnut-shaped cage (4" high, 12" diameter, 3" runway width) which had 4 hinged panels spaced equidistantly along the circumference of the wire mesh floor. Depression of a panel by a rat activated

a microswitch connected to an electric counter. Makes and breaks of the 4 microswitches were recorded for the first 15 minutes that an animal occupied the cage. The cage was located in a ventilated soundproof box without illumination.

Statistical tests of data obtained in the drug time studies and comparisons of stress effects to controls were carried out using Student's "t" test (Snedecor, 1956). Comparisons of data obtained for each drug pretreatment were carried out using analysis of variance and Duncan's new multiple-range test (Steel and Torrie, 1960).

RESULTS

Effects of reserpine and α-methyltyrosine on brain catecholamine and plasma corticosterone levels in nonstressed rats.

The time course of the effects of reserpine and α-methyltyrosine was examined in order to determine the optimum pretreatment times to be used in subsequent experiments.

Levels of brain catecholamines and plasma corticosterone were determined in rats at various times, up to 7 days, after the administration of reserpine (2 mg/kg). These results are depicted in Figure 3. A marked fall in brain norepinephrine and dopamine concentrations occurred within 3 hours and remained significantly below control levels for 7 days following the administration of the drug. This confirms previous reports (Pirch et al., 1967) that reserpine causes a sustained depletion of catecholamines in nervous tissue. Reserpine also caused a sharp increase in plasma corticosterone levels within 3 hours after its injection; this also confirms earlier reports (Montanari and Stockham, 1962). This effect was probably not due to nonspecific irritation resulting from the injection since the vehicle did not produce this effect. It may have been caused by an

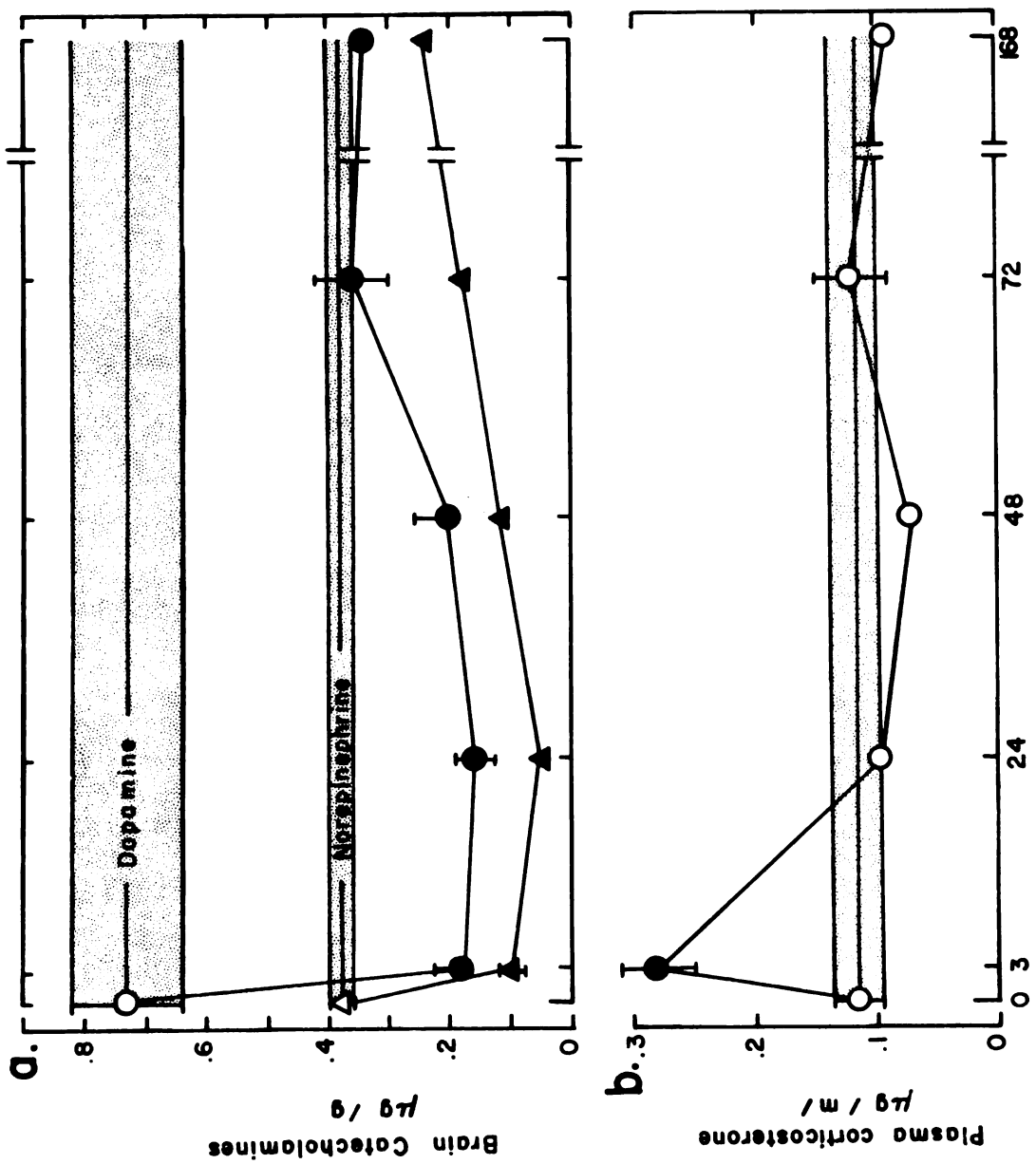
Figure 3a: Effects of a single intraperitoneal injection of reserpine (2 mg/kg) on the norepinephrine (△) and dopamine (○) content of rat brain.

The horizontal lines represent the mean catecholamine concentration and the shaded area is ± 1 standard error determined from 8 untreated animals. Each point is the mean value (vertical line ± 1 standard error) determined from 5-10 treated animals.

Figure 3b: Effects of a single intraperitoneal injection of reserpine (2 mg/kg) on the plasma levels of corticosterone (○).

The horizontal line represents the mean corticosterone concentration and the shaded area is ± 1 standard error determined from 8 untreated animals. Each point is the mean value (vertical line ± 1 standard error) determined from 5-10 treated animals.

Where no line is shown, the standard error is less than the radius of the point. Solid symbols represent values differing significantly from control ($p < .01$).



Figures 3a and 3b.

irritant action of the drug, by the release of peripheral or central catecholamines or serotonin or by some other effect of reserpine. The increase in plasma corticosterone levels was of short duration so that by 24 hours and thereafter the plasma corticosterone concentration remained at preinjection or control values. In order to minimize interference from the early effect of reserpine on the pituitary-adrenal system, animals were injected with this drug 24 hours prior to subjecting them to a stressful stimulus. At this time plasma corticosterone was at control levels while the brain catecholamines were markedly reduced.

A similar time study with α-methyltyrosine is depicted in Figure 4. Two hours after a single dose of α-methyltyrosine (50 mg/kg) a moderate but significant decrease in the brain content of norepinephrine and dopamine was noted; by 24 hours these amines had returned to control levels. The mean value of the plasma corticosterone concentration two hours after α-methyltyrosine was almost 3 times greater than control but due to the variation in values obtained, this difference was not significant. The increase in plasma corticosterone concentration, when it occurred, may have been due to an irritant action of the drug resulting from its low solubility. Because of the marked variability observed 2 hours following the injection, a pretreatment period of 4 hours was selected for α-methyltyrosine.

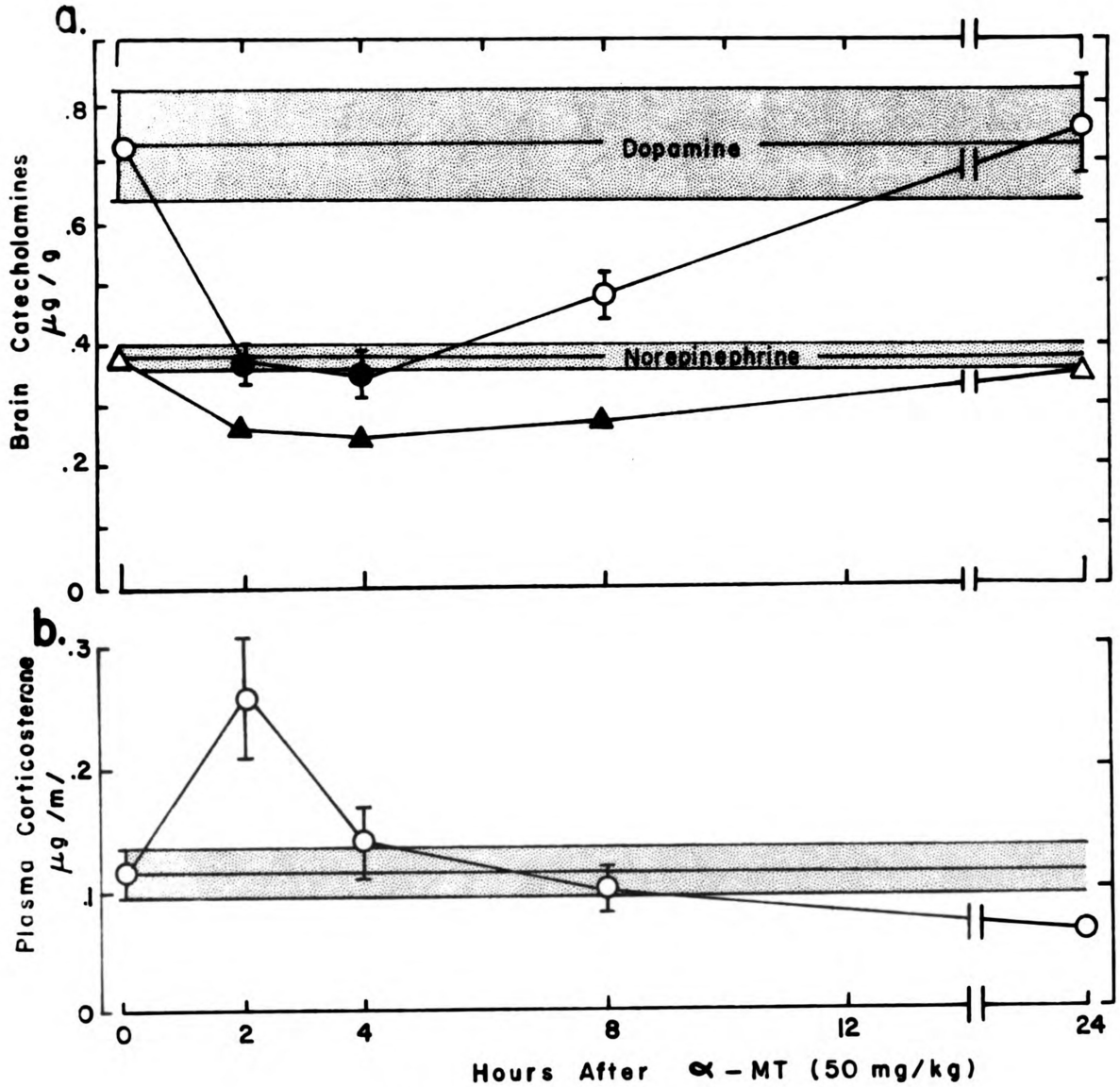
Figure 4a: Effects of a single intraperitoneal injection of α-methyltyrosine (50 mg/kg) on the norepinephrine (△) and dopamine (○) content of rat brain.

The horizontal lines represent the mean catecholamine concentration and the shaded area is ± 1 standard error determined from 8 untreated animals. Each point is the mean value (vertical line ± 1 standard error) determined from 6-10 treated animals.

Figure 4b: Effects of a single intraperitoneal injection of α-methyltyrosine (50 mg/kg) on the plasma levels of corticosterone (○).

The horizontal line represents the mean corticosterone concentration and the shaded area is ± 1 standard error determined from 8 untreated animals. Each point is the mean value (vertical line ± 1 standard error) determined from 5-10 treated animals.

Where no line is shown, the standard error is less than the radius of the point. Solid symbols represent values differing significantly from control ($p < .01$).



Figures 4a and 4b.

The effects of the 4 drug-vehicle pretreatment combinations on brain catecholamine and plasma corticosterone levels are summarized in Table 1. The control values (vehicle + saline) for levels of brain norepinephrine and dopamine and plasma corticosterone were comparable to recent reports (Goldstein and Nakajima, 1966; Dixit and Buckley, 1967). α-Methyltyrosine alone significantly reduced the brain content of norepinephrine and dopamine while reserpine alone caused an even greater reduction in the level of both amines. When these drugs were given in sequence the brain catecholamine levels were not significantly lower than those obtained with reserpine alone. The small concentration of catecholamine remaining in the brain might represent an extraneuronal or reserpine resistant pool. It is evident that with the time sequence selected (reserpine 24 hours and/or α-methyltyrosine 4 hours) neither drug, alone or in combination, had any effect on the levels of plasma corticosterone. In addition, reserpine had no significant effect on the concentration of α-methyltyrosine in the plasma. This indicated that hypothermia, hypotension, or some other action of reserpine did not alter the plasma levels of α-methyltyrosine.

Effects of reserpine and α-methyltyrosine on brain catecholamine and plasma corticosterone levels in stressed rats.

The effects of the drug-vehicle pretreatments on brain catecholamine levels after a variety of stresses can be seen

Table 1. Effect of drug pretreatments on brain catecholamine stores and plasma levels of corticosterone and α-methyltyrosine in nonstressed animals.

Treatment	N	Brain Norepinephrine	Brain Dopamine	Plasma Corticosterone	Plasma α-MT
		μg/g ± S.E.	μg/g ± S.E.	μg/ml ± S.E.	μg/ml ± S.E.
Saline + vehicle	8	.38 ± .02	.73 ± .09	.12 ± .02	--
α-MT + vehicle	8	.25 ± .01[a]	.35 ± .04[a]	.14 ± .03	15.0 ± 1.8
Saline + reserpine	10	.05 ± .01[b]	.16 ± .03[b]	.09 ± .01	--
α-MT + reserpine	10	.05 ± .02[b]	.13 ± .03[b]	.14 ± .04	19.6 ± 2.0

Reserpine (2 mg/kg) or vehicle was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given 4 hours before sacrificing the animals.

N Number of animals
a Significantly different from control ($p < .01$)
b Significantly different from control and α-MT + vehicle ($p < .01$)

in Tables 2 and 3. The various stresses did not alter the drug-induced pattern of brain catecholamine concentrations. That is, in all instances α-methyltyrosine significantly reduced the brain content of these amines; reserpine caused an even greater reduction. The brain catecholamine levels after the combination of reserpine and α-methyltyrosine were almost always lower than those after reserpine alone although the difference was not significant at the 1% level. This small difference may signify the remaining functional pool which is disrupted by α-methyltyrosine. Exceptions to the above pattern were observed with the histamine, ether and formalin stresses in which there was no significant difference in brain dopamine levels between the α-methyltyrosine and reserpine pretreatments. This was principally due to the variation in dopamine levels.

Brain norepinephrine, but not dopamine, levels were significantly lower after the formalin and restraint stresses in the control and α-methyltyrosine pretreatment groups. These results are comparable to previous studies in which various types of stress were employed (Moore and Lariviere, 1964; Bliss and Zwanziger, 1966).

The effects of α-methyltyrosine and reserpine on the plasma corticosterone concentration in stressed rats are given in Table 4. In control (vehicle + saline) animals,

Table 2. Effect of drug pretreatments on brain content of norepinephrine after "stress."

Stress	N	Saline + Vehicle	α-MT + Vehicle	Saline + Reserpine	α-MT + Reserpine	Coeficient of Variability
		μg/g ± S.E.	μg/g ± S.E.	μg/g ± S.E.	μg/g ± S.E.	
Control	8	.38 ± .02[a]	.25 ± .01	.05 ± .01	.05 ± .01	21.0%
Histamine	5	.35 ± .04	.19 ± .02	.03 ± .01	.02 ± .01	30.3%
New environment	5	.34 ± .02	.20 ± .02	.05 ± .01	.05 ± .01	27.5%
Motor activity	5	.34 ± .01	.20 ± .01	.05 ± .01	.04 ± .01	11.9%
Ether	5	.33 ± .02	.19 ± .01	.04 ± .01	.03 ± .01	21.4%
Formalin	5	.31 ± .01[a]	.15 ± .01[a]	.06 ± .01	.03 ± .01	16.2%
Restraint	5	.31 ± .01[a]	.15 ± .01[a]	.05 ± .01	.06 ± .01	22.9%

Reserpine (2 mg/kg) or vehicle was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given four hours prior to introduction of the stressful stimulus. Any two means in a horizontal row underscored by the same bar below the table are not significantly different. Any two means not underscored by the same bar are significantly different ($p < .01$).

N Number of animals

[a] Significantly different from control ($p < .01$)

Table 3. Effect of drug pretreatments on brain content of dopamine after "stress."

Stress	N	Saline + Vehicle	α-MT + Vehicle	Saline + Reserpine	α-MT + Reserpine	Coefficient of Variability
		μg/g ± S.E.	μg/g ± S.E.	μg/g ± S.E.	μg/g ± S.E.	
Control	8	.73 ± .09	.35 ± .04	.16 ± .03	.13 ± .03	29.0%
Histamine	5	.74 ± .09	.33 ± .05	.15 ± .02	.06 ± .03	37.4%
New environment	5	.61 ± .06	.33 ± .05	.06 ± .03	.04 ± .02	37.3%
Motor activity	5	.66 ± .06	.34 ± .03	.16 ± .03	.08 ± .02	27.3%
Ether	5	.74 ± .06	.22 ± .06	.24 ± .04	.05 ± .02	39.3%
Formalin	5	.70 ± .10	.32 ± .05	.15 ± .04	.10 ± .02	43.2%
Restraint	5	.79 ± .08	.29 ± .05	.11 ± .04	.02 ± .02	38.5%

Reserpine (2 mg/kg) or vehicle was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given four hours prior to introduction of the stressful stimulus. Any two means in a horizontal row underscored by the same bar below the table are not significantly different. Any two means in a horizontal row not underscored by the same bar below the table are significantly different ($p < .01$). Exceptions are underlined in the same manner in the body of the table.

N Number of animals

Table 4. Effect of drug pretreatments on plasma levels of corticosterone after "stress."

Stress	N	Saline + Vehicle	α-MT + Vehicle	Saline + Reserpine	α-MT + Reserpine	Coefficient of Variability
		µg/g ± S.E.	µg/g ± S.E.	µg/g ± S.E.	µg/g ± S.E.	
Control	8	.12 ± .02	.14 ± .03	.09 ± .01	.14 ± .04	77.9%
Motor activity	5	.25 ± .04	.33 ± .05	.31 ± .08	.32 ± .05	41.4%
New environment	5	.29 ± .04	.26 ± .02	.36 ± .05	.38 ± .02	30.3%
Ether	5	.36 ± .03	.36 ± .03	.36 ± .07	.42 ± .02	26.8%
Restraint	5	.38 ± .02	.41 ± .04	.47 ± .03	.35 ± .03	17.5%
Formalin	5	.45 ± .02	.43 ± .03	.44 ± .10	.48 ± .08	32.1%
Histamine	5	.51 ± .03	.54 ± .11	.51 ± .08	.54 ± .04	30.4%

Reserpine (2 mg/kg) or vehicle was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given four hours prior to introduction of the stressful stimulus. Any two means in a horizontal row underscored by the same bar below the table are not significantly different. Any two means in a horizontal row not underscored by the same bar are significantly different ($p < .01$). Plasma corticosterone levels from all stressed animals were significantly higher than the respective control values ($p < .01$).

each stress caused a significant increase in plasma corticosterone levels. None of the drug pretreatments altered these levels significantly. Evidently none of the pretreatments had altered the animals' ability to perceive or react to the stressful stimulus by secreting increased amounts of corticosterone.

As with the unstressed rats, reserpine pretreatment did not alter the plasma α-methyltyrosine levels. Moreover, none of the stresses significantly affected the plasma levels of α-methyltyrosine. These results eliminated the possibility that reserpine or stress could have altered the blood levels of α-methyltyrosine and thereby affect its intended action in this investigation (Table 5).

Effects of reserpine and α-methyltyrosine on motor activity.

Although depletion of brain catecholamines had no effect on the pituitary-adrenal response to stress, previous studies have shown that certain parameters of animal behavior are altered when subjected to the type of drug pretreatments employed in this investigation (Rech et al., 1967). This fact was verified by studying the effects of the 4 pretreatments on motor activity (Figure 5). Although both α-methyltyrosine and reserpine reduced motor activity, the combination of drugs reduced it to a greater extent than either drug alone. In contrast, there was no effect on plasma corticosterone levels in the same animals.

Table 5. Effect of reserpine and various stressful procedures on plasma α-methyltyrosine levels.

	N	α-MT + Vehicle	α-MT + Reserpine
		μg/ml ± S.E.	μg/ml ± S.E.
Control	8	15.0 ± 1.8	19.6 ± 2.0
Restraint	5	18.0 ± 0.9	12.2 ± 0.7
Motor activity	5	16.0 ± 2.0	16.0 ± 2.9
Histamine	5	14.2 ± 1.1	16.4 ± 1.4
New environment	5	13.8 ± 1.0	15.4 ± 1.3
Ether	5	12.2 ± 2.2	14.0 ± 1.9
Formalin	5	11.8 ± 1.6	14.6 ± 2.2

Reserpine (2 mg/kg) was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given four hours prior to introduction of the stressful stimulus.

N Number of animals

Figure 5: Effects of drug-vehicle pretreatments on motor activity and plasma corticosterone levels.

Each bar represents the mean of values (vertical lines above the bars represent one standard error) obtained from 5 animals. The lower unfilled portion of the bars depicting plasma corticosterone levels represents the mean of values obtained from 8 animals which received the drug-vehicle pretreatments but were not stressed. Reserpine (2 mg/kg) or vehicle was given 24 hours and α-methyltyrosine (50 mg/kg) or saline was given 4 hours prior to placement in the motor activity cage.

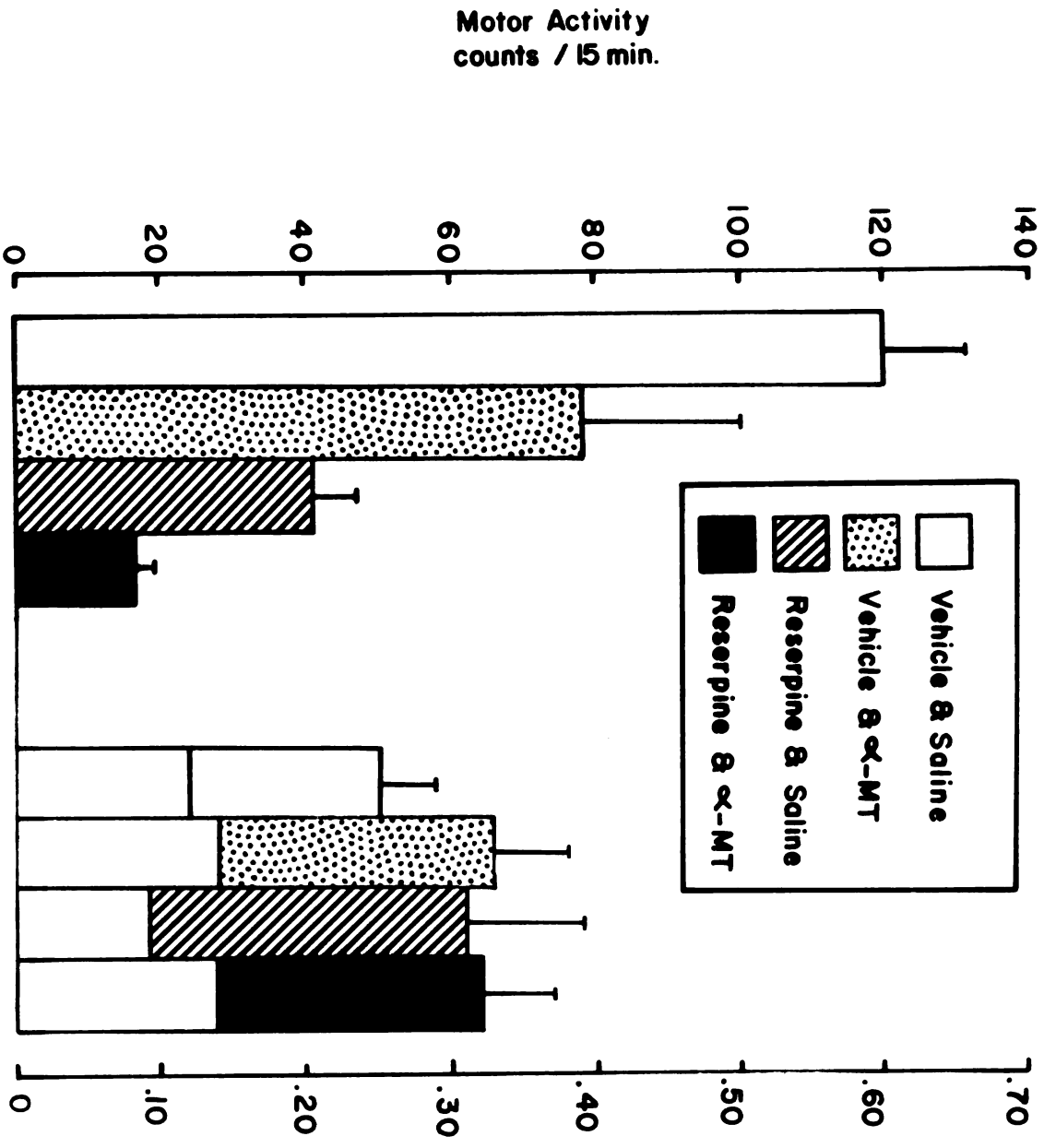

Motor Activity
counts / 15 min.
0
20
40
60
80
100
120
140
Vehicle & Saline
Vehicle & α-MT
Reserpine & Saline
Reserpine & α-MT
0
.10
.20
.30
.40
.50
.60
.70
Plasma Corticosterone
μg /ml

Figure 5.

Effect of reserpine, α-methyltyrosine and cholinergic blocking drugs on plasma corticosterone levels.

In spite of the fact that neither noradrenergic nor dopaminergic pathways appeared to play an essential role in the response of the pituitary-adrenal system to stress, it was speculated if cholinergic neurons in the central nervous system served such a purpose. Rats pretreated with the α-methyltyrosine-reserpine combination were also given atropine (2 mg/kg) or scopalamine (2 mg/kg) 45 minutes before being subjected to the new environment stress. There was no significant difference in plasma corticosterone levels between pretreated animals and controls (Table 6).

These results indicate that with the use of pharmacological tools which are currently available it is not possible to demonstrate the involvement of cholinergic, dopaminergic or noradrenergic neurons in the stress-induced activation of the pituitary-adrenal system.

Table 6. Effect of reserpine, α-methyltyrosine and cholinergic blocking drugs on response to new environment stress.

Treatment	Plasma Corticosterone
	μg/ml ± S.E.
Saline + vehicle	.29 ± .04
Reserpine + α-MT + atropine	.60 ± .05
Reserpine + α-MT + scopalamine	.40 ± .06

Reserpine (2 mg/kg) or vehicle was given 24 hours, α-methyltyrosine (50 mg/kg) or saline was given 4 hours, and atropine (2 mg/kg) or scopalamine (2 mg/kg) was given 45 minutes prior to introduction of the stressful stimulus.

DISCUSSION

Norepinephrine is firmly established as a chemical transmitter in the peripheral autonomic nervous system. However, the function of this amine in the central nervous system is less certain, primarily because an efficient barrier prevents appreciable amounts of all catecholamines from entering the brain from the systemic circulation (McLennan, 1963). Recent evidence indicates that norepinephrine and dopamine, the two catecholamines present in the brain, may function as chemical transmitters in certain neuronal pathways within the central nervous system. Much of the speculation on the functions of brain catecholamines is based upon anatomical evidence. Norepinephrine is concentrated in the brain stem (Vogt, 1954) while dopamine is primarily located in the basal ganglia (Carlsson, 1959). Both amines have been identified in the hypothalamus.

Evidence from anatomical, physiological, and pharmacological studies suggests that norepinephrine functions as a chemical mediator in hypothalamic sympathetic centers (Brodie and Shore, 1957), in the ascending activating system of the reticular formation (Vogt, 1957), in temperature-regulating centers (Cooper, 1966) and in a variety of other loosely defined brain systems. It has been suggested that dopamine serves as a

transmitter substance in the extrapyramidal system to control certain motor functions (van Rossum and Hurkmans, 1964).

It has also been suggested that both norepinephrine and dopamine may be concerned with the central regulation of the anterior pituitary gland. Most of the evidence supporting this thesis is derived from anatomical studies. Analysis of rather large areas (Carlsson, 1959) and, more recently, histochemical localization of these amines in discrete areas have demonstrated that a monoaminergic system exists in the hypothalamus (Fuxe, 1965). The dopaminergic and noradrenergic neurons in this region probably originate from both the reticular formation and the limbic system (Schute and Lewis, 1966). These amines have been identified in significant concentrations in the median eminence. This is precisely the same region where electrical stimulation and lesion studies have demonstrated the role of the hypothalamus in regulating the release of ACTH from the anterior pituitary (D'Angelo et al., 1964; Donovan, 1966).

It has been observed previously that the concentration of brain norepinephrine falls after subjecting animals to stressful stimuli which are known to increase plasma levels of corticosterone (Moore and Lariviere, 1964; Maynert and Levi, 1964; Bliss and Zwanziger, 1966). In control and α-methyltyrosine-pretreated rats, formalin and restraint significantly lowered brain concentrations of norepinephrine, but not dopamine. Since

norepinephrine is continuously being synthesized and metabolized, the actual level of this amine at any one time represents a steady state concentration. A variety of stresses probably accelerate the activity of neurons which transmit their impulses by releasing norepinephrine at synapses. As a result, norepinephrine is released and metabolized at a faster rate than it is synthesized. The other stressful procedures used in this study were probably not severe enough or of sufficient duration to cause any significant changes in the brain levels of norepinephrine.

The reason why emotional or chemical stress may deplete brain norepinephrine 20 to 50 percent but not affect dopamine levels is not clearly understood. One explanation may be the different anatomical distribution of these two amines in the brain. In the hypothalamus and brainstem, the regions most likely concerned with the stress response, norepinephrine is found in higher concentrations than dopamine and is considered to be the end product in the synthetic pathway. Dopamine, which is found most concentrated in the basal ganglia (Carlsson, 1959), structures which play little, if any, role in the stress response, would not be expected to be affected by the stressful procedures used in this study.

It is quite difficult at the present time to study the functions of central noradrenergic or dopaminergic nerves by

administering exogenous catecholamines. How then can their function be studied? One possibility is the method employed in this investigation. The object was to reduce the content of norepinephrine and/or dopamine in neurons to such an extent that the functions of these neurons are impaired. This approach has been used previously to study the role of catecholamines in regulating the release of various anterior pituitary hormones. For example, the induced release of growth hormone from the anterior pituitary by insulin hypoglycemia can be blocked by reserpine and other drugs which deplete the brain of catecholamines. If the animals are pretreated with inhibitors of monoamine oxidase, the intracellular catecholamine metabolizing enzyme, the reserpine blockade is prevented, presumably because functional pools of catecholamines remain intact (Müller et al., 1967). Reserpine and α-methyltyrosine have also been observed to affect the secretion of luteotropic hormone (LtH) and luteinizing hormone (LH) (Lippmann et al., 1967). LH secretion which normally occurs after injection of pregnant mare serum, is blocked when the animal is pretreated with reserpine. Secretion of LtH, normally inhibited, is stimulated after reserpine administration. If the catecholamine depletion is blocked by monoamine oxidase inhibitors, these effects are prevented. It was concluded from these studies that brain catecholamines regulate the secretion of growth hormone releasing factor (GRF), luteinizing hormone releasing factor (LRF) and prolactin inhibitory factor (PIF).

When rats which had been pretreated with reserpine and α-methyltyrosine were subjected to various stressful procedures in the present investigation, there was no significant disruption of the response by the pituitary-adrenal system to stress. It was confirmed by direct measurement that these drugs produced very low brain levels of norepinephrine and dopamine, yet they did not impair the ability of the various stresses to cause secretion of corticosterone from the adrenal cortex.

The conclusion that reserpine does not block the stress-induced release of ACTH is not in agreement with the reports of other investigators (Wells et al., 1956; Munson, 1963). However, in these studies, ACTH secretion was measured by means of adrenal ascorbic acid depletion which is presently only of historical significance because the method lacks sufficient sensitivity and specificity. For this reason, plasma corticosterone concentration was employed as a measure of pituitary-adrenal activation in the present study, since it is a much more labile and specific indicator (Mangil et al., 1966).

It has also been claimed that reserpine might cause a prolonged discharge of ACTH from the pituitary, severely lowering the pituitary content and explaining the failure for a pituitary-adrenal response to a second stress (Maickel et al., 1961). However, the pituitary response has been shown to be independent of pre-existing ACTH stores

(Vernikos-Danellis, 1963). It may be a persistent accumulation of adrenal steroids in central nervous system inhibitory centers which prevents the response (Mangili et al., 1966).

Other reports (Guillemin, 1957; Stockham and Montanari, 1962; Smelik, 1967) agree with the conclusions of this investigation in regard to the failure of reserpine pretreatment to block the pituitary-adrenal response to stress. Since it has been shown that a functional store remains after treatment with reserpine, it is felt that their conclusions may have been premature. However, with the techniques used in the present investigation, all functional stores would have been depleted.

The behavior of the animals after the drug treatments in this study was remarkably similar to that described by Guillemin (1957). The rats which received reserpine and the combination of reserpine and α-methyltyrosine appeared very indifferent to and unaffected by the stressful procedures. They remained motionless and apparently oblivious to their surroundings. In contrast, the rats which received the vehicles and α-methyltyrosine reacted with natural curiosity to the strange surroundings and often exhibited strong reactions to the chemical stresses. Thus it appears that these animals need not react outwardly in order to perceive stressful stimuli.

This dissociation between overt behavior and the pituitary-adrenal response to stress was further emphasized in the motor

activity experiments. The drug pretreatments clearly depressed normal inquisitive behavior upon being placed in a strange environment. They would remain quiet, sometimes not moving for 10 minutes. The reduction in motor activity correlated well with the depletion of brain catecholamines and confirmed the enhanced behavioral effects of α-methyltyrosine which have been reported in reserpine-treated rats (Rech et al., 1967).

Since cholinergic pathways are considered to be located in various areas of the brain (Schute and Lewis, 1966) and there is pharmacological evidence that acetylcholine causes vasopressin release by acting at the supraoptic nucleus (Sawyer and Mills, 1966), experiments were also conducted using cholinergic blocking drugs. However, rats pretreated with reserpine, α-methyltyrosine and atropine or scopalamine did not have lower plasma corticosterone levels than control rats after stress.

SUMMARY

An attempt was made to alter the pituitary-adrenal response to stressful procedures by depleting the brain of norepinephrine and dopamine. This depletion was accomplished in rats by administering reserpine, which destroys "reserve stores" of catecholamines, and α-methyltyrosine, which blocks catecholamine synthesis. A single injection of reserpine (2 mg/kg) caused a transient increase (at 3 hours) in the plasma corticosterone levels and lowered the brain content of norepinephrine and dopamine for at least 7 days. α-Methyltyrosine (50 mg/kg) did not significantly alter plasma levels of corticosterone but reduced brain levels of norepinephrine and dopamine for a short duration; the maximal effect occurred 2 to 4 hours after injection.

When administered in sequence to nonstressed rats, reserpine (for 24 hours) and α-methyltyrosine (for 4 hours) markedly reduced the brain content of catecholamines but did not alter the plasma levels of corticosterone. At this time, vehicle (control) and drug-pretreated animals were subjected to a variety of stressful procedures (administration of ether, histamine, or formalin; restraint or altered environment). Formalin and restraint stresses caused a significant reduction in the brain content of norepinephrine; the dopamine content was not altered by these stresses. Neither

reserpine nor the stressful procedures altered the plasma levels of α-methyltyrosine. All stresses increased plasma corticosterone levels; there was no difference between control and drug-pretreated animals.

The concentration of corticosterone increased significantly in the plasma of animals which were placed in motor activity cages. Reserpine and α-methyltyrosine pretreatment (alone and in combination) depressed locomotor activity in relation to the depletion of brain catecholamines; plasma corticosterone levels were unaffected by the drug pretreatments.

Pretreatment with centrally acting cholinergic blocking drugs, in combination with reserpine and α-methyltyrosine, did not prevent the rise in plasma corticosterone in response to the altered environment stress.

These results indicate that brain catecholamines do not play an essential role in stress-induced activation of the pituitary-adrenal system.

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