CENTRAL NERVOUS SYSTEM LESIONS BLOCKING THE RELEASE OF ACTH CAUSED BY TRAUMATIC STRESS

by

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GENERAL INTRODUCTION

Coordination of function in animals is accomplished by the nervous and endocrine systems. The nervous system is usually considered to control rapid adaptations with time courses ranging from milliseconds to seconds. The endocrine system, on the other hand, is usually regarded as integrating changes taking place more slowly, over hours to years. These generalities have a number of exceptions. For instance, memory enables the nervous system to influence behavior over many years, and the endocrine system, by means of the adrenal medulla or the hypothalamo-pituitary axis, can coordinate functions in seconds to minutes. The nervous system is intimately involved in mediating such rapid endocrine responses, but relatively little is known about the central nervous system pathways which carry the necessary information. This dissertation is involved with the study of one such functional pathway - that which mediates traumatic stress-induced release of ACTH.

HISTORICAL REVIEW

Innervation of the anterior pituitary

How the brain influences the anterior pituitary is still not completely settled. One question was whether the control was via direct innervation or by some neurohumoral mechanism. Anatomic studies have shown that the direct innervation of the anterior pitui-

tary is sparse and is primarily associated with blood vessels (1). There is still some controversy on this point, however. Metuzals (2) claimed that there are nerve fibers "which terminate probably among the gland cells" of the adenohypophysis. In contrast, Green has stated "no innervation of the pars distalis was found although (nerve) fibers and endings were seen in the pars intermedia, and fibers accompanying vessels in the pars tuberalis were common" (3). Green (4) listed several reasons why he doubts findings of nerve fibers in the anterior pituitary. Two important objections are: 1. Electron microscopy has not shown nerve fibers in the anterior pituitary. 2. Variations in most silver techniques may stain connective tissue fibers and make them appear to be nerve fibers.

Whether there are nerve fibers in the anterior pituitary is unimportant if they have no function. Physiological investigations have revealed only minimal influence of the cervical sympathetics on anterior pituitary function. Stimulation of these nerves in the rabbit resulted in ovulation, presumably due to an increase in luteinizing hormone secretion (5). No reports concerning the function of alleged nerve fibers reaching the anterior pituitary from the hypothalamus have been found.

Pseudopregnancy in the rat is a prolonged luteal phase of the estrous cycle which may be induced by stimuli to the cervix and vagina. It is caused by a sustained release of prolactin (6). Cervical sympathectomy interfered partially with the induction of pseudo-

pregnancy caused by electrical or glass rod stimulation of the cervix (7). However, mating a vasectomized male with a sympathectomized female almost invariably resulted in pseudopregnancy.

Finally, completely sympathectomized female rabbits ovulate following copulation and bear and raise completely normal litters (8). All of the authors quoted above stated that the effects of the sympathetics on pituitary function were minimal, were probably due to vasomotor changes, and that the control of the anterior pituitary must be by some other means.

Neurohumoral control of the anterior pituitary

If materials released in the brain are to be considered as modifiers of pituitary function, a pathway for their transport to the pituitary must be present. Older studies of pituitary circulation gave no clues to the existence of such a pathway. The pituitary portal circulation was discovered by Popa and Fielding, thus demonstrating a vascular link between the pituitary and the hypothalamus (9). However, they thought that blood flowed from the pituitary to the hypothalamus. Several years later Wislocki and King (10) stated, also on the basis of anatomical evidence, that blood flowed from the hypothalamus to the pituitary. But it was not until 1947 that Green confirmed by direct observation of the portal system in live amphibians that blood flowed from the hypothalamus to the pituitary (11).

Taubenhaus and Soskin were the first to suggest a function for the portal vessels (12). They thought that neurohumoral substances

such as acetylcholine might be released into the portal veins and thereby stimulate the anterior pituitary. Their experiments showed increased incidence of pseudopregnancy in rats which had acetylcholine and prostigmine (1.0 μg. and 0.5 μg. respectively) pipetted onto the pituitary in vivo. Their experimental results were fortuitous in that they may have been due to some non-specific activation of the pituitary by these substances, but their idea and approach were important.

More substantial proof of the function of the portal vessels was provided by Benoit and Assenmacher (13). They showed in the duck that cutting the portal supply but leaving the infundibulum intact resulted in testicular atrophy. However, cutting the infundibulum but leaving the portal vessels intact allowed maintenance of testicular size. Although the results of these experiments argue strongly that some agent necessary for pituitary function is supplied via the portal veins, there is no proof that the substance is not oxygen or some nutrient, since there was some degree of pituitary infarction after the destruction of the portal veins.

The hypothesis for neurohumoral control was strengthened in 1957, when substances which caused the release of ACTH from pituitaries both in vitro (14) and in vivo (15) were extracted from hypothalamic tissue. Soon thereafter, substances which inhibit or stimulate the release of the six well known anterior pituitary hormones were extracted and concentrated from hypothalamic tissue (16).

Although the evidence for the theory that the hypothalamus controls the anterior pituitary by releasing neurohumors into the portal veins is very good, it is still largely circumstantial. The structures of these neurohumors, otherwise known as "releasing factors", have not been determined although they are supposedly relatively simple substances with molecular weights below 2000 (17, 18). Investigators who concentrate releasing factors from vast numbers of hypothalami have not made any substantial amount available to other investigators to test. The hypothalamic A-V concentration difference of a releasing factor has never been measured. However, the theory of neurohumoral control of the anterior pituitary is consistent with the available data and there has been no better substantiated hypothesis.

The neural control of ACTH secretion

In 1950, it was shown independently by two groups that electrical stimulation of the hypothalamus increases ACTH secretion [1]

1 In the discussion to follow, the phrase ACTH secretion may be used in place of the actual parameter measured to estimate ACTH secretion. ACTH was rarely measured directly since it is difficult to measure minute amounts of a specific protein contained in a solution with a wide variety of proteins. Some of the parameters used for estimating ACTH are: lymphopenia, eosinopenia, adrenal ascorbic acid depletion, peripheral plasma corticosterone concentration, urinary 17-hydroxycorticoids, and adrenal corticoid secretion rate. Admittedly, many of these methods are not specific for ACTH. However, in the papers quoted, most of the principles were inferred from data involving probable gross changes in ACTH secretion for which the methods are most certainly valid.

and that lesions of the hypothalamus diminish or abolish ACTH secretion in response to stress (19, 20). The results from experiments involving discrete lesions of the hypothalamus in rats and dogs showed that an area of the brain very important for ACTH secretion is the median eminence (21, 22, 23). Lesions of the median eminence impaired adrenal function but at the same time did not impair the function of the gonads and thyroids. Thus, the inference has been made that the median eminence acts as a funnel, a final common path, for influences controlling ACTH release.

The next question, then, is what controls the median eminence?

Anatomical considerations

According to Nauta (24), the hypothalamus receives sensory input mainly from the non-specific thalamic nuclei and the mesencephalic reticular formation. The area of the mesencephalon importantly involved with direct connections to the hypothalamus has been defined as the limbic midbrain area and occupies an area in the midline. Included are the ventral part of the central gray, the ventral tegmental area, and the paramedian reticular nuclei of Bechterew and Gudden (25). The limbic midbrain area has extensive reciprocal connections with not only the hypothalamus but with limbic forebrain areas such as the hippocampus and amygdala. Nauta says (24) that from the anatomical connections observed it appears reasonable to assume that this limbic system midbrain circuit can be "activated or otherwise affected by a great variety of sensory modalities, especially

by all those that have wide access to the brainstem reticular formation". Most of the pathways in the limbic system midbrain circuit relay in the lateral hypothalamus but there are direct afferent connections with the medial hypothalamus. Fibers from the periventricular system of Schütz terminate in the caudal part of the medial hypothalamus and the stria terminalis distributes fibers from the amygdala to the rostral part of the medial hypothalamus. In addition, the medial hypothalamus has extensive collateral connections with the lateral hypothalamus and thereby with the limbic midbrain circuit. Thus many anatomical pathways are already known by which traumatic or emotional stress could activate the hypothalamus and initiate ACTH release.

Physiological considerations

The next part of this introduction will be devoted to physiological considerations concerning the means by which a peripheral stress such as shocking or burning a limb influences the median eminence to cause ACTH secretion.

The role of peripheral nerves:

It was shown by Gordon in 1950 that sectioning the sciatic and femoral nerves prevented the release of ACTH following breaking or mildly scalding that leg in the unanesthetized rat (26). He also found, however, that severe scalds of the "denervated" limb caused ACTH release. This latter finding might indicate the production of a substance at the site of the trauma (later termed a "wound hormone" by

Egdahl) which could cause ACTH release. Egdahl (27) suspected that not all of the nerves to the leg were cut and that the severe scalds stimulated the few remaining nerves enough to activate the neuronal system regulating ACTH secretion.

To distinguish whether the ACTH release in response to peripheral trauma was being mediated by nerves or the bloodstream, Egdahl prepared dogs whose legs had been completely severed at mid-thigh except for the femoral artery and vein and the sciatic nerve. Blood samples were collected from chronic catheters in the adrenal veins for the determination of 17-hydroxycorticoid secretion. The animals were tested for the ability to respond with ACTH secretion to trauma to the isolated leg from one to twenty-one days after surgery. With the blood vessels and nerve intact, large burns as well as subcutaneous ACTH or bacterial endotoxin injections to the isolated leg caused the expected rise in 17-hydroxycorticoids. However, with the nerve severed and the blood vessels intact only the ACTH and the endotoxin injections caused a rise. With the blood vessels cut but the nerve intact, only the burns caused a rise in 17-hydroxycorticoids. The ACTH and endotoxin injections served as a control for the integrity of the pituitary-adrenal axis as well as of the circulation to the isolated leg; if a wound factor were being produced by the burns, this control should show whether the factor could get back centrally to cause a rise in 17-hydroxycorticoids just as the ACTH or the endotoxin did. The conclusions from these

elegant experiments were that no wound hormone could be demonstrated after the trauma of burning and that activation of the pituitary-adrenal axis in response to burning a limb depends on having an intact nerve subserving the area of the burn.

The role of the spinal cord

It has been shown in both unanesthetized (28) and anesthetized (29) rats that section of the spinal cord at T-3 (28) or T-10 (29) prevents the ACTH release normally caused by shocking or breaking the hind legs, but has no effect on ACTH release caused by the same trauma to the forelegs. Other work has shown that cord section has no effect on the normal response to some emotional stress such as that produced by restraining the animal for several minutes (30). The fact that cord section does not block the response to emotional stress is not surprising since this stress need not involve information carried by the spinal cord.

The role of midbrain structures

Slusher and Hyde (31) reported that stimulation of the dorsal midbrain tegmentum in unanesthetized, unrestrained cats caused ACTH release and ventral stimulation inhibited it. However, Endröczi and Lissak (32) reported just the converse. Neither of these studies is entirely convincing, and they are not directly comparable. Slusher and Hyde stimulated unanesthetized, unrestrained cats and bioassayed for ACTH in rats, a perfectly acceptable procedure. However, prestimulation levels of ACTH in their cats were undetectable, and the

maximum observed levels were at the lower limit of detection in their assay. Endröczi and Lissak also stimulated unanesthetized, unrestrained cats, but they anesthetized the cats 45 minutes after stimulation, performed a laparotomy, and collected adrenal venous blood for an hour. (It is known that, in rats at least, ACTH levels are maximal in three minutes after stress (33) and adrenal vein corticosteroids are maximal soon after and remain high for the duration of the laparotomy[1]). Therefore, Slusher and Hyde were measuring the effects of electrical stimulation on ACTH secretion, whereas Endröczi and Lissak were probably measuring the effect of previous stimulation on the release of ACTH caused by laparotomy and adrenal vein cannulation under anesthesia. Slusher (34) reported that in the rat some anterior pontine lesions increased ACTH secretion caused by laparotomy and adrenal vein cannulation, whereas adjacent lesions caused a decrease under the same conditions. She also found that larger adjacent lesions in the midbrain were without effect. No reports confirming or denying the validity of her results have been found.

Experiments involving complete transection of the midbrain have also resulted in some conflicting conclusions. Martini et al reported that complete midbrain transection in the rat blocked ACTH release in response to laparotomy and to ether, both of which pro-

1 J.W. Kendall, personal communication, 1967.

duce a maximum response in intact animals (35). A later paper from the same laboratory stated that the plasma corticoids were elevated after midbrain transection (time after transection not stated) and that dexamethasone, a potent synthetic glucocorticoid, had to be given in amounts of 100 μg/100g body weight to significantly, but not completely, depress the plasma corticosterone levels (36). In intact rats a significant effect was obtained with 3 μg/100g body weight. Their conclusions were that the pathways to the hypothalamus which mediated the stress-induced release of ACTH were cut by the transection and that a steroid-sensitive feedback area was located in the midbrain caudal to the transection.

The conclusion about the feedback area in the midbrain was placed in doubt by some experiments performed by Kendall et al (37). They showed that by waiting 72 hours after midbrain transection the blood corticoid levels dropped down to those of normal unstressed rats. When tested three days postoperatively, the rats did respond to ether with an increased ACTH secretion. Also at that time a 25 μg/100g body weight dose of dexamethasone suppressed the blood corticoid levels to those of hypophysectomized rats. These authors felt that the initially high levels of ACTH and lack of responsiveness to dexamethasone and ether found by Martini et al, might have been the result of some stress caused by the transection itself. Only when the high ACTH levels from this stress subsided could the effects of other stressors be tested.

More confusion concerning the results of midbrain transection was provided by Egdahl (38). He reported that midbrain transection in the dog did not prevent the ACTH release normally seen following sciatic nerve stimulation. To reconcile some of these data, Egdahl postulated a "hindbrain factor" which will be dealt with in the next section.

Results from brain removal studies

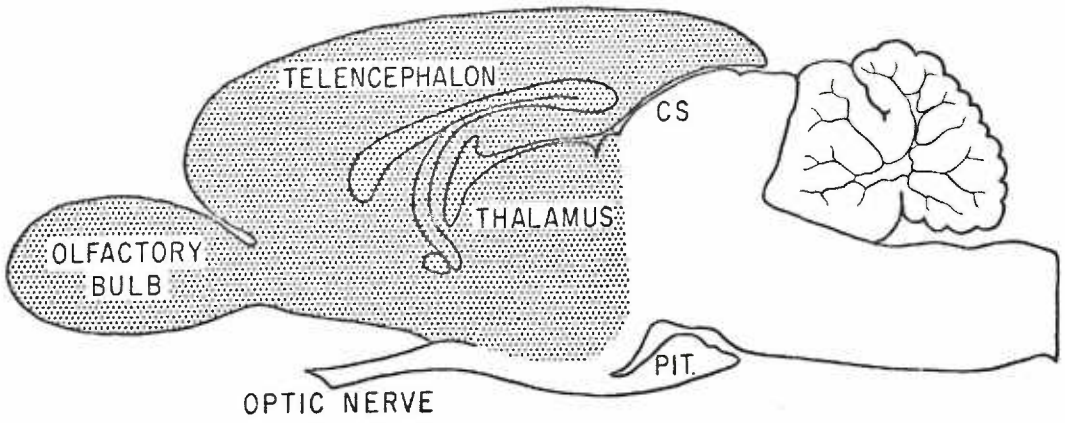
Total forebrain removal to the superior colliculus leaving the pituitary intact (pituitary island) has been reported to result in high levels of ACTH secretion in the dog. ACTH release in response to the trauma of burning in such animals has also been claimed (39). Since Egdahl had previously reported that ACTH release in response to peripheral trauma was blocked by cord section but not by midbrain transection, he suggested the existence of a hindbrain factor. This was postulated to be a substance released from the CNS above C-7 and below the mid-collicular level in response to a traumatic stress, such as burning. The substance was presumed to act on the pituitary to cause ACTH secretion. The release of the substance was thought to be tonically inhibited by higher neural structures. However, the validity of the "hindbrain factor" hypothesis was placed in doubt when high resting ACTH levels were found in dogs with their entire brains removed but with the pituitary left in situ (40). Egdahl subsequently showed that even total CNS removal resulted in high resting ACTH secretion in the dog if the pituitary were left in place (41).

I have not found reports in the subsequent literature showing increased ACTH after sciatic nerve stimulation in midbrain transected dogs. The monkey (42) may be similar to the dog in having high resting ACTH levels when the brain is removed. One interpretation of these findings is that some animals have higher inhibitory areas tonically preventing the release of ACTH.

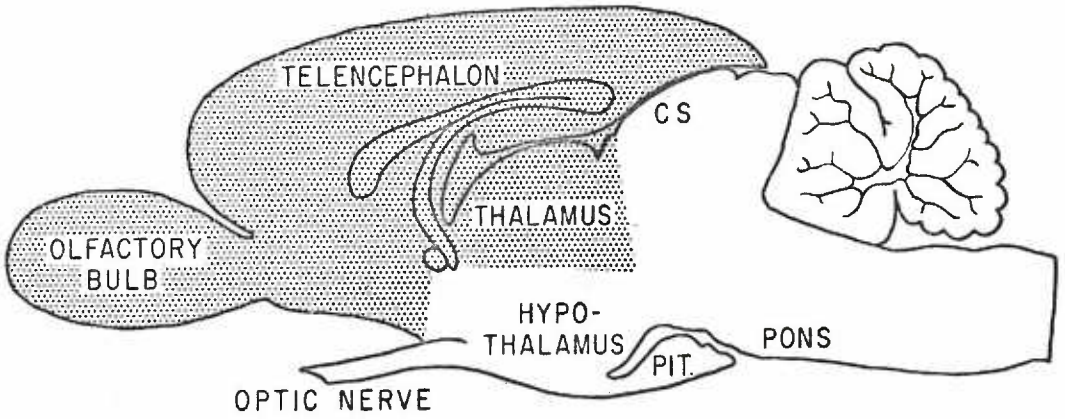
In order to establish which areas of the brain are necessary for secretion of ACTH following ether stress or foreleg fracture, Matsuda et al (29, 43) performed several interesting brain removal experiments. With regard to ether stress, they have shown that rats with a median eminence island (brain removal to the superior colliculus except for the median eminence and pituitary) will respond to ether with an increase in corticosterone secretion. Since rats with a pituitary island or with pituitaries transplanted to a heterotopic site (44) do not respond to ether, the ether must act directly on the median eminence to cause ACTH release. Ether may also act elsewhere since corticosterone secretion was higher following ether in intact rats than in rats with a median eminence island. Experiments involving foreleg fracture under pentobarbital anesthesia in rats with graded removal of the forebrain showed the following: Pituitary island, median eminence island, and hypothalamic island rats, as well as median eminence peninsula and small hypothalamic peninsula rats, (See figure 1) failed to respond with an increase in ACTH secretion. Intact rats showed a doubling of adrenal corticosterone secretion rate

Figure 1: Schematic drawings from Matsuda et al (29) showing from top to bottom: median eminence peninsula, small hypothalamic peninsula and large hypothalamic peninsula. The area in stipple represents brain removed.

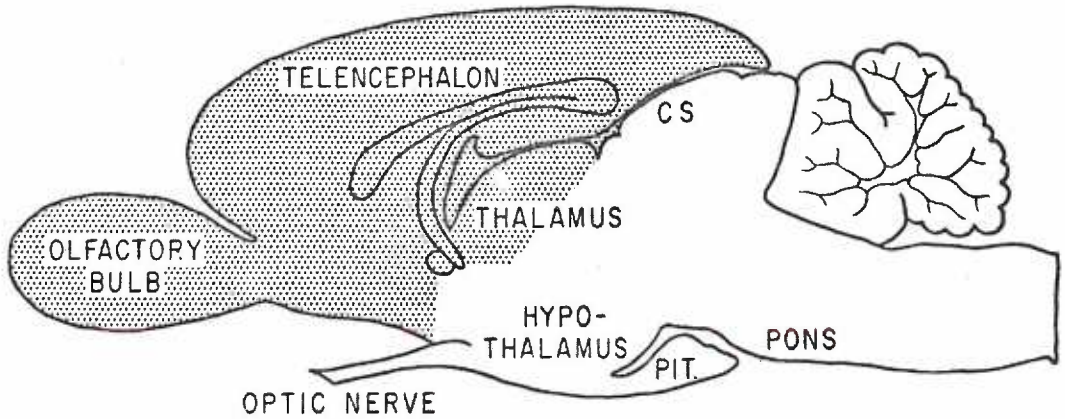
MEDIAN EMINENCE PENINSULA



SMALL HYPOTHALAMIC PENINSULA



LARGE HYPOTHALAMIC PENINSULA



in response to foreleg fracture as did animals with large hypothalamic peninsulas and thalamic peninsulas. Their conclusion was that a direct neural pathway to the hypothalamus not involving higher structures was sufficient to enable the stress of a foreleg fracture to elicit ACTH release since the response occurred in rats lacking all of the cortex and part of the thalamus.

Electrophysiological evidence for pathways

There is also electrophysiological evidence for the existence of nervous pathways from the leg to the hypothalamus (45). Studies of potentials evoked in the hypothalami of cats by contralateral sciatic nerve stimulation have indicated that there may be two routes by which stimuli travel. There was a short latency (7-10 ms.) pathway to the posterolateral hypothalamus thought to involve the medial lemniscus, and a long latency (20-35 ms.) pathway thought to course through the reticular formation. The effect of ipsilateral stimulation was not stated.

Conclusions from the literature

Several conclusions can be drawn from the literature about neural pathways involved in the control of ACTH secretion: 1. ACTH secretion in response to trauma to a limb depends on the integrity of a neural pathway to the hypothalamus. 2. A functional pathway goes from the limb via the spinal cord to the hypothalamus without involving higher structures. 3. Facilitatory and inhibitory areas may exist which can modify ACTH secretion in response to trauma.

Consideration of the problem

I decided that the anatomical characteristics and location of the pathway carrying information controlling ACTH secretion could best be investigated by using the stress of tibial fracture in the pentobarbital-anesthetized rat. This stress is easily duplicated and has been demonstrated to depend on an intact neural pathway. Emotional stimulation of ACTH secretion is probably blocked by pentobarbital anesthesia, permitting a relatively low and reproducible basal level of plasma corticosterone. If a restricted neural pathway exists, it should be possible to interrupt it by the placement of lesions in the central nervous system. A small stereotaxic instrument (46, 47) capable of placing lesions accurately in rat brains was available as was a rat stereotaxic brain atlas.

Since ACTH itself is very difficult to measure, the fluorescence of plasma corticosterone (Kendall's compound "B") may be used as an index of ACTH secretion. Although there are many valid sounding reasons (48) why plasma corticosterone might not accurately reflect ACTH secretion, it has been shown to be a fairly reliable method for estimation of ACTH secretion in the rat (49). Guillemin showed that resting levels of "B" in the intact male rat varied between 9 and 15 micrograms per 100 ml. of plasma (μg%). After stress the values increased up to five times resting values. After total adrenalectomy or hypophysectomy the fluorescence corresponded to 3.5 - 6.5 μg% of "B". There was no change in fluorescence upon stressing

adrenalectomized or hypophysectomized animals. Injection of from 0.2 milliunits to 1.0 milliunits of U. S. P. standard ACTH increased the fluorescence in a linear log-dose relationship. After adrenalectomy the injection of ACTH was without effect on the plasma fluorescence. In female rats the phase of the estrous cycle had no relationship to the fluorescence. These facts show that the fluorescence of peripheral plasma reflects at least the gross changes in corticosterone which may be presumed to be due to alterations in ACTH secretion thought to occur after the above experimental procedures.

The hypothesis for this investigation is that there is a discrete neural pathway from the leg to the hypothalamus which mediates the increase in ACTH secretion caused by a traumatic stress such as a tibial fracture.

MATERIALS AND METHODS

In general, lesions of the central nervous system were made and their effectiveness in blocking ACTH secretion was tested in anesthetized rats by fracturing their tibias and measuring changes in their peripheral plasma corticosterone in response to the tibial fracture.

Animals and their care

Female Sprague-Dawley rats were obtained from Pacord Laboratories, Beaverton, Oregon, or Berkeley-Pacific Laboratories, Berkeley, California. At the time of lesion placement the rats weighed between 130 and 170 g. to conform approximately to the size of rat for which the brain atlas of Konig and Klippel was constructed (50). The animals were housed in wire mesh cages approximately 18x25x40 cm., three to six per cage, in a light and temperature controlled room. The temperature varied between 22 and 25 degrees C., and the lights were on 12 hours per day beginning at 6:00 a.m. They received tap water to drink and Purina lab chow to eat. In all instances at least a week elapsed between the time of receipt of the rats and their sacrifice.

Surgical procedures

Spinal Cord Hemisections

The animals were anesthetized with ether and an incision was made in the skin of the back. The muscles were dissected away from the vertebral column and the neural arch of vertebra T-9 or T-10 was

was removed with rongeurs. A sharp, pointed scalpel was inserted perpendicularly into the cord just lateral to the midline, pushed ventrally down to the bone, and then pulled laterally. Bleeding was often encountered, and hemostasis was facilitated with Gelfoam. The procedure for sham transections was identical except that the cord was not cut.

Partial Medullary Hemisections

The animals were anesthetized with ether, and a midline incision about two cm. long was made in the skin over the skull and neck. The muscles were dissected away to expose the atlanto-occipital joint, and an incision was made in the atlanto-occipital membrane and dura. A sharp, curved scalpel was inserted into the medulla about a millimeter from the midline and directed laterally and ventrally to the floor of the skull. The transection was then completed by pulling the blade laterally. If done properly, the transection remained lateral to the vertebral artery, and in 48 hours the animal was overtly normal.

Placement of Lesions

Prior to mounting on the stereotaxic device the rats were anesthetized with ether, the hair was clipped from the tops of their heads, and a cut was made 3 to 5 mm. long beginning in the external auditory meatus and extending ventrally. This cut facilitated a good fit between the ear bars and the external auditory meatus. After the head was firmly affixed to the stereotaxic device, an incision about one cm.

long was made in the skin over the point of entry to the brain, and the skull was cleaned of adhering tissue. A hole in the skull about 2 mm. in diameter was made with a dental burr, and the electrode was inserted to the desired point with a micromanipulator. The electrode was unipolar and made from 27 gauge stainless steel tubing insulated except for the tip with four coats of formvar enamel baked for 15 minutes at 230 degrees C. between successive coats. The tip exposure was about 0.6 mm. With the stereotaxic device as the indifferent electrode, about 2 ma. were passed through the electrode for seven seconds from a Grass radiofrequency lesion maker. After several seconds of current passage there was normally a muffled pop from inside the braincase caused by the boiling of brain tissue at the electrode tip. The size of the lesions depended on: 1) the amount of tip exposure; 2) the amount of current. The current was turned on, the lesion made, and the electrode removed and cleaned within 12 seconds. Occasionally the location of the lesion necessitated puncturing the superior sagittal sinus or the transverse sinus, and some bleeding would occur. When the bleeding had stopped, the skin was joined with wound clips and the animal replaced in its cage. The procedure for making sham lesions was identical except that no current was passed through the electrode.

Experimental procedure

All experiments were performed between 8:00 and 10:00 a.m. with a minimum of noise and prior disturbance in the animal room.

All animals were anesthetized with intraperitoneal injections of sodium pentobarbital (Nembutal, Abbott Laboratories) 3.5 - 4.5 mg. per 100 g.body weight. The pentobarbital was diluted with 0.9% NaCl to a concentration of 3.5 mg./ml., and the animals received about one ml. per 100 g. body weight. Ten or fifteen minutes later the animals were checked for level of anesthesia. Those which were not unconscious and in a state of muscular relaxation received an additional 0.5 ml. of pentobarbital. Thirty to forty-five minutes after the initial injection, one or both tibias were broken by hand.

Although efforts were made to keep conditions identical for all experiments, a certain variation was possible in noise, degree of anesthesia, responsiveness to a tibial fracture, etc. Therefore all experiments were run as individual entities including the control group and all of the independent variables being tested under the particular conditions of the experiment. Results from several identical experiments were then pooled and the differences between groups tested for statistical significance by appropriate analysis (51).

Sample collection and treatment

Exactly twenty minutes after tibial fracture (TF) the animals were decapitated and trunk blood was collected in 50 ml. beakers containing approximately 100 units of heparin each. The blood was centrifuged, and one milliliter of plasma was obtained from each sample for analysis. Plasma not analyzed within 24 hours was frozen at -25° C. until just before analysis. Frozen samples were analyzed within

one week. Freezing the plasma for this length of time has no effect on the fluorescence[1].

The analysis for corticosterone was a modification of the method of Mattingly (52). The one ml. of plasma was placed into a 40 ml. centrifuge tube and extracted with 15 ml. of dichloromethane (Merck, filtered through a silica gel column to remove non-specific fluorogens). The extraction took about 15 seconds with a Vortex Junior mixer. To speed the separation of the organic and aqueous phases, the mixture was centrifuged at about 200 times gravity for five minutes; the aqueous phase was sucked off and discarded. Exactly 10 ml. of the dichloromethane (now containing corticosterone) were extracted for 15 seconds on a Vortex Junior mixer with a mixture of 75% conc. H_2SO_4 (Mallinckrodt, reagent grade) and 25% absolute ethanol (Rossville, Gold Shield, Commercial Solvents Co.) in a 15 ml. glass stoppered centrifuge tube. Several minutes later the dichloromethane phase was sucked off and discarded. Exactly 12 minutes after mixing, the H_2SO_4 - ethanol phase was placed in a quartz cuvette and its fluorrescence measured in an Aminco-Bowman Spectrophotofluorometer. The sample was activated at 470 mμ and read at 525 mμ. This reading was compared with both a reagent blank containing one ml. of water in place of the plasma and with a corticosterone standard (usually 0.50 μg. in one ml. of water). It will be shown in the results that

1 J.W. Kendall, Personal Communication, 1967.

there is a satisfactorily linear relationship between fluorescence and concentration of corticosterone from 0.01 to 10.00 μg./ml.

Confirmation of placement of lesions and hemisections

No attempt at histological confirmation of the spinal cord hemisections was made. Instead, a simple physiological test was applied: The hemisection was regarded as complete if the animal's right rear leg were paralyzed and the left were functional - the hemisection having been made on the right side. Results from animals not meeting these criteria were discarded.

All other lesions were inspected. The heads were skinned, marked, then immersed in 10% formalin. After several weeks they were transferred to a formic acid-citrate solution for decalcification. Representative brains were infiltrated with and imbedded in paraffin, then sectioned at 10 microns and stained with toluidin blue to ascertain the exact extent of the lesions. The rest of the brains were then sliced with a razor blade by hand and inspected under a 16x dissecting microscope for the presence and location of the lesions. It was possible to adequately judge the position and size of the lesion under the dissecting microscope. Less than 1% of the brains were found to have unsatisfactory lesions. The lesions were regarded as satisfactory if they were within a millimeter of the desired location and if they were larger than a half-millimeter in diameter. The partial medullary hemisections were regarded as satisfactory if they cut at

least the lateral quarter of the medulla.

RESULTS

Validation of Methods

The validity of the analytic technique in my hands was tested by dissolving corticosterone (Mann Research Labs. Inc., New York) in ethanol and then diluting the solution to various concentrations with water. One ml. samples of the solutions containing various concentrations of corticosterone were subjected to analysis for fluorescence as previously outlined. The results are shown in figure 2. Part A shows on a log-log scale that there is a good linear relationship between the concentration of corticosterone ("B") and its fluroescence in the H_2SO_4 - ethanol solution. In the range of 1 to 1000 μg./100 ml. (μg%), the coefficient of linear correlation was .999 ($t=78.6$, $df=12$, $p<<0.001$). Part B shows on a linear scale the repeatability of four measurements of fluorescence on each of three different concentrations of corticosterone. The linear correlation coefficient was .987 ($t=19.4$, $df=10$, $p<<0.001$). The 95% confidence limits are shown in stipple on both graphs.

Spinal Cord Hemisection

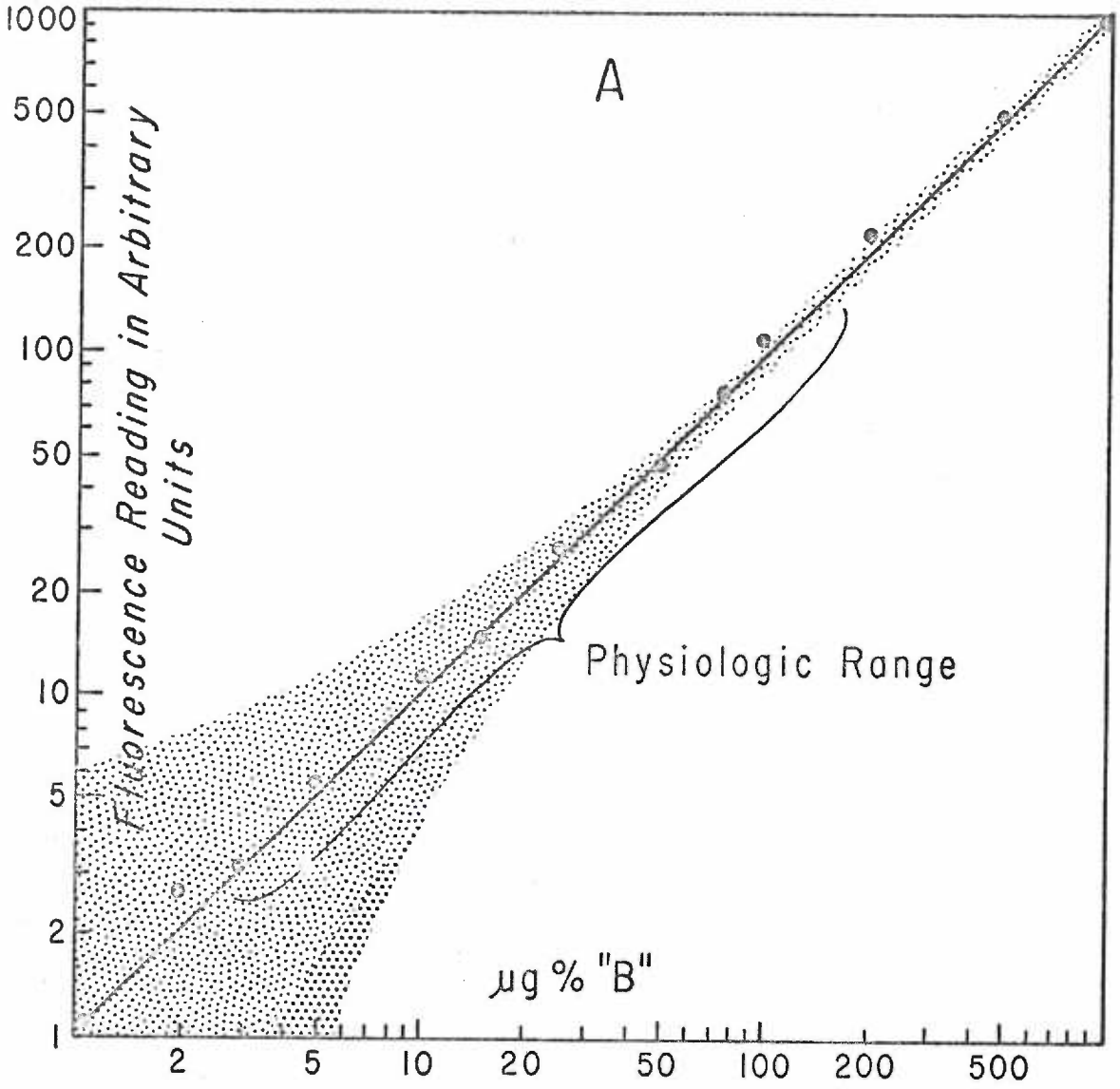
Three experiments involving hemisection of the spinal cord at the level of T-10 were performed. The purpose was to find on which side of the cord the impulses travel that carry information causing the increase in ACTH secretion in response to a tibial fracture. The results pooled from the three experiments are shown in figure 3. A right hemisection at T-10 blocked the rise in corticosterone nor-

Figure 2: Relations between concentration of corticosterone ("B") in micrograms per 100 ml. of water (μg%) and fluorescence reading in arbitrary units.

Part A shows the fluorescence of samples ranging in concentration from one to 1000 μg% on a log-log plot.

Part B shows the fluorescence of four different samples at each of three concentrations of corticosterone on a linear scale.

In both cases the lines are drawn by the method of least squares, and the 95% confidence intervals are shown in stipple.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200
500

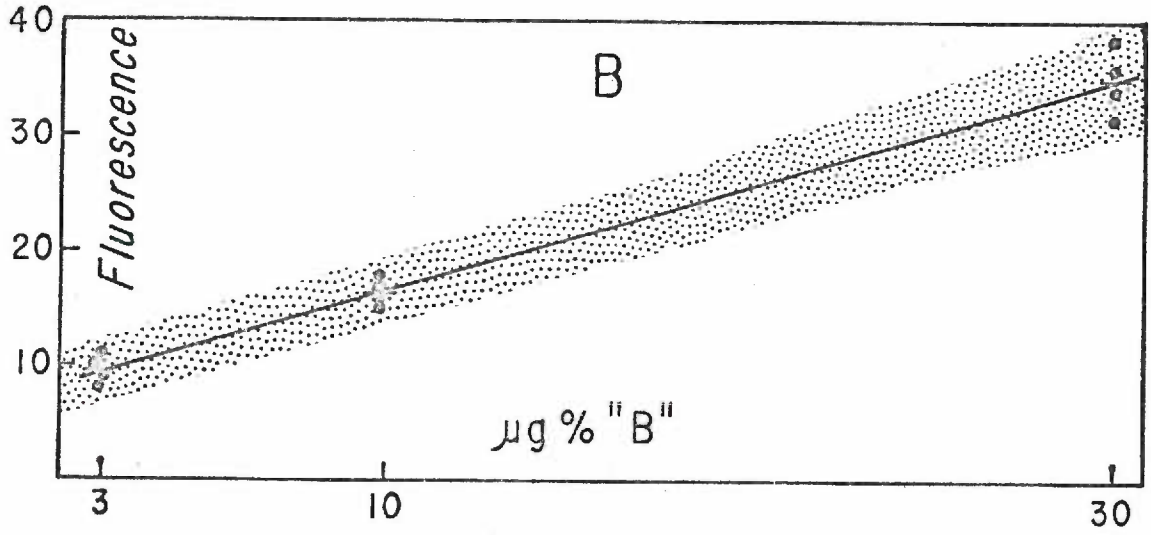
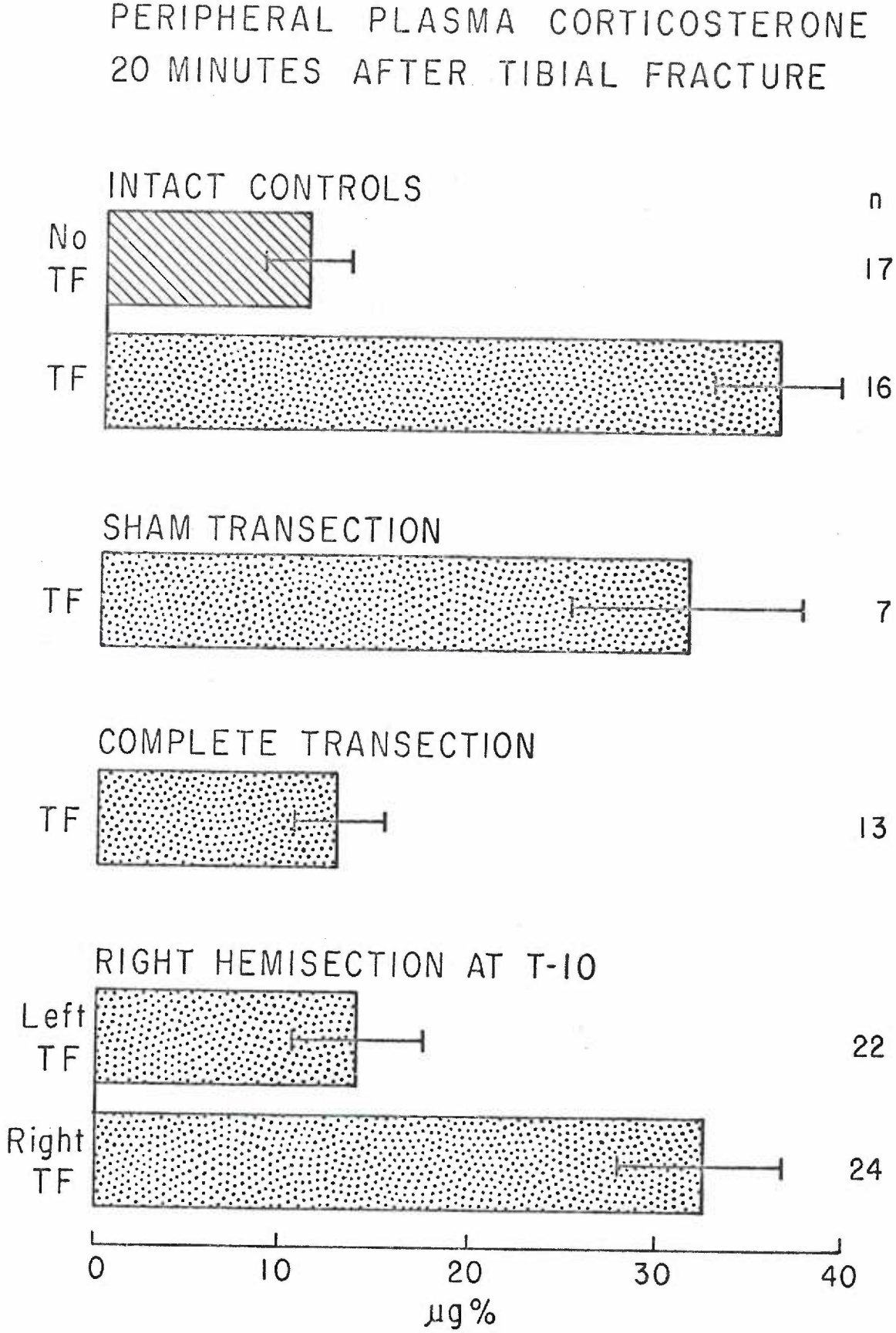

B
40
30
20
10
Fluorescence
µg % "B"
3
10
30

Figure 3: The results from the spinal cord hemisection experiments are shown. The bars represent mean peripheral plasma corticosterone in micrograms per 100 ml. (μg%). The interval on the right side of each bar is the standard error. The number of animals is shown in the right side.


PERIPHERAL PLASMA CORTICOSTERONE
20 MINUTES AFTER TIBIAL FRACTURE
INTACT CONTROLS
n
No TF
17
TF
16
SHAM TRANSECTION
TF
7
COMPLETE TRANSECTION
TF
13
RIGHT HEMISECTION AT T-10
Left TF
22
Right TF
24
0
10
20
30
40
µg%

mally caused by a left tibial fracture (TF), whereas the same hemisection had little effect on the response to a right TF.

Partial Medullary Transections

In an effort to extend the results concerning the laterality of the pathway in the spinal cord, partial medullary transections were made on the right side and the difference between a right and a left TF tested 48 hours after surgery. A histological section of a representative medullary transection is shown in figure 4. The results are shown in figure 5.

The data show that there was a significant difference between the plasma corticosterone levels caused by a right and a left TF ($p<0.05$ in both cases). A partial medullary hemisection on the right side decreased the response to a left TF more than to a right TF.

Unilateral Medullary Lesions

In order to trace the pathway more anteriorly, lesions had to be made inside the braincase. Since transections there are difficult, radio-frequency lesions were placed stereotaxically. The lesions were 2 mm. lateral to the midline, 2 mm. posterior to the interaural line, and 4 mm. ventral to the zero horizontal plane on the right side (-2-4 R2 lesions). (See figure 6). The effectiveness of these lesions was tested at 24 and 48 hours after placement. When the results from the two groups were compared using the t test, the difference was found to be insignificant ($t=.89$, $p>0.3$). The results from the 24 and 48 hour experiments were therefore pooled and are

Figure 4: Shown is a frontal section of a rat medulla bearing a partial transection on the right side. The transection involves approximately the right one-quarter of this picture and is shown by the dashed line. The stain is toluidin blue and the magnification is 23x.

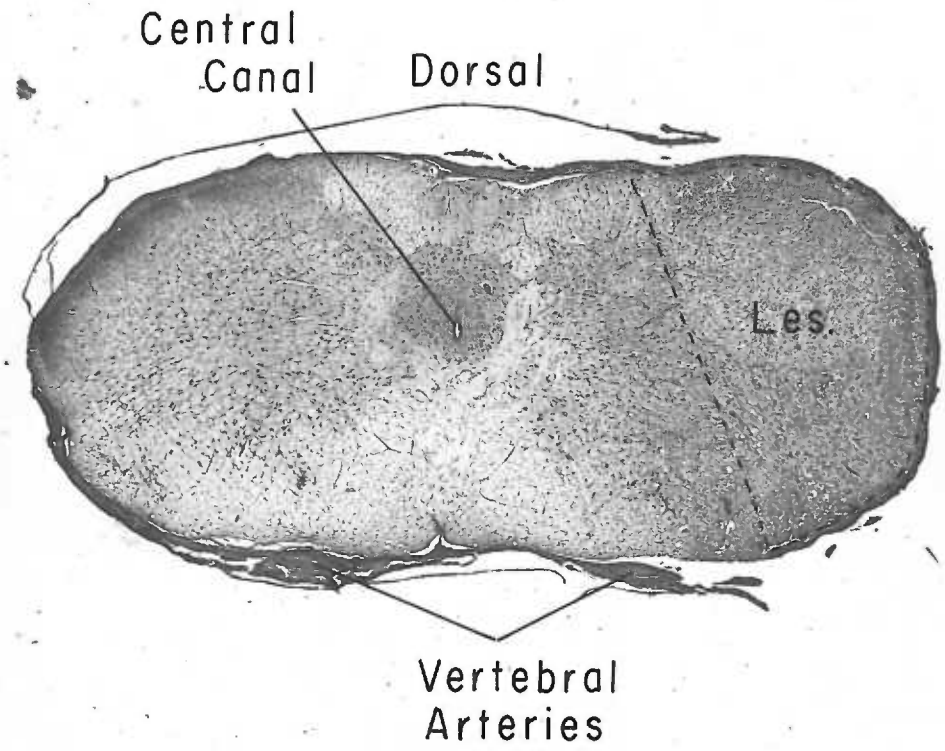

Central
Canal
Dorsal
Les.
Vertebral
Arteries

Figure 5: Shown are the pooled results from the partial medullary hemisection experiments. The bars represent mean peripheral plasma corticosterone ("B") in micrograms per 100 ml. (μg%). The standard error is represented by the lines on the right end of the bars. The number of animals is shown on the right . The upper two bars represent the results from males and the lower two bars from females.

PERIPHERAL PLASMA CORTICOSTERONE

PARTIAL RIGHT MEDULLARY HEMISECTION

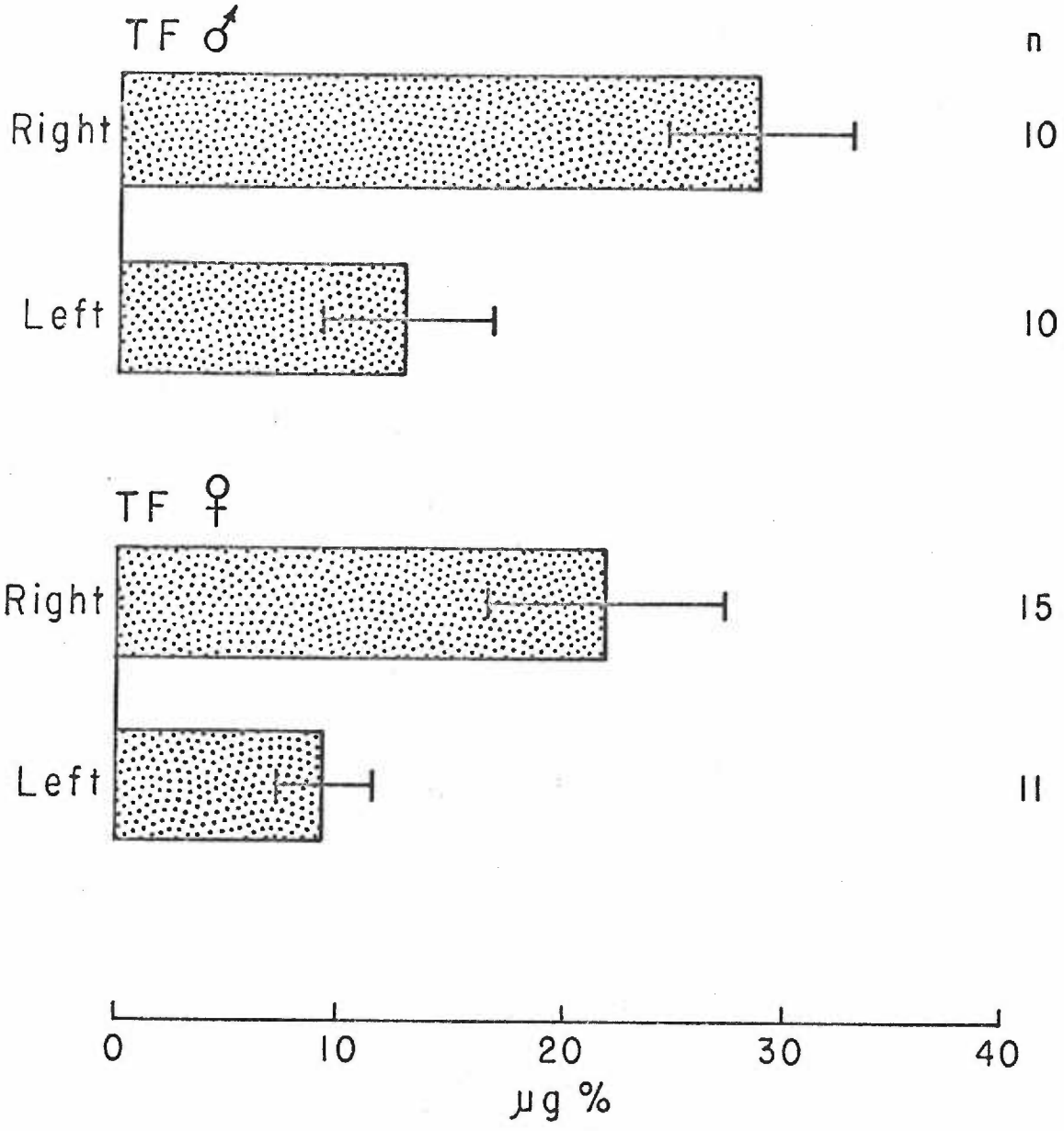
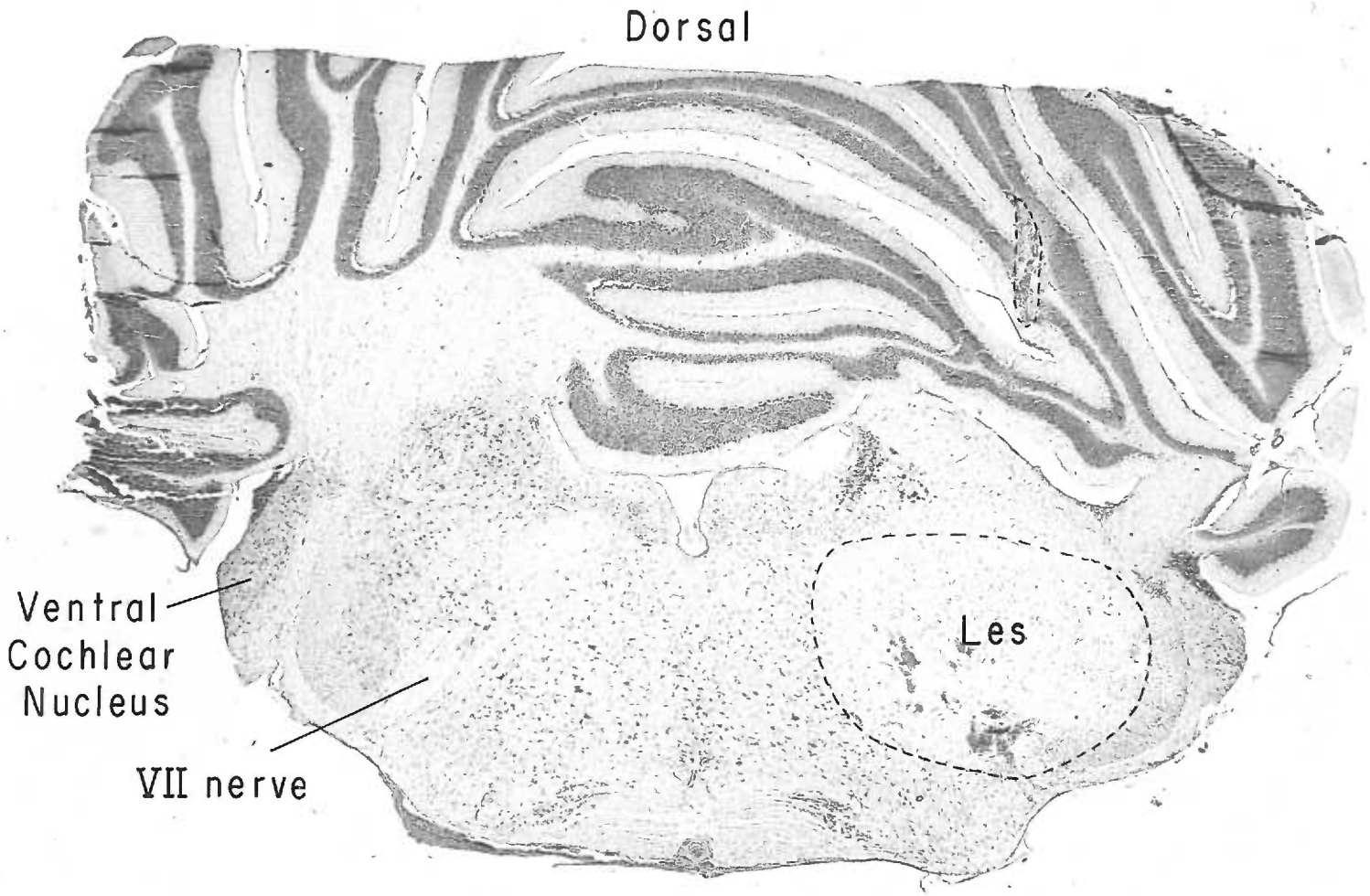


Figure 6: Shown is a frontal section from the medulla approximately 2 mm. posterior to the interaural line. On the left side may be seen the VII nerve and on the right side the lesion occupying approximately two-thirds of the right side of the medulla. The stain is toluidin blue and the magnification 23x.


Dorsal
Ventral
Cochlear
Nucleus
VII nerve
Les

shown in Table I.

Table I

The effect of a unilateral medullary lesion on the response to TF

	Lesion		Control
	Right TF	Left TF	No TF
Peripheral Plasma "B" in μg%, Mean ± S. E.	11.3±2.8	7.8±1.0	8.3±2.1
(# of animals / group)	(18)	(18)	(7)

t=1.2, N.S.

The results pooled from three separate experiments showed no difference between the response caused by a right TF, a left TF, and no TF. Two possible explanations were that the information normally causing ACTH release in response to a TF was reaching the median eminence but was somehow failing to cause a rise in plasma corticosterone or that the information was not reaching the median eminence. If the latter explanation were true there are again at least two possibilities: 1. The lesion was causing a generalized depression of nervous transmission. 2. The lesion had damaged an area specifically necessary for transmission of information controlling ACTH secretion.

Response to ether in rats with medullary lesions

It was possible that the lesion, through some unknown mechanism, had lowered pituitary or adrenal responsiveness thereby preventing the detection of the arrival of signals from the leg to the median eminence. In order to test the integrity of the pituitary-

adrenal axis in the rats with a unilateral medullary lesion, seven rats were anesthetized with ether 20 minutes before sacrifice. The animals receiving ether averaged 73.3±5.4 μg% of corticosterone compared to an average of 9.5±1.8 μg% for the animals receiving TF, as shown in Table I. Even with bilateral medullary lesions (at -2-4, R2 and L2), the animals receiving ether responded with a large and obviously significant corticosterone secretion (78.8±1.5 μg%).

Since ether is thought to act directly on the median eminence to cause ACTH release, it was clear that the median eminence-pituitary-adrenal axis was functioning normally. Therefore the depression of ACTH release in response to a TF in the rats with lesions must have been due to a block occurring between the leg and the median eminence.

Comparison between medullary lesions on the right and left side

Among the possible explanations as to how a unilateral lesion well off the midline can block transmission of nervous information from either leg is that the pathway carrying the information is not bilaterally symmetrical. In order to test the possibility that the nervous impulses caused by fracturing either leg were traveling on the right side only, an experiment comparing the effects of lesions on either side of the medulla was run. Six rats received unilateral lesions 2 mm. to the left of the midline (at -2-4, L2) and six received lesions 2 mm. to the right (at -2-4, R2). Twenty-four hours later both groups received bilateral TF under pentobarbital. The results are shown in Table II.

Table II

Comparison between a Medullary Lesion on the Right and Left side

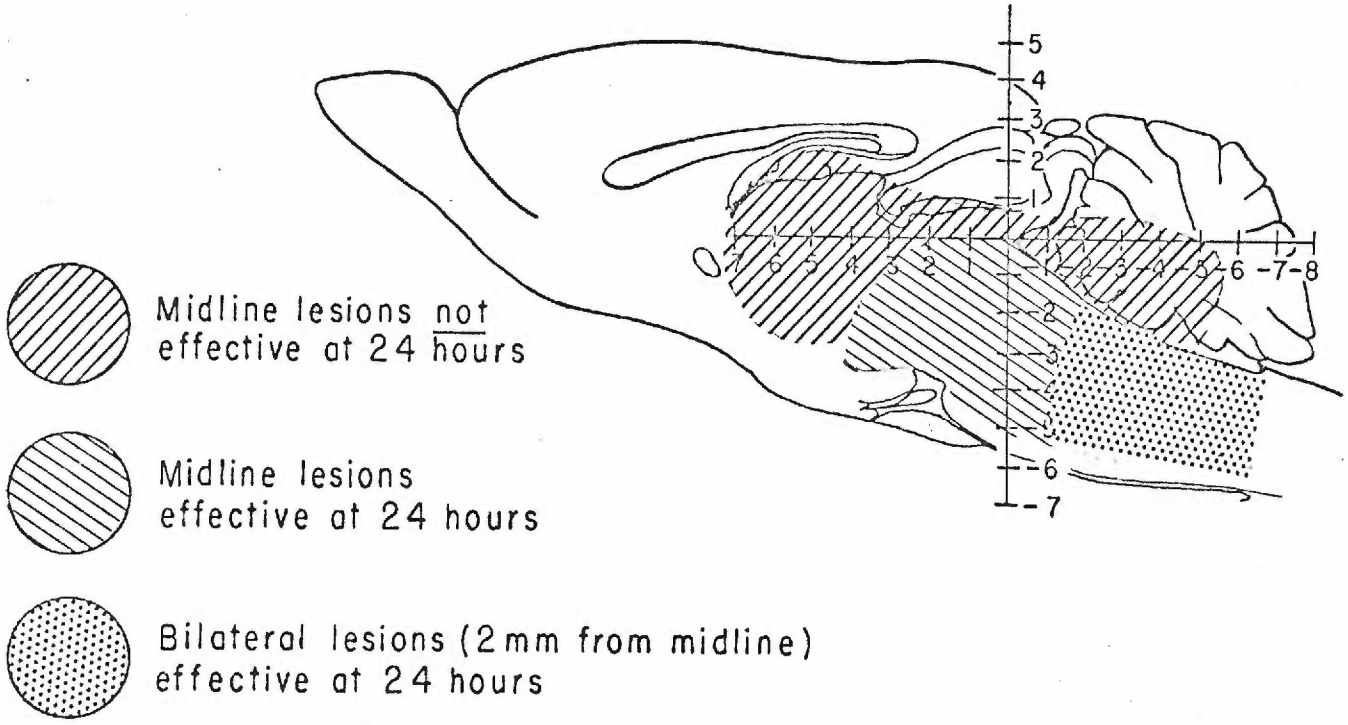
	Left Lesion	Right Lesion
Peripheral Plasma "B" in μg%, Mean ± S. E.	8. 8 ± 0. 7	9. 6 ± 1. 9
(# of animals/group)	(6)	(6)

There was no significant difference between the groups, both failing to respond to TF; thus the hypothesis of bilateral asymmetry of the pathway is not supported, whereas this evidence is consistent with the hypotheses for a generalized CNS depression and for the existence of a bilateral facilitatory area.

Midline Brain Lesions

Various large midline lesions from the thalamus to the anterior medulla (See figure 7) were placed to gain a rough idea of the areas involved in transmitting information mediating the TF-induced release of ACTH. The lesions were approximately one to three mm. in diameter. Their effectiveness in blocking the TF-induced release of ACTH was tested 24 hours later and the results are shown in figure 7. The criterion for effectiveness of a lesion was that three of the four animals in the group must have had plasma corticosterone concentrations below 15 μg% following TF. Animals receiving midline lesions 3 mm. or more behind the interaural line died immediately, and animals receiving midline lesions between 2 and 3 mm. behind the interaural line died upon administration of the usual anesthetic dose of pentobarbital. Death seemed to be caused by respiratory failure. Bilateral lesions 3 to 6 mm. posterior to the interaural line and 2 mm. lateral to the

Figure 7: Shown is a schematic drawing of a midsagittal view of a rat brain. The approximate areas receiving lesions are shown by hatching and stipple. Other areas were not successfully tested.


5
4
3
2
1
-6 -7 -8
-6
-7
Midline lesions not effective at 24 hours
Midline lesions effective at 24 hours
Bilateral lesions (2mm from midline) effective at 24 hours

midline in the medulla were effective by the aforementioned criterion in blocking ACTH release after TF without rendering the animals prone to respiratory failure under anesthesia.

Long term effects of unilateral pontine lesions

Since almost all lesions placed in the brainstem from the medulla to the midbrain would block the release of ACTH after a TF when tested 24 hours after placement, it was possible that the block was due to some non-specific effects of the lesions which disappear with time. Accordingly, a series of animals was tested at different time intervals following lesion placement.

Unfortunately, animals with the unilateral medullary lesions (at -2-4, R2) were incapacitated insofar as feeding and caring for themselves because of a pronounced postural instability. The lesion destroyed some of the vestibular nuclei on the right side and caused the animal to rotate itself in a counterclockwise direction (from the front) on an axis parallel with the length of the animal. The deficit would have necessitated force-feeding the animals for the duration of any long experiment thus adding another variable. Therefore, exploratory lesions were made in an effort to find one which blocked the response to a bilateral tibial fracture for one or two days but which did not incapacitate the animal.

Lesions were placed in the anterior medulla (-2-3, R2) the pons (-1-4, R2), and the anterior pons-midbrain (0-3, 0-4, R2). All lesions were two mm. from the midline. The only group which

showed signs of equilibrium disturbance was the one that received a lesion at -2-3, R2. It had the same "rolling syndrome" as the -2-4, R2 group. The others were slightly ataxic 24 hours later but seemed able to care for themselves. All animals received bilateral tibial fractures under pentobarbital anesthesia 24 hours after placement of the lesions. The results are shown in Table III.

Table III

The effects of various brainstem lesions on the response to TF

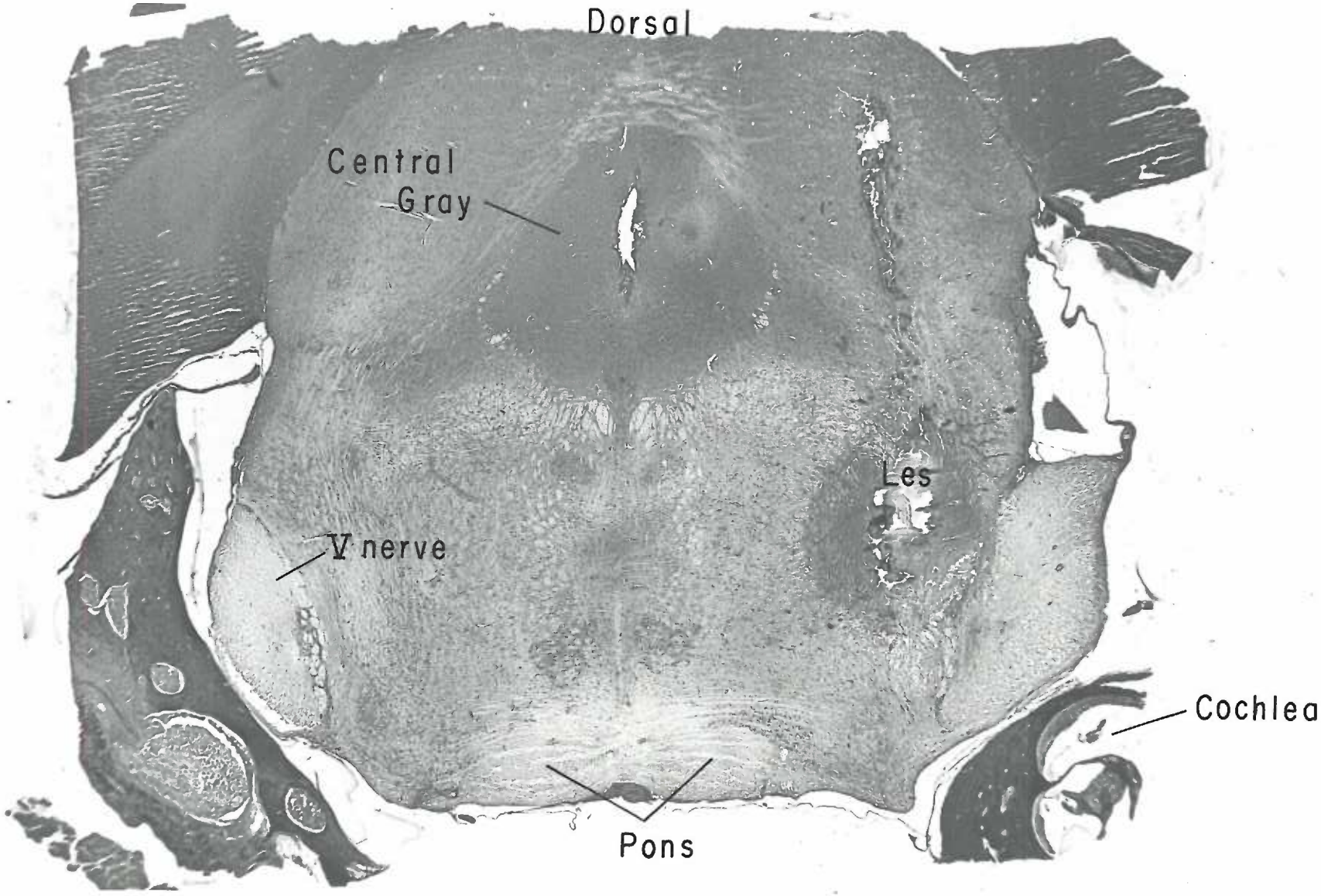
	-2-3R2	-1-4R2	0-4R2	0-3R2
Peripheral plasma "B" in μg%, Mean ± S. E.	4.4±1.4	32.5±10.5	15.2±7.5	3.5±1.2
(# animals/group)	(6)	(6)	(6)	(6)

Since anterior pontine lesions (0-3, R2) depressed the response to TF without causing the "rolling syndrome", animals bearing this lesion were selected for further study. A section of a representative lesion at 0-3, R2 is shown in figure 8.

Lesions were made in groups of rats at various times. Then the different groups were all sacrificed on the same day under identical conditions, the only variable being the length of time from placement of the lesion to sacrifice. Animals were tested at one, four, eight to ten, and greater than 20 days after placement of the lesion. This time schedule was selected because preliminary experiments had suggested the following:

1. There was an initial depression of the response at one day.

Figure 8: Shown is a frontal section in the pons at the level of the interaural line. The lesion and the electrode track are clearly visible on the right side. The stain is toluidin blue and the magnification 23x.


Dorsal
Central
Gray
Les
V nerve
Cochlea
Pons

2. There was a partial recovery of ACTH secretion at 4 days.

3. There was a subsequent depression at 8-10 days after placement.

4. There was a final recovery of the response to tibial fracture at periods greater than 20 days after placement of the lesion. The data from the preliminary experiments were discarded because of a flaw in the protocol which introduced additional, unnecessary variation.

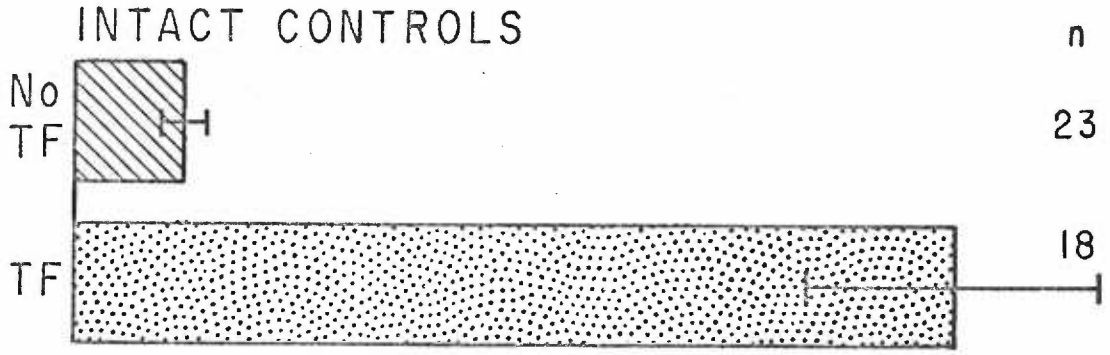
The results from four separate experiments involving 116 rats are pooled in figure 9. The following abbreviations are used: Controls with no TF = CNTF, controls with TF = CTF, animals receiving lesions at one day = les. 1, animals receiving lesions at four days = les. 4, animals receiving lesions at 8-10 days = les. 8-10, and animals receiving lesions at more than 20 days = les. 20. Statistical analysis by the Neuman-Keuls Multiple Comparisons test (51) confirms the following:

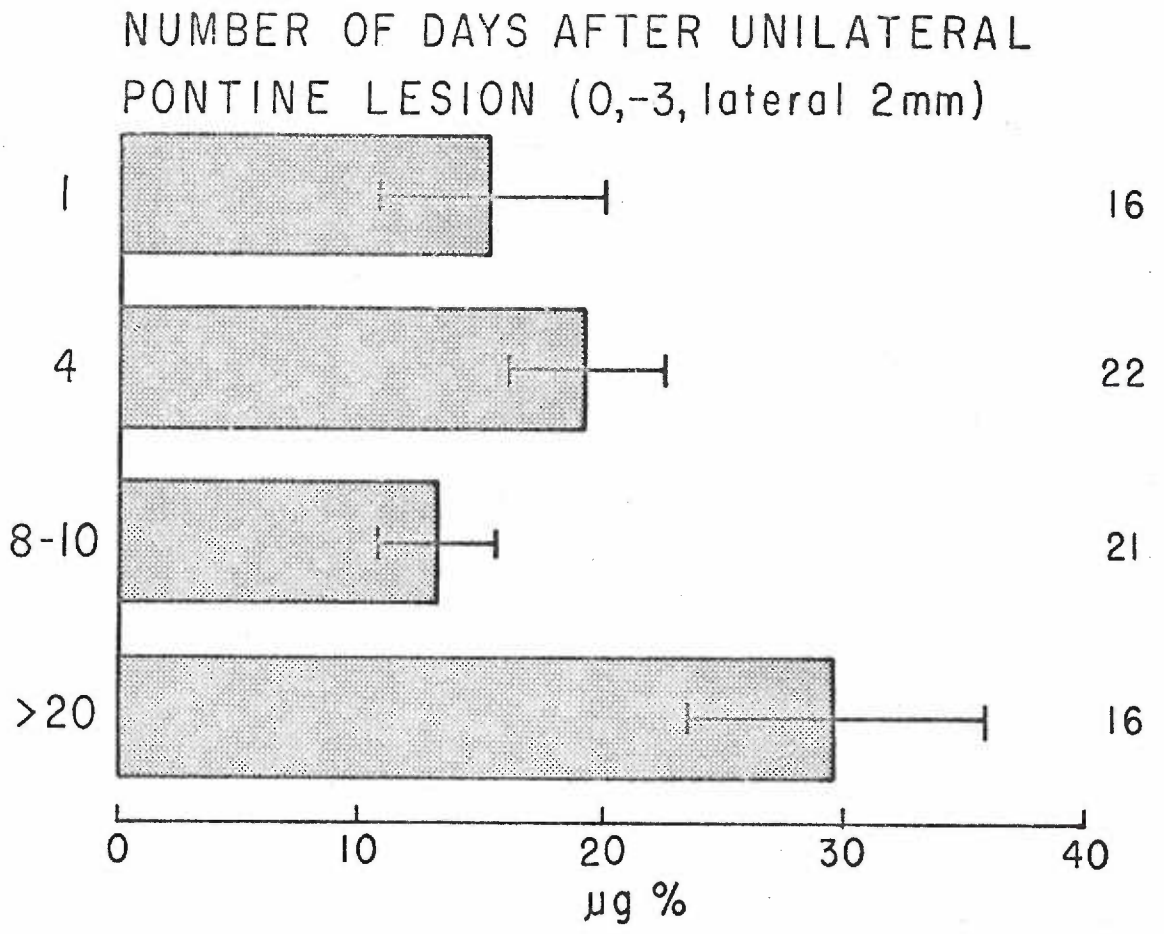
CNTF	<	CTF, les. 20	CNTF	<	CTF, les. 20, les 4
les. 1	<	CTF	les. 1	<	CTF, les. 20
les. 4	<	CTF	les. 4	<	CTF
les. 8-10	<	CTF	les. 8-10	<	CTF, les. 20
p	<	0.01	p	<	0.05

The results indicate that there is a significant depression, lasting at least 8-10 days, of the response to bilateral TF. They also show that there is a significant recovery from this depression at >20 days after lesioning.

Figure 9: Shown are the results from the experiments testing the effect of time after lesion placement on the response to TF. The bars represent peripheral plasma corticosterone in micrograms per 100 ml. (μg%). The lines on the right ends of the bars represent the standard error. The number of animals is shown on the right.

PERIPHERAL PLASMA CORTICOSTERONE
20 MINUTES AFTER TIBIAL FRACTURE





Sham Lesions

It was possible that the depression of the response to TF was due to the surgical procedures necessary for the placement of the lesions rather than to the effects of the lesions themselves. In order to separate the effects caused by the surgical procedures from the effects of a large lesion in the pons, sham lesions were made. The procedure was identical to that for making a real lesion except that no current was passed through the electrode. Thus the term "sham lesion" is not quite accurate, since the electrode did cause some damage when inserted into the brain. Figure 10 shows a section of the pons with a sham lesion made four days before sacrifice. Figure 11 shows the results of one experiment comparing animals with real and sham lesions. Unfortunately, in this experiment, the controls with TF did not respond normally, four of the five animals failing to show a substantial increase in plasma corticosterone. Nevertheless, I feel the experiment is valid. Animals with real lesions showed a depressed response to TF at one, four, and nine days after placement. Animals with sham lesions were also depressed at one and four days, but were significantly ($p<0.05$ by the Neuman-Keuls Multiple Comparisons test) more responsive to TF at nine days after surgery than animals with real lesions. There were no other statistically significant differences between the effects of real and sham lesions. The conclusion is that merely going through the surgical procedures and inserting the electrode into the pons is enough to

Figure 10: Shown is a frontal section in the pons at the level of the interaural line. The sham lesion is shown as the curved electrode track on the right side. The cause of the curve may be an artifact of the fixing and imbedding procedure. The stain is toluidin blue and the magnification is 23x.

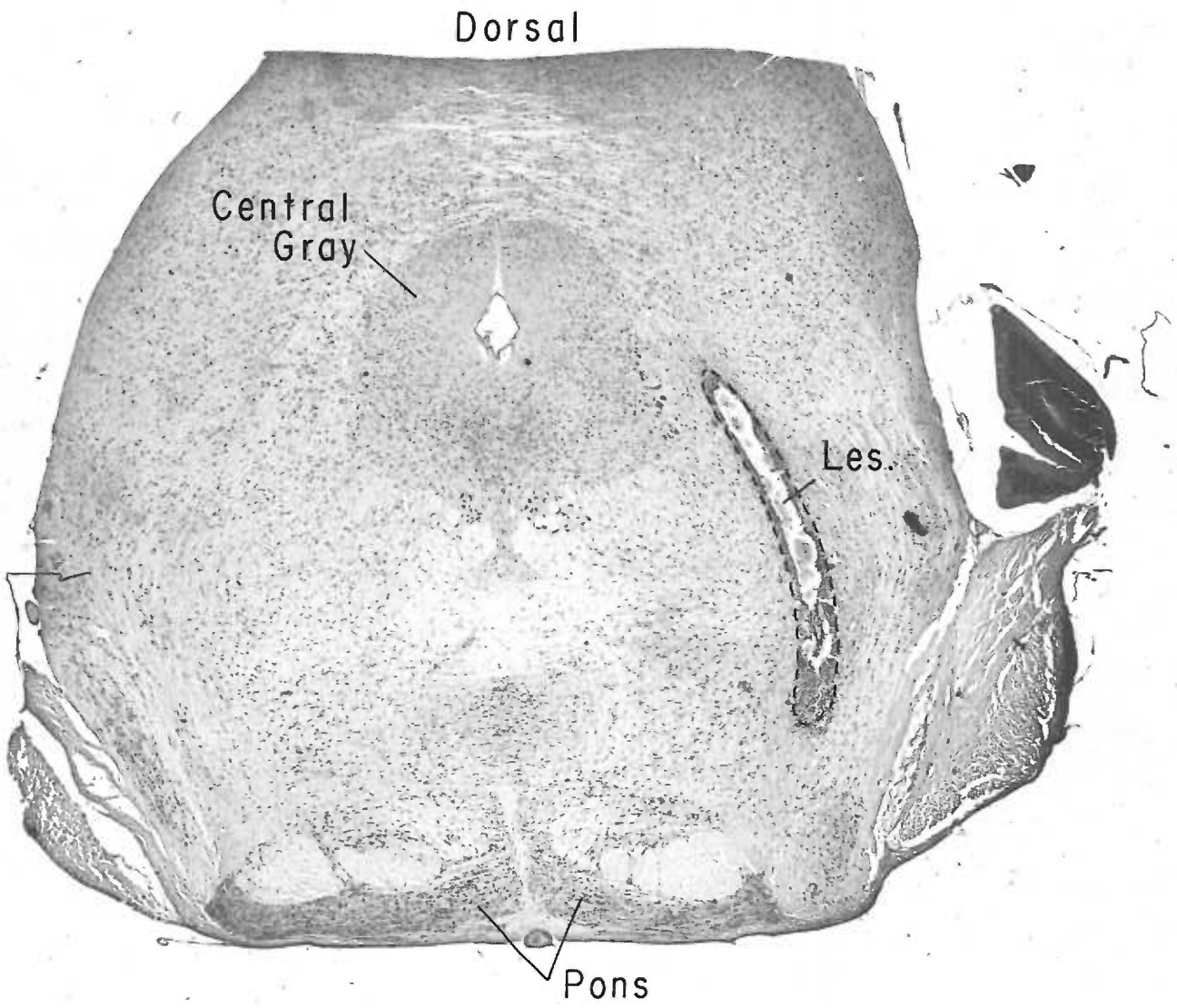
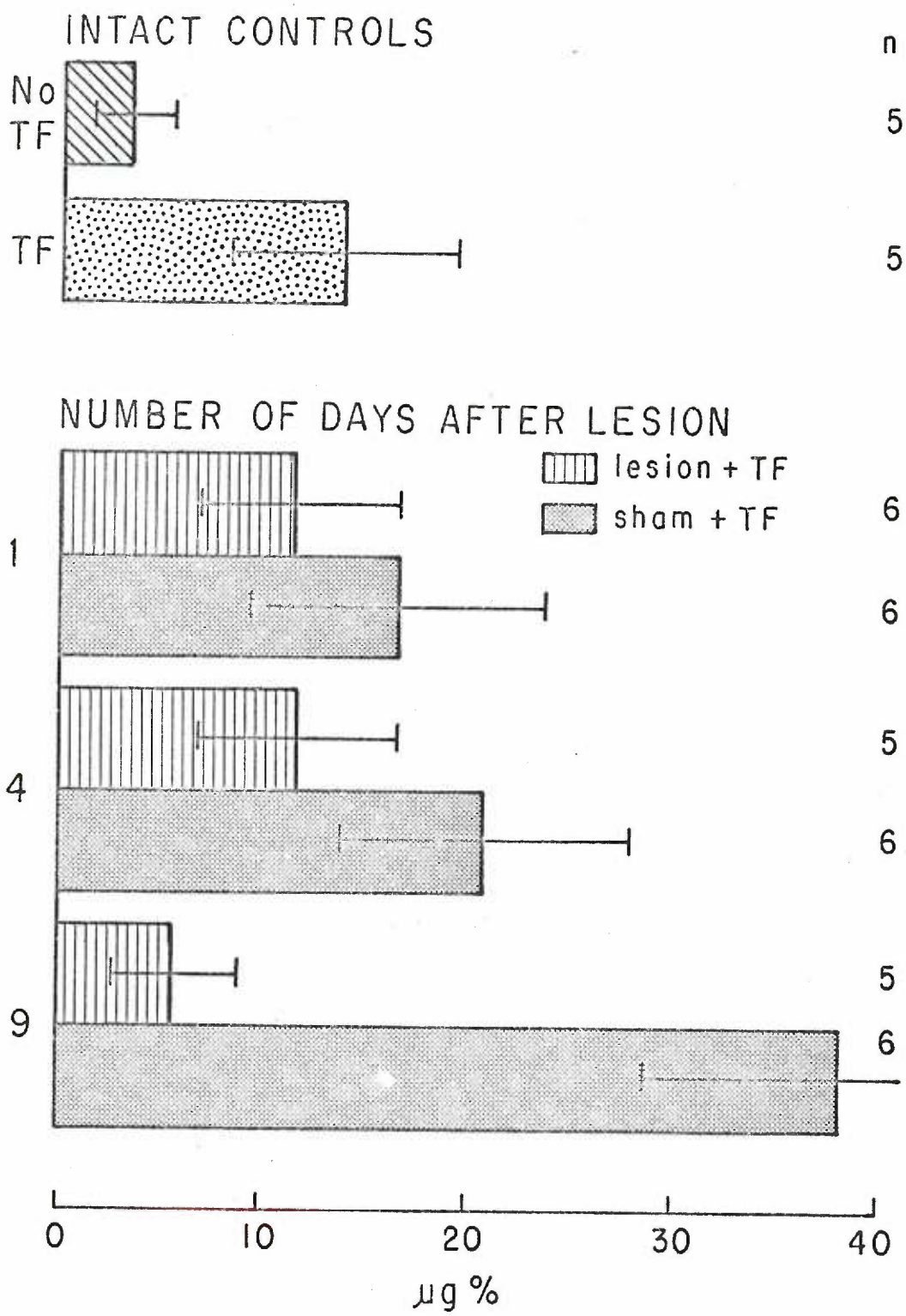

Dorsal
Central
Gray
Les.
Pons

Figure 11: Shown are the results from an experiment comparing real and sham lesions. The bars represent mean peripheral plasma corticosterone in micrograms per 100 ml. (μg%), and the lines on the right side of the bars represent the standard error. The number of animals is shown on the right.

PERIPHERAL PLASMA CORTICOSTERONE



depress ACTH secretion in response to a TF for several days, and that the depression is gone by nine days after the surgery.

Comparison between one and two tibial fractures in intact rats

If the unilateral pontine lesion were damaging part of the pathway over which impulses controlling ACTH secretion traveled, the response to bilateral TF might be impaired in proportion to the number of fibers damaged. However, if a certain minimum number, say half, of the fibers is sufficient to allow a maximum response, then there should be no diminution in the response until more than half of the fibers have been damaged.

In an intact rat a bilateral TF would presumably activate twice as many fibers to the brain as a unilateral TF. In order to determine if one TF could cause as much corticosterone secretion as bilateral TF, an experiment comparing the two was run with intact rats. The results are shown in Table IV.

Table IV

Comparison between one and two tibial fractures in intact rats

	Unilateral TF	Bilateral TF	No TF
Peripheral Plasma "B" in μg%, Mean ± S. E.	35.6±3.3	34.8±3.4	3.9±0.5
(# of animals/group)	(18)	(18)	(7)

t=0.24, N.S.

There was no difference between the response caused by one and that caused by two tibial fractures in intact rats. A unilateral TF can

activate sufficient numbers of fibers to cause a maximum response of ACTH secretion under the conditions of the experiment. Therefore, apparently no more than half of the fibers from the tibias to the pons need be activated to cause this maximum response.

The effect of various pontine lesions on the response to bilateral tibial fracture

If all lesions in the pons depressed the response to bilateral TF equally, then the depression might be ascribed to some sort of general interference with nervous transmission through the pons. However, if lesions in certain areas of the pons depressed the response whereas others did not, the implication might be that the certain areas had something specifically to do with ACTH secretion. Lesions were made in several areas of the pons and their effect on ACTH secretion in response to a bilateral TF was tested 10 days later. The period of 10 days was chosen because some lesions in the pons (See figure 8) significantly depressed ACTH secretion when tested at that time, and I wanted to see if that depression were due to damage to an area specific for ACTH secretion. Figure 12 is a schematic drawing of a section in the pons at the level of the interaural line and shows the areas where lesions were made. The results are in the legend. Lesions at "B" and "C" significantly depressed ACTH secretion in response to a TF when compared to the response of animals with no lesion. Lesions in areas "A", "D", "E", and "F" did not depress the response, although the group with the lesion at "A" was

Figure 12: Shown is a schematic drawing of a frontal section in the pons at the level of the interaural line. The approximate areas with lesions are shown in hatching. The peripheral plasma corticosterone following TF of rats bearing the various lesions is as follows: (number of animals per group in parentheses) The numbers are the mean ± S.E. in micrograms per 100 ml. (μg%).

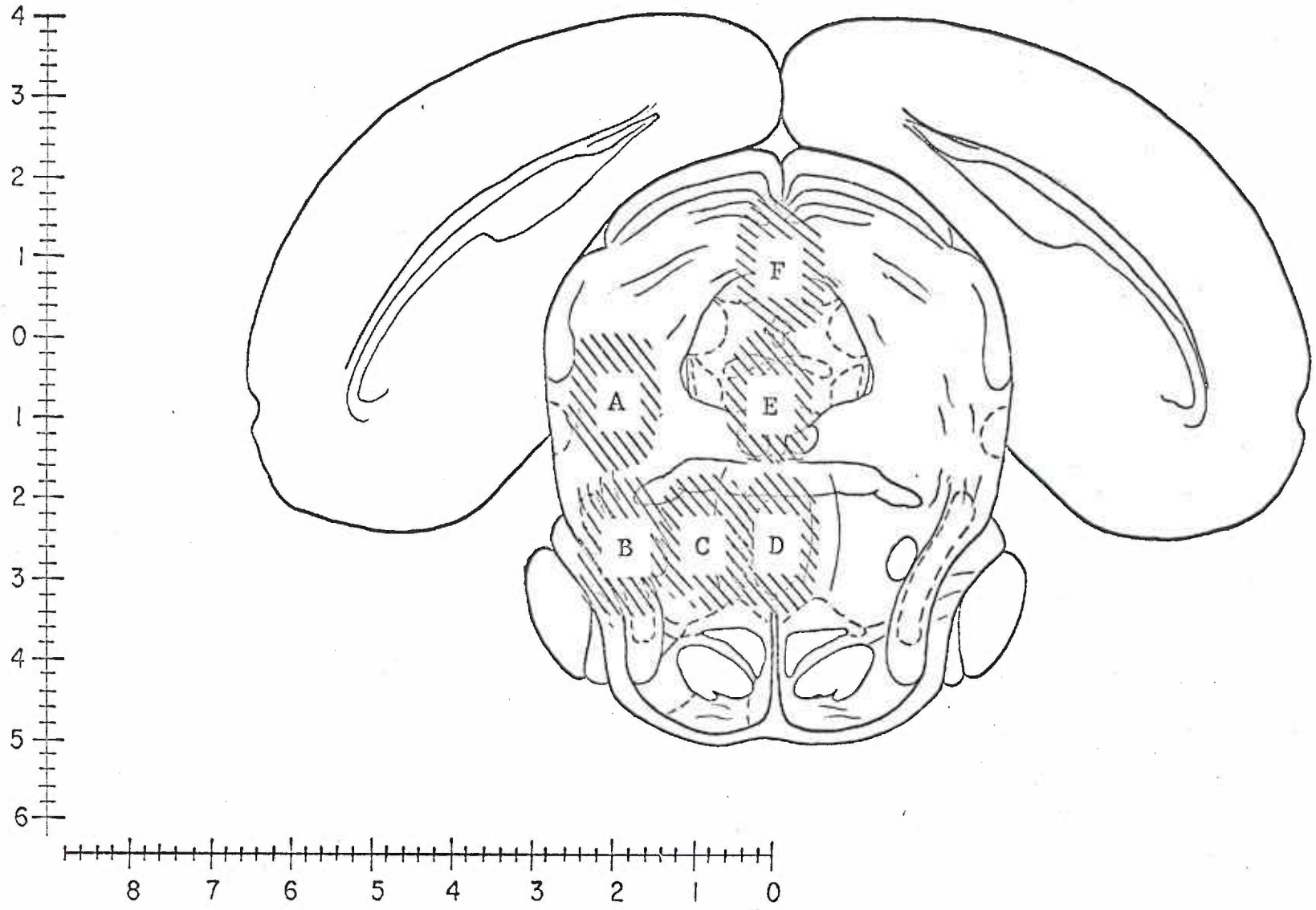
"A" = 29.3±4.6 (21) "D" = 34.9±3.3 (35)

"B" = 22.1±3.1 (38) "E" = 42.1±6.5 (19)

"C" = 21.7±3.1 (16) "F" = 36.1±6.1 (22)

Controls TF = 45.3±4.2 (24)

Controls No TF = 6.0±0.6 (20)


4
3
2
1
0
1
2
3
4
5
6
8
7
6
5
4
3
2
1
0
A
B
C
D
E
F

close to the statistical criterion of $p<0.05$. The lesions at "B" are in the same area used previously (See figure 8), and they did significantly ($p<0.01$) depress the response to a TF compared to lesions at "D" 2 mm. away. (See figure 13). Thus not all lesions in the pons depress the response to TF when the animals are tested 10 days after placement of the lesion. And therefore, the depression caused by the unilateral lesion (0-3, R2) is probably not due to a generalized interference with nervous transmission through the pons.

The effect of right tibial fracture compared to a left tibial fracture at 10 and >30 days after placement of a unilateral pontine lesion

Since a generalized depression of CNS function is probably not present 10 days after lesion placement, it should be possible to learn on which side of the pons the pathway(s) is (are) located by comparing the effects of a right and a left TF. Unilateral pontine lesions similar to the one shown in figure 8 were used. The difference was tested both at 10 days after placement of lesions when there was significant depression of the response to bilateral TF and at more than 30 days when there was no significant depression. Figure 14 shows the results obtained at 10 days after placement of lesions and figure 15 shows the results at more than 30 days. Analysis by the t test shows that for both time periods the probability that the difference between a right and a left TF was due to chance alone is less than 0.01. Since with a lesion on the right side a left TF causes no response, and a right TF causes the same response as a bilateral TF,

Figure 13: Shown is a section in the pons at the level of the interaural line. The lesion is in the midline just ventral to the central gray. The stain is toluidin blue and the magnification 23x.

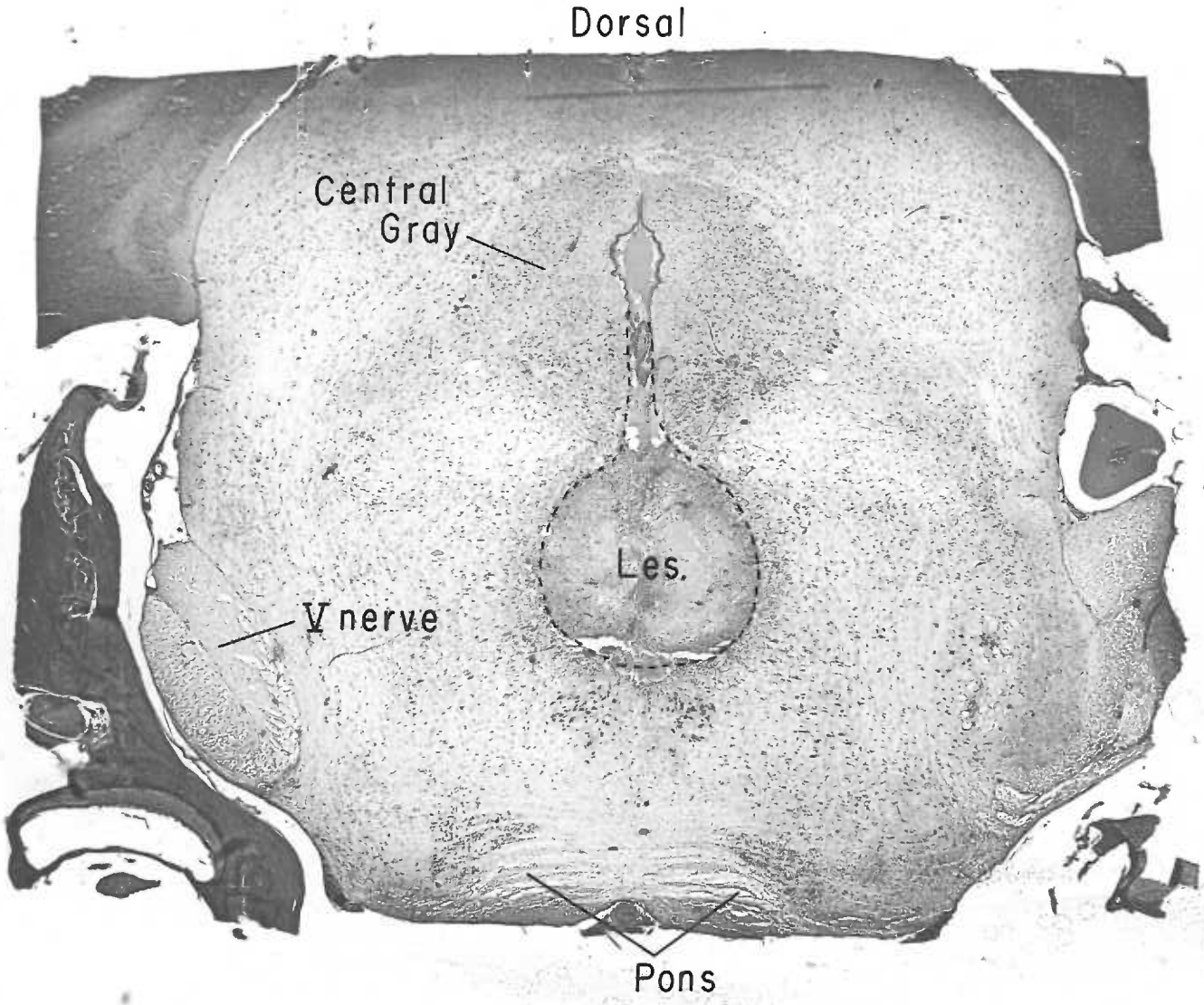

Dorsal
Central
Gray
Les.
V nerve
Pons

Figure 14: Shown are the results comparing the effects of right and left TF with a right unilateral pontine lesion placed 10 days before sacrifice. The bars represent peripheral plasma corticosterone in micrograms per 100 ml. (μg%). The lines on the right end of the bars represent the standard error. The number of animals is shown on the right.

PERIPHERAL PLASMA CORTICOSTERONE

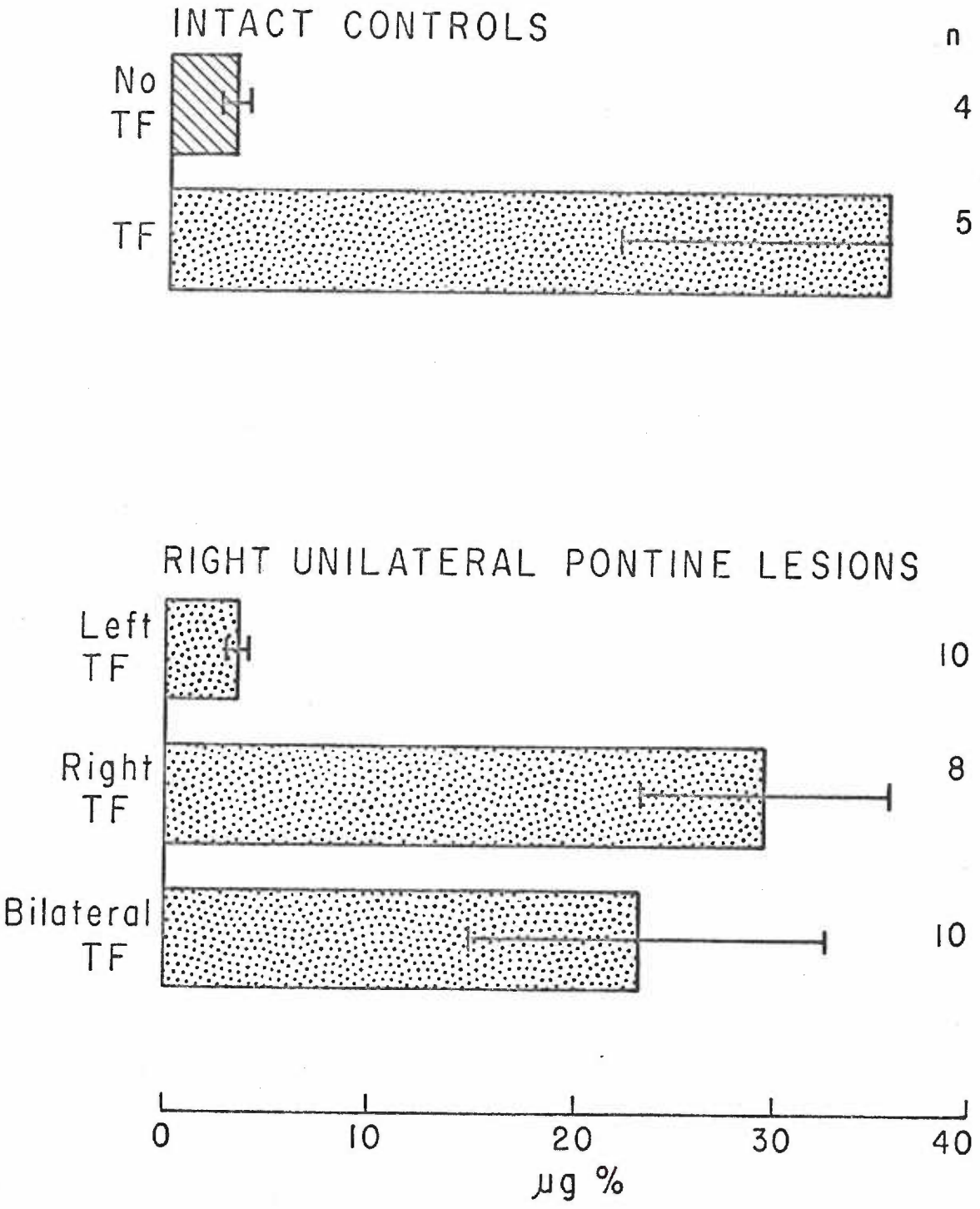
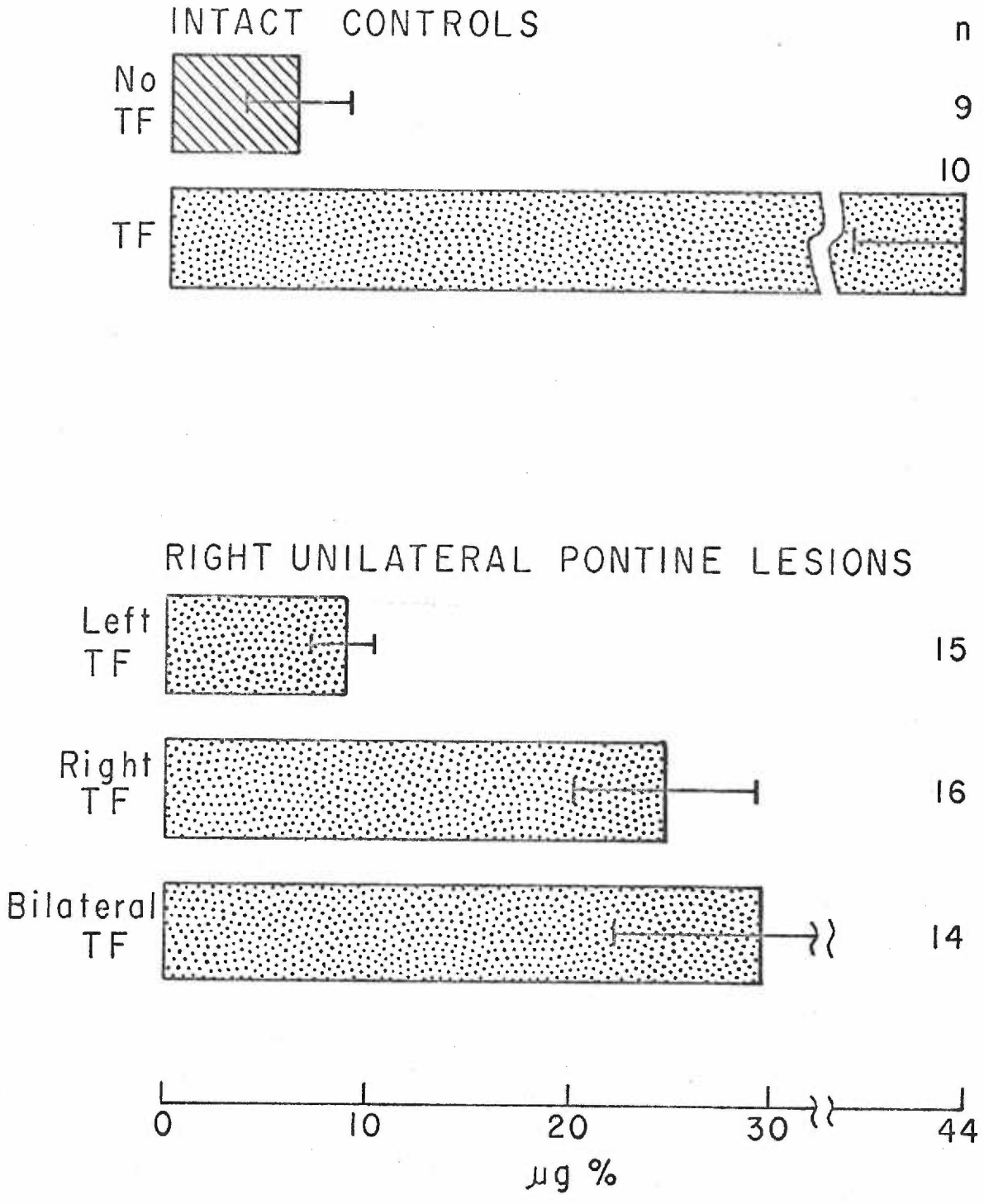


Figure 15: Shown are the results of two experiments comparing the results of right and left TF with a right unilateral pontine lesion placed more than 30 days before sacrifice. The bars represent mean peripheral plasma corticosterone in micrograms per 100 ml. (μg%). The lines on the right of the bars represent the standard error. The number of animals is shown on the right.

PERIPHERAL PLASMA CORTICOSTERONE



the pathway must (a) be almost entirely contralateral to the fractured tibia and (b) pass through the pons near the lesion (located at 0-3, R2).

Localization of the pathway(s)

From the latter series of experiments it appeared that the pathway must pass near the locus 0-3, 2 mm. lateral to the midline, but it was possible that other contralateral lesions in the pons would have similar effects. To make sure that the pathway was confined near the previously mentioned locus, an experiment was performed comparing the effects of various lesions on the release of ACTH caused by either a right or a left TF. Figure 16 is a schematic drawing of a section at the level of the interaural line showing where lesions were placed 10 days before sacrifice. The results shown in figure 17 indicate that the two lesions in the reticular formation ("A" and "B") depressed the response to tibial fracture and also tended to show a difference between the response caused by a right and a left TF. These differences between the responses to left and right TF were barely significant ($t=2.16$, $df=5$, and $t=2.01$, $df=9$ respectively, $p<0.05$ in both cases) even when using the more lenient one-tailed test. However, the use of the one-tailed t test in this instance is justified, since the previous set of experiments showed me that the pathway is contralateral, and therefore I would expect the response to a right TF to be higher than to a left TF. The lesion at "C" caused a significant ($t=2.41$, $df=20$,

Figure 16: Shown is a schematic drawing of a frontal section in the pons at the level of the interaural line. The areas receiving lesions are shown in hatching. The results are shown in Figure 17.

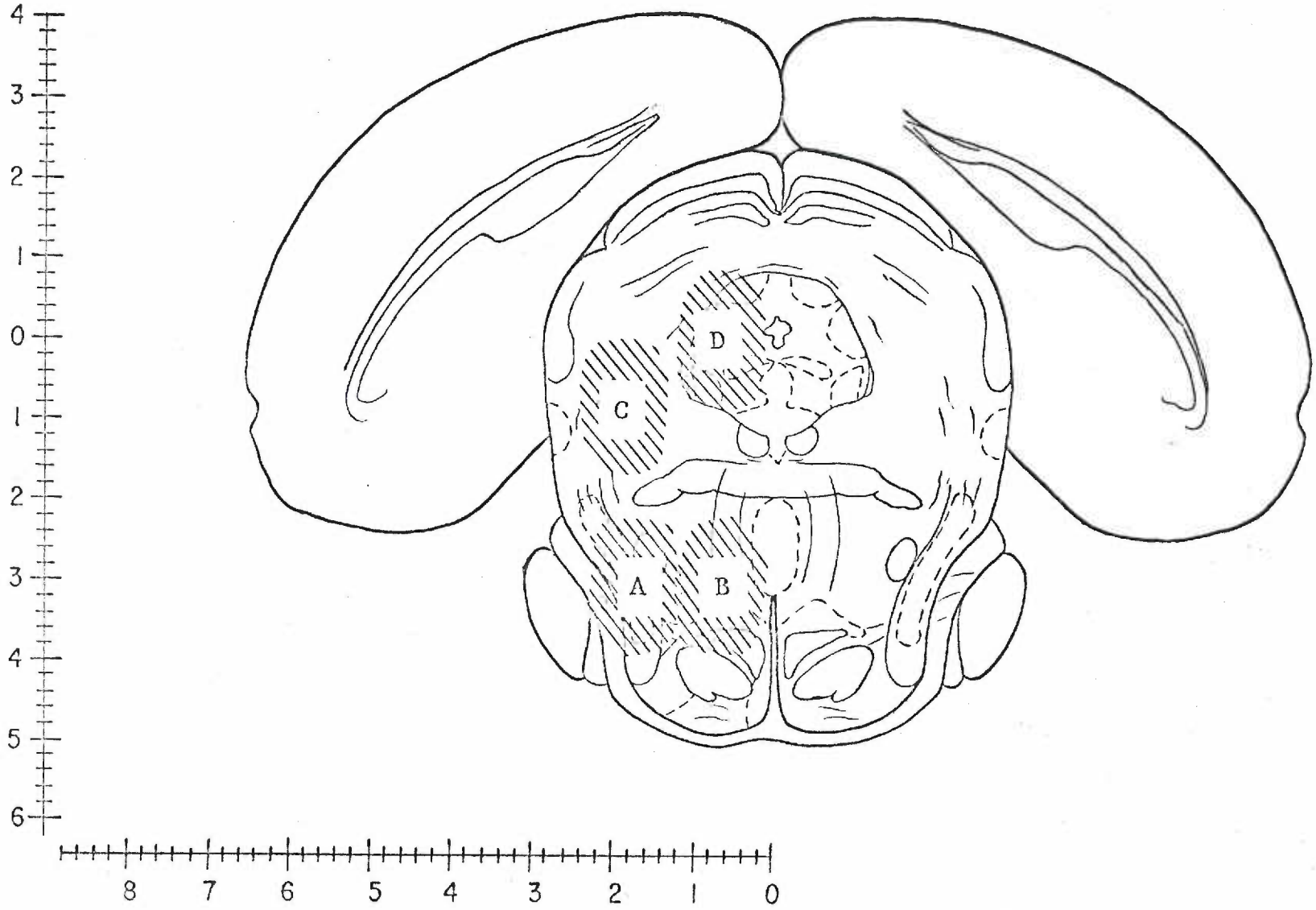
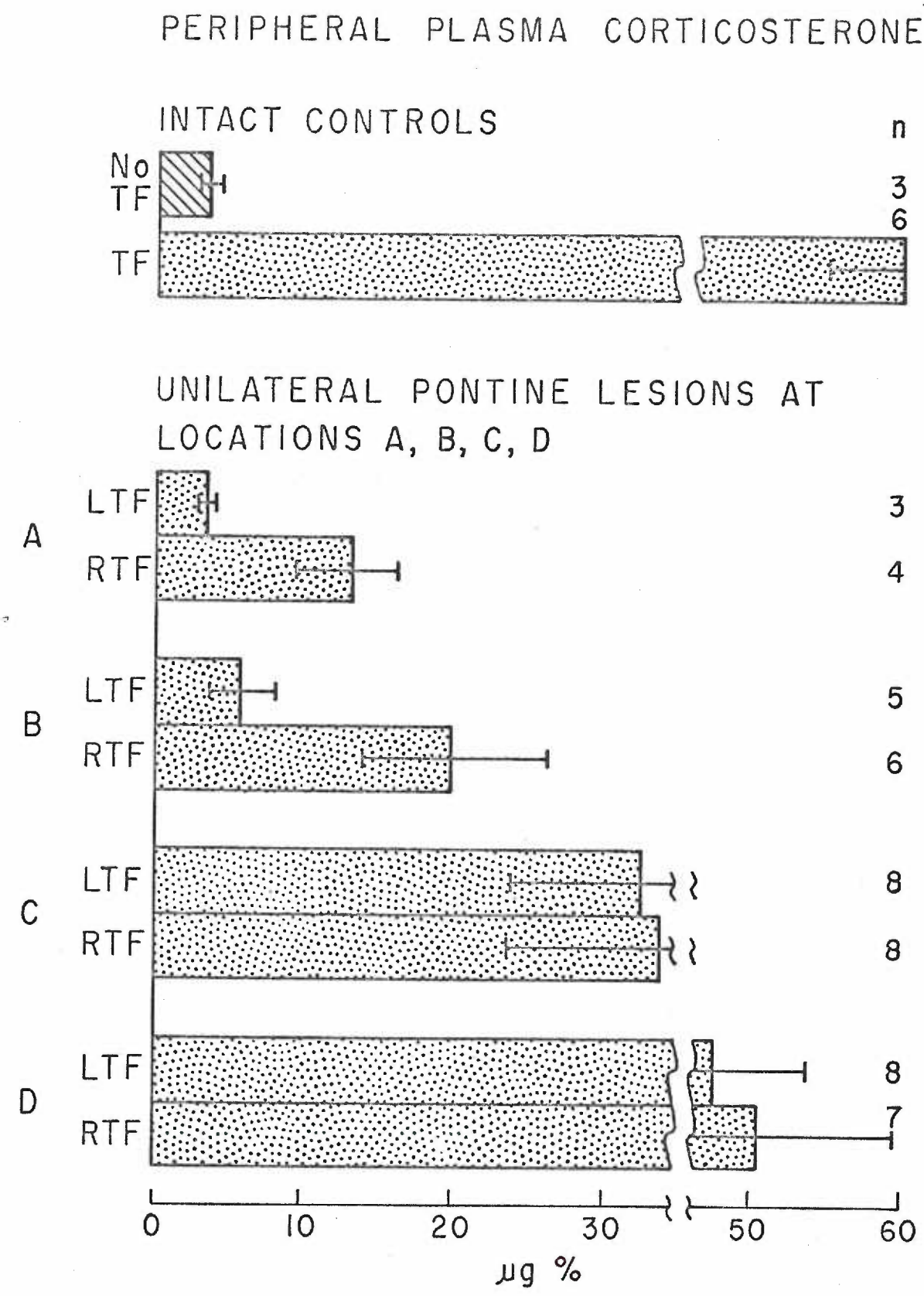

4
3
2
1
0
1
2
3
4
5
6
8
7
6
5
4
3
2
1
0
D
C
A
B

Figure 17: Shown are the results of an experiment performed 10 days after placement of lesions at locations "A" through "D" shown in Figure 16. The response to right TF was compared to that for left TF with each lesion. The bars represent mean peripheral plasma corticosterone in micrograms per 100 ml. (μg%). The lines on the right end of the bars represent the standard error. The number of animals is shown on the right.


PERIPHERAL PLASMA CORTICOSTERONE
INTACT CONTROLS
n
No TF
3
TF
6
UNILATERAL PONTINE LESIONS AT LOCATIONS A, B, C, D
A
LTF
3
RTF
4
B
LTF
5
RTF
6
C
LTF
8
RTF
8
D
LTF
8
RTF
7
0
10
20
30
50
60
µg %

$p<0.05$) depression of the response but there was no difference between a right and a left TF. The lesion at "D" did not significantly depress the response ($t=1.24$, $df=19$, $p<0.2$) and there was no difference between a right and a left TF.

One might conclude that the lesions "A" and "B" had damaged the pathway because they produced a difference between a right and a left TF, and they also caused some sort of damage which depressed ACTH secretion. The lesion at "C" was probably not in the pathway since there was no difference between a right and a left TF, but it did cause depression of ACTH secretion in response to TF. Lesions in area "D" did not seem to affect ACTH secretion.

Effects of Pentobarbital

Three experiments were done to test the effect that pentobarbital had on the response to TF in rats with lesions. The lesions used were bilateral in the anterior medulla (-2-4, R2.5, L2.5) 2.5 mm. from the midline. Unanesthetized animals that did not receive TFs were handled 20 minutes before sacrifice as a control for the handling necessary for TF. The animals had been handled twice a day for the week preceding the experiment to minimize the rises in ACTH caused by handling. The results are shown in Table V.

Table V

The effects of pentobarbital on the response to tibial fracture

		Mean Plasma "B" ± S.E. in µg% (# animals/group)			
		Intact		Lesion	
Pento-barbital	No TF	8.2±2.6 (6)	=p<0.01	7.5 ±1.4 (6)	±N.S.
	TF	35.4±6.3 (6)		10.0±1.6 (6)	
No Pento-barbital	No TF	38.7±7.3 (8)	=p<0.01	49.3±3.3 (12)	=p<0.01
	TF	70.6±4.4 (7)		75.9±3.9 (15)	

The results indicate that these anterior medullary lesions are effective only when the animals are anesthetized with pentobarbital. In addition, since all groups receiving pentobarbital responded less than any non-anesthetized group, it is possible that the non-anesthetized animals were receiving input to the hypothalamus over more channels than the anesthetized animals.

DISCUSSION

The results from these experiments confirm and extend the observations of previous investigators that an intact nervous pathway is necessary to carry impulses mediating the release of ACTH in response to traumatic stress under pentobarbital anesthesia. Experiments involving hemisections of the spinal cord and medulla showed that a pathway mediating the response to tibial fracture is contralateral to the fractured tibia. Unilateral radio-frequency lesions placed in the medulla and pons depressed the response to bilateral TF when performed 24 hours after lesion placement. The depression lasted for at least 10 days and was partially recovered at twenty days. Then it was shown by experiments conducted 10 days after lesion placement that destruction of certain areas of the pons resulted in the depression, but lesions several mm. away were not effective. By testing the animals at 10 and more than 30 days after lesion placement, it was demonstrated that the pathway in the pons was also contralateral to the fractured tibia.

Thus destruction of a certain area in the pons completely blocked the response to a contralateral TF and caused a depression of the response to bilateral or ipsilateral TF. This depression cannot be explained as being due to a general depression of nervous transmission through the pons or as being due to elimination of some of the fibers transmitting impulses from the leg to the pons. Finally, it was shown that the medullary lesions were effective only when the animals

were anesthetized with pentobarbital.

Throughout the results it may have been noticed that there was a wide variance in the corticosterone levels both in control and experimental groups. Some days the animals seemed more responsive to TF, on other days they were less responsive. It is possible that some of this variation was due to errors in analyzing for corticosterone. However, I feel that it is at least partially a reflection of the extreme lability of the response. It is true that the animals were anesthetized with pentobarbital at least an hour before they were sacrificed and that all precautions were made to ensure quiet in the animal room before and during the experiment, but even these measures were apparently not adequate to equalize the response for every experiment.

Interpretation of the effects of pentobarbital

The fact that the lesions are effective only in pentobarbital anesthetized animals implies that there may be two pathways by which TF can elicit pituitary-adrenal activation - one sensitive to pentobarbital and one not. It further implies that the lesion is not interfering with transmission of nervous activity in the pathway sensitive to pentobarbital.

The limbic system has been implicated in the release of ACTH following emotional stress and could be the pentobarbital sensitive component. Ganong (48) made lesions in the amygdalae of dogs and found that ACTH release from the trauma of surgery was normal, but

the release of ACTH after the stress of immobilization was impaired. A similar impairment was produced by lesions in the pathway from the amygdala to the tuberal region of the hypothalamus. In addition, Mason (53) has shown a rise in plasma 17-hydroxycorticoids following electrical stimulation of the amygdala in monkeys.

Even with large bilateral lesions in the medulla, conscious rats seemed able to interpret noxious stimuli as pain. Therefore in conscious rats with lesions pain may have acted as an emotional stimulus. The emotional stimulus could then have excited the pathway from the amygdala to the hypothalamus, possibly bypassing another more direct pathway to the hypothalamus. It has been shown by Matsuda et al that a foreleg fracture under pentobarbital can activate the pituitary by a relatively direct pathway (29). They found that corticosterone secretion increased following foreleg fracture in rats with the cortex and some of the thalamus removed. If the pathway involving the amygdala were sensitive to pentobarbital, whereas the direct pathway were not, and if the pontine lesions were blocking function of the direct pathway, then my results would be explained.

The effect of pentobarbital also provides some insight for interpreting Feldman's (45, 54) electrophysiological studies of pathways from the sciatic nerve to the hypothalamus. He showed that there is a short-latency (7-10ms) evoked potential in the posterolateral hypothalamus caused by impulses ascending in the medial lemniscus and a long-latency (20-35 ms) evoked potential in the

ventromedian hypothalamus caused by impulses traveling through the reticular formation. However, these evoked potentials may not be related to ACTH secretion for two reasons: 1. Feldman showed that pentobarbital (30 mg/Kg) abolished the long-latency potential evoked in the ventromedian hypothalamus (55), although certain doses of pentobarbital (between 25 and 40 mg/Kg) promoted the appearance of a very long-latency (50-90 ms) evoked potentials in all of the hypothalamus and other forebrain structures. This latter potential was compared by Feldman to the "secondary response" originally described by Derbyshire, et al (56). It will be shown below that it is probably not related to ACTH secretion. Since pentobarbital completely eliminates the long-latency evoked potential in the ventromedian area of the hypothalamus, and ACTH is still secreted under pentobarbital in response to TF, ACTH secretion may not be elicited by whatever causes this potential. 2. Feldman also showed that lesions of the midbrain reticular formation sparing the lateral portions of the medial lemniscus greatly depress the long-latency response in the ventromedian area but do not decrease the short-latency response in the posterolateral hypothalamus. In my experiments, however, almost any brainstem lesion would block for several days ACTH release in response to a TF. The lesions in his experiments were produced either during the electrophysiological studies or one or two days before them, and varying amounts of pentobarbital were used during the stimulation and recording. In addition, the very long

latency response promoted by certain doses of pentobarbital was still present, although with the lesions the dose necessary to produce the effect was lower (about 10 mg/Kg). Thus in Feldman's experiments midbrain reticular formation lesions did not decrease the evoked potential in the posterolateral hypothalamus, whereas I found that any lesion in the medulla, pons, and midbrain would for several days prevent ACTH release after a TF. Therefore, the cause of this potential may not be related to the cause of ACTH secretion in response to a TF. Also, since the very long latency potential does not disappear with the lesions, it is probably not associated with ACTH release.

Thus, the evoked potential in the posterolateral hypothalamus is present and actually increased under conditions similar to those I found to depress ACTH secretion in response to a TF, and the evoked potential in the ventromedian hypothalamus is depressed under conditions when ACTH secretion is still significantly high after TF. Circumstantially, at least, it appears that the mechanisms responsible for these evoked potentials are not likely to be associated with ACTH release in response to a TF. Indeed, the possibility that neither potential is associated with ACTH release is not too surprising. Feldman took precautions to see that the stimulus intensity at the sciatic nerve was low enough not to activate the small pain conducting fibers since many of his experiments involved unanesthetized animals.

Distinction between a facilitatory area and a tract

A tract in the nervous system may be defined as an accumulation of functionally related fibers in a relatively small cross-section. The tract is usually long in relation to its cross-section, and the fibers generally conduct bits of information from one point to another. My results are consistent with the idea that TF induces ACTH secretion by causing information to be sent along a tract to the hypothalamus. However, due to the small size of the rat brain and the comparatively large size of the lesions it was not demonstrated that the fibers were limited to a relatively small zone of cross-section.

A facilitatory area, on the other hand, may be regarded as an accumulation of functionally related cells which increase the response to information transmitted over a tract. In contrast to a tract, a facilitatory area may not be absolutely necessary for ascending information to cause a response. My results are also consistent with the theory that there is an area in the pons which facilitates the release of ACTH after TF.

Interpretation of evidence concerning a facilitatory area in the pons

The fact that one or two days after placement, any lesion in the brainstem can block the release of ACTH in response to a TF, leads me to believe that the block is due to some nonspecific effect of the lesion such as edema or some kind of generalized depression.

However, 10 days after lesion placement, when the animals were nearly normal in their overt behavior, there was a difference

in the effects of the lesions that is related to their position in the pons. Unilateral lesions 2 mm. lateral to the midline and 3 mm. ventral to the interaural line significantly depressed the response compared to midline lesions 3 mm. ventral to the interaural line.

It was possible that the lesion had cut some of the nerve fibers from the leg to the hypothalamus which conducted impulses mediating ACTH release; and it was therefore necessary to know what percent of these fibers reaching the hypothalamus from the leg must be activated to cause a maximum ACTH release. The results showed that a bilateral TF in an intact rat caused no more ACTH secretion than a single TF. In fact, a single TF under pentobarbital can cause as much ACTH secretion as can the administration of ether[1], a powerful and consistent stimulus of ACTH release. The evidence also shows that the pathway is contralateral to the fractured tibia and not near the midline. Therefore it follows that a bilateral TF must be activating twice as many fibers, at least to the pons, as a unilateral TF, yet there is no difference in the response. Therefore, activation of no more than half of the available fibers is sufficient to cause maximum ACTH release under the conditions of the experiment. If the lesion is unilateral and well off the midline (See figure 8) it cannot, assuming bilateral symmetry, block more than half of the fibers in any tract. Therefore, a bilateral TF in a unilaterally lesioned animal,

1 M.A. Greer, Personal Communication, 1967.

which should activate at least as many fibers as a single TF in an intact rat, should maximally activate ACTH release; but it does not.

Since the depression could not be explained on the basis of damage to the tract nor on the basis of a general depression of brain function, another explanation had to be found. It was possible that the lesion had damaged an area which facilitates the release of ACTH. Slusher and Hyde (31) and Endröczi and Lissak (32) have attempted to show areas of the midbrain in cats which facilitate or inhibit the release of ACTH. My results are not directly comparable to either of these studies although Endröczi and Lissak did seem to demonstrate a facilitatory area in the ventral midbrain tegmentum. Slusher and Hyde (31), however, thought there were inhibitory components there.

The reticular activating system of the brainstem is known to increase the level of excitability of the cortex to afferent stimuli (57). The unilateral pontine lesion (0-3, R2) does destroy part of the pontine reticular formation. It is thus conceivable that this area in the pons increases the level of excitability of the hypothalamus in a fashion analogous to that by which the reticular activating system increases the level of excitability in the cortex.

Interpretation of evidence concerning the location of a neural pathway carrying impulses mediating ACTH release

The results from experiments involving hemisection of the spinal cord are clear-cut. Since there was no difference between

the response caused by a TF with a complete transection and that caused by a left TF with a right hemisection, and conversely, no difference between the response caused by a TF in a rat with a sham transected cord and that caused by a right TF in a rat with a right hemisection, it appears that the pathway is mainly, if not entirely, contralateral in the cord.

The results from experiments involving partial hemisection of the medulla, although not as consistent as those from the spinal cord hemisection experiments, nevertheless demonstrate that the pathway is still contralateral in the medulla. Since the partial hemisections were limited to the lateral portion of the medulla (See figure 4), it is possible that some of the fibers mediating the response were medial to the cut, which would blur the difference between a right and left TF.

The results from experiments involving lesions in the pons at 10 and greater than 30 days are also clear-cut. With a lesion on the right side, a right TF causes the same response as a bilateral TF, whereas a left TF does not cause pituitary-adrenal response. Again, it appears as though the pathway is entirely contralateral to the fractured tibia.

Although from the above results it appeared that the lesion was actually located in the pathway involved with ACTH secretion in response to traumatic stress, it was still possible that any lesion contralateral to the fractured tibia could block the release of ACTH.

However, the experiment comparing the effects of various unilateral lesions on the difference between a right and a left TF (See figure 17) implied that the pathway is limited to an area more than 2 mm. ventral to the interaural line and more than 1 mm. lateral to the midline.

The pathway carrying impulses mediating the TF induced release of ACTH thus seems to travel in the central nervous system in the vicinity of the spinothalamic tract, at least up through the pons (58, 59).

How the pathway(s) goes from the pons to the hypothalamus was not investigated during work for this thesis. Nauta has shown both direct and indirect connections from the paramedian area of the midbrain to the hypothalamus (24). He also thinks that stimuli which have wide access to the reticular formation can activate these connections to the hypothalamus. Since (a) the spinothalamic tract has numerous connections with the reticular formation (60), (b) the spinothalamic tract carries impulses caused by noxious stimuli, and (c) lesions in the vicinity of the spinothalamic tract block ACTH release caused by contralateral TF, some of my observations are consistent with the possibility that the medial area in the midbrain connecting with the hypothalamus is important in ACTH secretion. Midline lesions in the pons at 0-3 (See figure 13) which extend into the areas in the midbrain described by Nauta did cause some depression of the response to TF when compared to animals with no lesions. The statistical significance however was borderline ($t=1.96$, $df=57$, $p<0.1$,

2 tailed, $p<0.05$, 1 tailed). In addition these lesions were no more effective than any other lesion in the pons (See figure 12) and the slight depression obtained could have been due to non-specific effects of the lesion. Therefore, I do not think it is possible from my data to confirm or deny the possible function of the anatomically identified pathways to the hypothalamus.

A point concerning recovery of function of the structures involved in the traumatic stress-induced release of ACTH was noticed when statistically analyzing the results. The response to bilateral TF with a unilateral pontine lesion was partially recovered at periods greater than 20 days after lesion placement (See figure 9). On the other hand, the response to contralateral TF showed no tendency to recover even at 30 days after placement (See figures 14 and 15). The latter finding is consistent with the idea that the lesion had interrupted a direct pathway whose function could not be rerouted. However, the recovery of the response to bilateral TF is consistent with a more complex and plastic organization of function than a direct pathway. Since the lesion did destroy part of the reticular formation, it is not unreasonable to speculate that function could in time shift to an undamaged area of this highly complicated and intertwined structure. Many further speculations are possible but the available data are not sufficient to warrant them.

SUMMARY AND CONCLUSIONS

Using the specific stress of a tibial fracture under pentobarbital anesthesia and the technique of stereotaxic placement of brain lesions, a direct nervous pathway from the leg to the pons has been demonstrated which mediates the stress-induced release of ACTH in animals anesthetized with pentobarbital. Lesions, partial hemisections, or full hemisections in the brainstem and spinal cord contralateral to a fractured tibia block the ACTH release which would occur in intact rats, whereas lesions or hemisections ipsilateral to the fractured tibia have lesser effects. Since a unilateral lesion in the pontine reticular formation significantly reduced the response of ACTH secretion to bilateral TF in an otherwise normal rat for as long as 10 days, whereas other lesions in the pons did not, it was postulated that the lesion was in an area necessary for the facilitation of tibial fracture-induced ACTH release.

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