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Noradrenergic regulation of serotonergic neurons in the dorsal raphe : physiological, pharmacological, and anatomical studies

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ABSTRACT

NORADRENERGIC REGULATION OF SEROTONERGIC NEURONS IN THE DORSAL RAPHE: PHYSIOLOGICAL, PHARMACOLOGICAL AND ANATOMICAL STUDIES

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Recent pharmacological studies suggest that a central noradrenergic (NE) system regulates the firing activity of the serotonin (5-HT)-containing neurons in the dorsal raphe nucleus. Reduction of noradrenergic tone by a variety of pharmacological interventions leads to a suppression of 5-HT cell firing (Svensson et al., 1975). It has been suggested that an alpha-adrenoceptor mediates NE's effects since systemic administration of an alpha-adrenoceptor antagonist, piperoxan, reduces 5-HT cell firing (Gallager and Aghajanian, 1976a). Since the dorsal raphe receives a prominent noradrenergic innervation (Fuxe, 1965), these noradrenergic terminals could mediate the noradrenergic regulation of 5-HT neuron firing activity.

This dissertation will present studies which are aimed at: 1)demonstrating that the NE innervation of the dorsal raphe does regulate 5-HT cell firing, 2) characterizing the pharmacology of the noradrenergic control of 5-HT cell activity, and 3) elucidating the local synaptic circuitry underlying NE's action on 5-HT cells of the dorsal raphe.

The first set of experiments provides evidence that the activity of 5-HT cells is regulated by the NE innervation of the dorsal raphe. Earlier studies showed that reserpine suppresses 5-HT cell firing activity (Aghajanian and Haigler, 1972). Studies

will be presented which indicate that this action of reserpine is due to its interference with central noradrenergic transmission. In order to determine whether restoration of NE tone within the dorsal raphe could restore 5-HT cell firing, NE was administered by iontophoresis in the vicinity of 5-HT cells during their suppression by reserpine. Small amounts of NE restored 5-HT cell firing to normal or above normal levels. This observation suggests that NE released locally in the dorsal raphe maintains the tonic activity of 5-HT cells.

A second series of experiments characterizes pharmacologically the receptor mediating NE's activation of 5-HT cell activity. Alpha-adrenoceptor antagonists were found to block NE's activation of 5-HT cell firing. Also, these drugs suppressed spontaneous firing of 5-HT cells when applied iontophoretically. These findings suggest that alpha-adrenoceptors present in the dorsal raphe mediate NE's action on 5-HT cells. The measurement of the relative potencies of a series of agonists in activating 5-HT cells suggests that the alpha-adrenoceptor mediating NE's action can be classified in the alpha-1-adrenoceptor category.

The results of earlier physiological studies led to the proposal that GABA interneurons present in the dorsal raphe mediate NE's regulation of 5-HT cell firing (Gallager and Aghajanian, 1976b). Another group of experiments tests this hypothesis by examining the effect of GABA receptor antagonists on NE's regulation of 5-HT cell firing. Application of these drugs by iontophoresis did not interfere with the suppression of 5-HT cell firing produced by alpha-adrenoceptor antagonists. This finding suggests that NE's regulation

of 5-HT cell firing is not mediated by GABA.

The dorsal raphe contains both non-serotonergic, as well as serotonergic neurons (Aghajanian et al., 1978). Therefore, the effects of NE on 5-HT cells could stem from a direct innervation of 5-HT cells, or alternatively, could be mediated indirectly by non-serotonergic neuronal elements. Anatomical experiments will be presented which provide evidence that NE's activation of 5-HT cells results from a direct NE innervation. NE terminals in the dorsal raphe were identified by electron microscopic autoradiography. Futhermore, neuronal elements were classified as either serotonergic or non-serotonergic following treatment with the selective serotonin neurotoxin, 5,7-dihydroxytryptamine. A majority of the post-synaptic elements innervated by NE terminals displayed signs of degeneration. This observation suggests that NE terminals directly innervate 5-HT neurons of the dorsal raphe.

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To Liba

and my parents

PREFACE

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INTRODUCTION

I. Rationale

Despite disagreement over the exact criteria which define a neurotransmitter, there is overwhelming experimental support for the assertion that serotonin (5-HT) is a transmitter in the central nervous system. Since the detection of 5-HT in mammalian brain, (Twarog and Page, 1953; Amin et al., 1954) the brain's ability to synthesize serotonin from tryptophan (Gal et al., 1963; Grahame-Smith, 1964, 1967) to release it upon electrical stimulation (Chase et al., 1969; Holman and Vogt, 1972), and to inactivate it by a high-affinity uptake system (Blackburn et al., 1967; Ross and Renyi, 1967) have been well established. A major advance in the study of central 5-HT systems followed upon the application of the formaldehyde-condensation histochemical method (Falck et. al., 1962) to brain. This technique allowed the visualization of 5-HT cell bodies (Dahlstrom and Fuxe, 1964) terminals (Aghajanian et al., 1973, Fuxe, 1965) and pathways (Anden et al., 1966). This anatomical information directed study to the physiological effect of 5-HT in areas which receive a dense and uniform 5-HT innervation. Exogenous application of 5-HT in densely innervated forebrain areas, invariably inhibited neuronal firing (Bloom et al., 1972b; Haigler and Aghajanian, 1974b). Recent studies of the effect of 5-HT on facial motoneurons have demonstrated that 5-HT enhances the excitability of these cells by a receptor mediated mechanism (McCall and Aghajanian, 1979).

Perhaps, the most essential criterion which a transmitter

candidate must fulfill is the demonstration that the exogenously applied putative transmitter "mimics" the effect of pathway stimulation. Since histofluorescence (Aghajanian et al., 1973; Anden et al., 1966) and autoradiographic studies (Conrad et al., 1974; Bobillier et al., 1976) had identified the presence of a 5-HT pathway from the midbrain raphe to the amygdala, this criterion could be examined. Stimulation of the afferent 5-HT pathway to the amygdala markedly depressed neuronal firing, effectively mimicking the inhibitory action of exogenously applied 5-HT (Wang and Aghajanian, 1977d). In that study, the authors provide strong support for the idea that the release of 5-HT by pathway stimulation produced the inhibition. Selective destruction of 5-HT fibers by injection of 5,7-dihydroxytryptamine (5,7-DHT), a 5-HT neurotoxin (Baumgarten and Lachenmeyer, 1972) prevented the stimulation-induced inhibition, as did depletion of serotonin levels by pretreatment with parachlorophenylalanine, PCPA, an inhibitor of 5-HT synthesis (Jequier et al., 1967). In addition, application of a 5-HT high-affinity uptake blocker, chlorimipramine, (Carlsson, 1970; Ross and Renyi, 1969) prolonged the effects of both 5-HT pathway stimulation and application of exogenous 5-HT. Taken together, the findings described above provide evidence that serotonin acts as a neurotransmitter in the mammalian brain.

5-HT-containing neurons of the brain are located primarily within the brain stem raphe nuclei (Dahlstrom and Fuxe, 1964) and project widely throughout the central nervous system (Fuxe, 1965). Substantial evidence suggests that these neurons play an important

role in a wide range of physiological functions. Several studies indicate that the 5-HT system regulates the release of a variety of pituitary hormones including FSH, LH, prolactin and ACTH (Chase and Murphy, 1973). The remarkable finding that 5-HT cells retain a tonic firing rate except during periods of REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979) suggests that they play a special role in the physiology of the sleep-waking cycle. In addition, behavioral studies support the notion that increased 5-HT tone suppresses pain perception (Chase and Murphy, 1973).

The relationship of the serotonin system to behavioral disorders has received special attention. Administration of PCPA, which blocks 5-HT synthesis, unmasks aggressive and hypersexual behavior (Sheard, 1969). The hypothesis that decreased serotonergic transmission underlies endogenous depression in a subgroup of patients has gained acceptance (Coppen et al., 1972); drugs which enhance serotonergic transmission alleviate depressive symptoms, lending support to this theory (Coppen et al., 1963, 1967). The proposal that decreased 5-HT transmission may be related to psychotic behavior was inspired by studies which demonstrated an antagonism of 5-HT's action by LSD and related hallucinogens (Wooley and Shaw, 1954; Kety, 1959). These observations suggest that disruptions of the 5-HT system may play an important role in certain behavioral disorders.

In the hope of gaining greater insight into the physiology and pathophysiology of the 5-HT system, it is important to elucidate the manner in which other neuronal systems influence the activity

of the 5-HT system. In particular, the experiments presented in this dissertation have focussed on the regulation of the 5-HT system by the noradrenergic systems located within the brain.

II. NE Regulation of the 5-HT System: Anatomical and Biochemical Studies

Fluorescent histochemical studies demonstrated the close apposition of catecholamine nerve terminals and 5-HT cell bodies (Fuxe, 1965). Even though the cell bodies of the serotonin cells are distributed throughout the brain stem among the midbrain raphe nuclei, many of these nuclei were observed to contain catecholamine terminals. These studies first led to the suggestion that central noradrenergic systems influence the 5-HT system. More recent biochemical measurements have confirmed the presence of relatively high NE concentrations in the raphe nuclei (Saavedra et al., 1976; Levitt and Moore, 1979).

The development of a biochemical index of 5-HT cell activity allowed further study of a possible functional link between these systems. The concept that increased 5-HT neuronal firing activity produced elevated levels of 5-hydroxyindole acetic acid (5-HIAA), a metabolite of 5-HT, had received experimental support from the studies of Sheard and Aghajanian (1968). They found that electrical stimulation of the 5-HT cells of the midbrain raphe, which project to the forebrain (Anden et al., 1966), produced marked elevations of 5-HIAA there. Therefore, the effects on 5-HIAA levels of a variety of treatments which interfere with NE activity were measured. Lesions of the locus coeruleus, which projects extensively to the

forebrain (Anden et al., 1966) and administration of 6-OHDA, a selective catecholamine neurotoxin (Malmfors and Thoenen, 1971) were used to deplete NE levels in the brain. Both these treatments produced increased levels of 5-HIAA (Kostowski et al., 1974; Blondaux et al., 1973). Other pharmacological manipulations of the NE system produced a similar result. Treatment with either a tyrosine hydroxylase inhibitor, alpha-methyl-paratyrosine (Stein et al., 1974) or a dopamine beta hydroxylase inhibitor (Johnson et al., 1972) also increased 5-HIAA levels in the forebrain areas. The general consensus of these studies suggests that decreased NE tone tends to produce elevations of 5-HT turnover. However, it must be emphasized that even though stimulation of 5-HT pathways leads to elevated 5-HIAA, the converse is not assured, i.e., increased 5-HIAA levels do not necessarily imply an increase in functional activity of the 5-HT system. For example, administration of tryptophan suppresses 5-HT cell firing (Aghajanian, 1972b) yet elevates 5-HIAA levels (Moir and Eccleston, 1968). Indeed, in two of the four studies mentioned above, levels of brain tryptophan were measured and were elevated (Blondaux et al., 1973; Stein et al., 1974), suggesting that the elevations of 5-HIAA might be related to increased levels of precursor tryptophan, rather than the increased impulse flow.

A more promising biochemical approach to determining the functional activity of the 5-HT system involves measuring the disappearance of 5-HT from brain following inhibition of its synthetic enzyme, tryptophan hydroxylase. When this index of 5-HT utilization was monitored, clonidine, an alpha-adrenergic agonist which reduces

NE turnover, also slowed the disappearance of 5-HT, suggesting that 5-HT activity decreases in parallel with NE activity (Anden et al., 1970; Scheel-Kruger, 1974).

III. Neurophysiological Studies of 5-HT Neurons

1. Identification of 5-HT Neurons

Histofluorescence studies demonstrated the presence of a dense cluster of 5-HT cells in the dorsal raphe nucleus (Dahlstrom and Fuxe, 1964). The successful application of extracellular recording techniques to these 5-HT neurons (Aghajanian, 1972a) allowed the direct measurement of impulse activity in the 5-HT system. These cells display a characteristic slow (approximately 1 spike/sec), regular firing rate and a wide action potential (1-2 msec) which allow them to be tentatively identified as 5-HT cells during recording experiments. Routine histological examination of dye delivered from recording electrodes demonstrated that the distribution of these cells corresponds to that of 5-HT cells identified in histofluorescent studies. In addition, both physiological and anatomical methods have been used to confirm the serotonergic identity of these neurons. Iontophoretic ejection of l-tryptophan from recording electrodes in the vicinity of presumptively identified 5-HT neurons produced highly fluorescent spots when the tissue was processed for histofluorescence (Aghajanian and Haigler, 1974). This finding suggests that the ejected tryptophan was taken up into 5-HT cells, since only these neurons in brain are capable of converting l-tryptophan to its highly fluorescent endproduct 5-HT (Aghajanian and Asher, 1971). Further evidence that these neurons are serotonergic was provided

by electrophysiological techniques. The course of efferent 5-HT fibers emerging from the dorsal raphe had been mapped by the histofluorescence method (Anden et al., 1966). Therefore, this bundle could be stimulated electrically by an electrode stereotaxically positioned in its vicinity. Stimulation of these fibers initiates action potentials which travel both orthodromically as well as antidromically back to the cell bodies. Therefore, a recording electrode located within the dorsal raphe can detect the antidromically elicited spike. In this way, it was found that the presumptively identified 5-HT neurons can be antidromically activated by stimulation of the 5-HT pathway demonstrating that they are serotonergic (Wang and Aghajanian, 1977c). In a later study, Aghajanian et al. (1978) found that following 5,7-DHT treatment, which selectively damaged 5-HT cells, the activity of slow and regularly firing cells of the dorsal raphe could not be detected in routine recording experiments, further verifying the serotonergic nature of these cells. This body of evidence allows the identification of serotonergic neurons in the dorsal raphe with a high degree of certainty.

2. Autoinhibition of 5-HT Cells

The 5-HT cells of the dorsal raphe possess a tonic, regular firing rate. In an effort to gain a greater understanding of the function of the 5-HT system, a variety of studies have been aimed at identifying factors that affect 5-HT cell firing activity. To this end, the actions on 5-HT cells of both systemically and locally applied drugs have been examined. Since 5-HT cells fire

at a remarkably constant rate, it is possible to ascertain the base-line firing rate of an individual cell, administer a drug systemically or iontophoretically, and monitor the cell's response to the drug treatment.

Systemically applied drugs which increase the availability of 5-HT at 5-HT receptors lead to a suppression of 5-HT cell firing. Systemic administration of tryptophan accelerates the synthesis of 5-HT (Eccleston et al., 1965), presumably because tryptophan hydroxylase, the rate limiting enzyme in 5-HT synthesis, is not normally saturated with its substrate tryptophan (Green and Grahame-Smith, 1975). This treatment reduces 5-HT cell activity (Aghajanian, 1972b). Also, administration of either 5-HT uptake inhibitors, which are thought to increase the amount of 5-HT available to stimulate post-synaptic receptors, or parachloramphetamine, which releases 5-HT, silences these cells (Sheard et al., 1972; Bramwell, 1974; Mosko and Jacobs, 1977). Therefore, in general, the 5-HT system possesses a self-limiting regulatory mechanism, i.e., an increase in 5-HT 'availability' leads to a reduction of 5-HT cell firing activity.

Biochemical evidence had suggested that the indole hallucinogen, LSD, decreases 5-HT cell firing activity (Aghajanian, 1972c). Indeed, this drug when applied systemically invariably inhibits 5-HT cell firing (Aghajanian et al., 1968). Perhaps, LSD inhibits 5-HT cell firing by stimulating 5-HT receptors.

Since 5-HT cell bodies are inhibited by iontophoretic application of the indole hallucinogens, as well as 5-HT (Haigler and

Aghajanian, 1974a; Aghajanian and Haigler, 1975), it has been suggested that an increase in 5-HT tone at 5-HT receptors within the dorsal raphe could underlie this feedback regulation. The concept of a local feedback circuit has been supported by physiological studies. Stimulation of the 5-HT fibers emerging from the dorsal raphe initiates an antidromic action potential which invades the 5-HT cell bodies of the dorsal raphe. Antidromic stimulation of the 5-HT cells in this manner markedly inhibits 5-HT cell firing during the post-stimulus period (Wang and Aghajanian, 1977b). These authors propose that antidromic stimulation releases 5-HT from a network of collateral processes which communicate among 5-HT cells. In this way, increased 5-HT availability at inhibitory 5-HT autoreceptors within the dorsal raphe may dampen 5-HT cell activity.

3. GABA-ergic Inhibition of 5-HT Cells

Examination of afferent pathways to the dorsal raphe has produced evidence that GABA-ergic neurons exert a prominent inhibitory influence on the activity of 5-HT cells located there. Application of the horseradish peroxidase retrograde tracing technique to the dorsal raphe, demonstrated that the lateral habenula sends a major projection to the dorsal raphe (Aghajanian and Wang, 1977). Electrical stimulation of this pathway produced a pronounced inhibition of 5-HT cell firing (Wang and Aghajanian, 1977c). In an attempt to identify the transmitter mediating this inhibtion, it was found that systemic or iontophoretic application of picrotoxin, a GABA antagonist, reduced the post-stimulus inhibitory period. This finding suggests that GABA-ergic neurons mediate the observed inhibition.

GABA is implicated in the effects of a second afferent pathway. Stimulation of the pontine reticular formation also inhibited 5-HT cell activity (Wang et al., 1976). Again, application of picrotoxin but not strychnine, a glycine antagonist, blocked the inhibition. These studies provide evidence that GABA plays an important role in regulating 5-HT cell activity. Experiments to be presented below have examined the relationship of this GABA-ergic influence to the NE regulation of 5-HT cell activity, discussed in the next section.

4. NE-Regulation of 5-HT Neurons

The identification of 5-HT neurons in the dorsal raphe by extracellular single unit recording techniques provided a more direct approach to examining the functional activity of the 5-HT system. The ability to record from 5-HT neurons prompted several studies which examined the effect of electrical stimulation of NE systems on 5-HT cell activity in the dorsal raphe. Stimulation of the locus coeruleus NE system did not markedly affect 5-HT cell firing (Wang et al., 1976; Anderson et al., 1977). In order to explore the possibility that the medullary NE system (Dahlstrom and Fuxe, 1964) which projects to several brain stem areas was involved in the regulation of 5-HT cell activity, stimulation of these cells was also undertaken. However, only weak and variable effects on 5-HT cell activity were obtained (Stern et al., 1980). It is interesting to note that amphetamine, which increases NE tone by releasing NE (Glowinski and Axelord, 1965) does not markedly increase baseline 5-HT cell firing activity either (Foote et al., 1969). Therefore, these physiological studies suggest that an increase in

NE tone does not affect 5-HT cell firing, whereas, the biochemical studies with clonidine mentioned above, indicate that a decrease in NE tone decreases 5-HT activity. These apparently conflicting observations can be interpreted as suggesting that under the conditions of these studies the NE system exerts a near maximal effect on the 5-HT system; therefore further stimulation of the NE system produces little effect, yet a reduction in NE tone could produce a marked decrease in 5-HT cell activity.

Further physiological studies examined the effect of decreased NE tone on 5-HT cell firing activity (Svensson et al., 1975). These studies demonstrated that low doses of clonidine which totally inhibited the firing of NE cells in the locus coeruleus also suppressed the firing of the overwhelming majority of 5-HT cells in the dorsal raphe. Clonidine's effect on 5-HT cells was attributed to an indirect action mediated by its effect on NE systems, since local application of clonidine in the vicinity of 5-HT cells did not produce an inhibition of firing. This finding implies that a decrease in NE tone produces a parallel decrease in 5-HT cell activity. Several observations support this interpretation. Administration of 6-OHDA intraventricularly produced a suppression of 5-HT cell activity. Administration of alpha-methyl-para-tyrosine, an inhibitor of tyrosine hydroxylase, slowed the firing of 5-HT cells. Furthermore, the suppression of firing produced by clonidine could be reversed by amphetamine, which releases NE.

Further confirmation of this concept that decreased NE tone decreases 5-HT cell activity came from studies with a variety of

antipsychotic drugs. Of several antipsychotic drugs tested only some suppressed 5-HT cell activity (Gallager and Aghajanian, 1976a). The alpha-adrenoceptor blocking potency of these drugs (Keller et al., 1973; Burki et al., 1975) was found to correlate with their ability to suppress 5-HT cell firing suggesting that blockade of NE transmission produces a suppression of 5-HT cell firing. Furthermore, since piperoxan, an alpha-adrenoceptor antagonist, but neither sotalol, a beta-adrenoceptor antagonist, nor haloperidol, a dopamine antagonist, is effective in reducing 5-HT cell firing, it was suggested that an alpha-adrenoceptor mediates the NE regulation of 5-HT cell firing.

5. Pharmacology of Central Alpha-Adrenoceptors

While peripheral alpha-adrenoceptors have been well characterized pharmacologically (Furchgott, 1972), the difficulties encountered in studying central neurons have hindered the examination of central alpha-adrenoceptors. Many of the classical agonists and antagonists do not readily enter the brain when applied systemically. Furthermore, the effects of agents which do gain access to the brain may not be the result of a direct interaction with the neuron under study, since actions of the drug at distant sites may be relayed by afferent neuronal pathways. For these reasons, the microiontophoretic application of drugs which allows their delivery to a limited area enjoys important advantages. This technique has been used extensively in the studies presented below which examined the pharmacology of the NE regulation of 5-HT neuronal firing activity.

Evidence has recently accumulated for the existence of two

different types of alpha-adrenoceptors; alpha-1 and alpha-2 adrenoceptors (Langer, 1974; Starke et al., 1975). The classical peripheral post-synaptic alpha-adrenoceptor has been designated the alpha-1 adrenoceptor, while the recently described 'pre-synaptic' alpha-adrenoceptor which regulates the amount of transmitter released from NE terminals has been classified as an alpha-2 adrenoceptor. The pharmacological differentiation of the two receptor types is based on the different rank order potency of agonists, as well as the selectivity of antagonists. For example, while NE stimulates both alpha-1 and alpha-2 adrenoceptors, alpha-methylnorepinephrine is more potent than NE at alpha-2 adrenoceptors but less potent at alpha-1 adrenoceptors. In addition, antagonists have been identified which show markedly different potencies at these two receptors. WB-4101, a potent alpha-1 receptor antagonist possesses little activity at alpha-2 receptors while yohimbine exerts selective antagonism at alpha-2 receptors (Kapur and Mottram, 1978; Drew, 1976, 1977; Marshall et al., 1978).

The presence of both alpha-1 and alpha-2 adrenceptors in brain has been demonstrated by in vitro binding studies. These studies have demonstrated that clonidine, a potent alpha-2 agonist, and WB-4101, an alpha-1 antagonist bind selectively to distinct alpha-adrenergic receptor sites (U'Prichard et al., 1977; U'Prichard and Snyder, 1979). Even though alpha-2 adrenoceptors were originally described as pre-synaptic in location, these studies have indicated that both alpha-1 and alpha-2 receptor types are located post-synaptically. Therefore, these receptor categories should be used only to refer to the pharmacology of the receptor and not to their anatomical localization.

The iontophoretic technique has been used to examine neuronal responses to NE which are mediated by alpha-adrenoceptors. Bevan and co-workers (Bevan et al., 1977; Szabadi, 1979) have examined the response of unidentified cortical neurons to NE. They found that stimulation of alpha-adrenoceptors produces excitatory responses. Their studies of the effects of several adrenergic agonists suggest that these receptors can be classified in the alpha-1-adrenoceptor category. Rogawski and Aghajanian (1980) have demonstrated on the basis of extensive pharmacological studies that the activation of lateral geniculate neurons by NE is mediated by alpha-1-adrenoceptors. Cedarbaum and Aghajanian (1977) characterized pharmacologically the inhibitory action of NE on NE neurons in the locus coeruleus. The rank order of the potencies of adrenergic agonists indicated that NE's inhibitory action on locus coeruleus neurons is mediated by alpha-2-adrenoceptors. The pharmacology of the adrenoceptor mediating NE's regulation of 5-HT cell firing will be examined in experiments presented below (Experimental Studies, Section II).

IV. Neuronal Organization of the Dorsal Raphe

1. Non-Serotonergic Neurons of the Dorsal Raphe

Physiological experiments have demonstrated the presence of both non-serotonergic neurons as well as 5-HT neurons in the dorsal raphe. A population of normally quiescent non-serotonergic interneurons present in the dorsal raphe was detected during a study of the effect of stimulation of peripheral sensory nerves on 5-HT cell firing. While 5-HT cells were inhibited by peripheral nerve stimulation, these neurons were transiently activated

(Aghajanian et al., 1978). The neurons which were activated by peripheral nerve stimulation can be distinguished from the 5-HT neurons with confidence since they are not activated antidromically by stimulation of the ascending 5-HT fiber bundle. Furthermore, these interneurons were still activated by peripheral nerve stimulation following destruction of 5-HT neurons by 5,7-DHT pretreatment.

The presence of non-serotonergic neurons in the dorsal raphe has also been suggested by anatomical experiments. Descarries et al. (1979) have examined the dorsal raphe with H^3-5-HT autoradiography. This technique selectively labels the serotonergic neurons present in the dorsal raphe. Of note, two-thirds of the neurons in the dorsal raphe were not labelled by H^3-5-HT. This finding confirms the presence of non-serotonergic neurons in the dorsal raphe.

Several lines of evidence suggest that many of the non-serotonergic neurons present within the dorsal raphe are GABA-ergic. The dorsal raphe contains one of the highest levels of GABA and its synthetic enzyme, glutamate decarboxylase, in brain (Tappaz et al., 1979). Kainic acid lesions of the dorsal raphe which are thought to selectively damage cell bodies produced a marked decline in GABA levels there indicating that the dorsal raphe contains intrinsic GABA neurons (McGeer et al., 1979). Anatomical evidence for the presence of GABA-ergic neurons in the dorsal raphe has also been provided by electron-microscopic autoradiographic studies with tritiated GABA. Following infusion into the ventricular system, tritiated GABA is taken up by GABA neurons near the ventricular surface which can be detected by autoradiography. Examination of

sections of the dorsal raphe processed for electron-microscopic autoradiography demonstrated that GABA-ergic neurons are present in the dorsal raphe (Gamrani et al., 1980). This combination of anatomical and biochemical experiments suggest that GABA-ergic interneurons are located in the dorsal raphe.

These local GABA-ergic interneurons could mediate the inhibition of 5-HT cells produced by stimulation of afferent pathways. Iontophoretic administration of picrotoxin in the vicinity of 5-HT cells blocks the inhibition produced by stimulation of the lateral habenula (Wang and Aghajanian, 1977c). However, lesions of the lateral habenula did not markedly reduce the concentration of GABA and glutamate decarboxylase in the dorsal raphe (McGeer et al., 1979), indicating that 'long' GABA-ergic afferents from the habenula to the dorsal raphe do not mediate this inhibition. Alternatively, if local GABA neurons were activated by habenular stimulation, this could produce the picrotoxin sensitive inhibition of 5-HT cell firing, that was described by Wang and Aghajanian (1977c).

Similarly, the inhibition of 5-HT cell firing produced by stimulation of ascending fibers is also blocked by picrotoxin (Wang et al., 1976). In addition, local interneurons in the dorsal raphe are activated by sensory stimulation and display a reciprocal firing pattern to that of the 5-HT cells (Aghajanian et al., 1978). These observations are consistent with the proposal that local GABA-ergic interneurons when activated by afferent stimulation, exert a prominent inhibitory influence on 5-HT cell activity.

The dorsal raphe receives a prominent NE innervation which has been demonstrated by histofluorescence (Fuxe, 1965), biochemical

(Levitt and Moore, 1979) and immunocytochemical (Grzanna and Molliver, 1980) techniques. Experiments to be presented below (Experimental Studies, sections I and II) suggest that this NE innervation, as well as the GABA-ergic input, exerts a dramatic control over 5-HT cell activity.

These experiments demonstrate that reduction of NE transmission within the dorsal raphe can totally suppress 5-HT cell firing activity. Since other afferent inhibitory influences on 5-HT cell firing appear to be mediated by local GABA interneurons, NE might exert its control over 5-HT cell firing in a similar fashion. That is, the reduction of inhibitory NE tone could allow normally quiescent GABA-ergic interneurons within the dorsal raphe to fire. These activated cells could then, in turn, directly inhibit 5-HT cell firing. Alternatively, NE terminals might terminate directly on 5-HT cells; interruption of NE transmission could then block a tonic NE activation of 5-HT cell firing producing the observed cessation of firing.

Physiological experiments which will be presented below (Experimental Studies, sections III) were aimed at determining whether the NE regulation of 5-HT cells is mediated by local GABA-ergic neurons or not. As well, anatomical experiments (Experimental Studies, section IV) were conducted which were aimed at determining whether 5-HT or non-5-HT neurons receive the NE innervation in order to distinguish between the alternative circuits which could underlie NE's action.

2. <u>The Origin of the NE Innervation of the Dorsal Raphe</u>

Prior to a discussion of the methods used to examine the target of the NE innervation, it is of interest to consider its origins. Several studies have examined the possibility that NE cells of the locus coeruleus project to the dorsal raphe. While an initial report suggested that the locus coeruleus innervates the dorsal raphe (Loizu, 1969), recent studies involving more specific lesions of the locus coeruleus have not found either a decrease in the density of fluorescent terminals or in NE levels in the dorsal raphe (Levitt and Moore, 1979). These findings argue strongly against a locus coeruleus projection to the dorsal raphe. In support of these findings, retrograde tracing studies of the dorsal raphe afferents labelled only occasional cells in the locus coeruleus (Aghajanian and Wang, 1977). Anterograde tracing studies of locus coeruleus efferents also did not identify a projection to the dorsal raphe (Jones and Moore, 1977). Furthermore, lesions of the locus coeruleus did not obviously decrease 5-HT cell firing (Gallager and Aghajanian, 1976a) suggesting that the NE regulation of 5-HT cells does not stem primarily from the locus coeruleus. The other NE cell nuclei are less compact than the locus coeruleus cluster and therefore more difficult to manipulate experimentally. However, retrograde tracing of dorsal raphe afferents did reveal afferents in the vicinity of the A1 and A2 cells in the medulla (Aghajanian and Wang, 1977), suggesting that medullary NE cells may contribute to the NE innervation of the dorsal raphe. Of note, evidence has emerged recently from immunocytochemical and autoradiographic studies demonstrating the presence of an anterior portion of the

locus coeruleus which extends into the midbrain central grey (Grzanna et al., 1980; Nowaczyk et al., 1978). The proximity of these NE cells to the dorsal raphe requires that these cells be considered a potential source of the NE input to the dorsal raphe.

3. Ultrastructural Localization of NE

The visualization of NE terminals in the dorsal raphe by fluoresence histochemistry indicates presumed sites of transmitter release. However, because of the limited resolution of the light microscope, electron microscopic methods were needed in order to identify the neurons of the dorsal raphe receiving the NE innervation. The development of electron-microscopic autoradiography for H^3-NE has allowed ultrastructural identification of NE neuronal elements. Briefly, small amounts of tritiated NE, administered intraventricularly, diffuse throughout the ventricular system and into the brain parenchyma. NE neurons which possess a high affinity uptake system for NE (Iversen, 1971) can then selectively concentrate the H^3-NE. The tissue is then fixed and processed for autoradiography at both the light and electron-microscopic level.

This technique was first applied to the central nervous system by Aghajanian and Bloom (1966), who successfully visualized NE terminals in the paraventricular nucleus by this method. NE terminals located there contained both small agranular and larger granular vesicles similar to those described in peripheral NE terminals (Grillo, 1966). However, in contrast to peripheral NE terminals which do not form classical synaptic junctions, central

NE terminals were found to frequently participate in classical synaptic junctions.

In more recent studies, Descarries and co-workers (Beaudet and Descarries, 1978) have applied this method to cortical areas. NE terminals and axons were successfully identified by this technique. In these studies, the selectivity of the labelling was confirmed by two methods. First, addition of non-tritiated NE to the perfusate which should saturate the high-affinity NE uptake system drastically reduced the labelling observed, as did pretreatment of animals with 6-OHDA, the selective catecholamine neurotoxin. In view of its selectivity and widespread applicability, NE EM-autoradiography provided a method by which NE terminals in the dorsal raphe could be visualized in order to examine the nature of the post-synaptic elements receiving the NE innervation.

V. Scope of Experiments

Experimental studies were conducted which examined the noradrenergic regulation of the 5-HT cells of the dorsal raphe. First, experiments were performed which indicated that reserpine, which suppresses 5-HT cell firing (Aghajanian and Haigler, 1972) does so by interfering with adrenergic transmission. Furthermore, it was found that NE, iontophoretically applied in the vicinity of 5-HT cells, reverses reserpine's suppression of 5-HT cell firing. This finding suggests that the dense NE innervation of the dorsal raphe regulates the activity of 5-HT cells located there. Second, the effect of a variety of adrenergic agonists and antagonists on 5-HT cell firing was examined in order to characterize the

receptor mediating the NE control of 5-HT cell activity. Third, since the studies outlined above indicated that NE released from terminals in the dorsal raphe exerts a dramatic control over 5-HT cell firing, the relationship of NE's regulation of 5-HT cell firing to the influence of GABA-ergic afferents to these cells was examined. Finally, the synaptic circuitry underlying the regulation of 5-HT cells by the NE innervation of the dorsal raphe was investigated using the techniques of electron-microscopic autoradiography.

METHODS

I. Neurophysiological Techniques

1. Introduction

In the four studies to be presented below both neurophysiological and anatomical approaches have been used. The first three experimenal sections rely heavily on the physiological techniques of single unit recording and microiontophoresis. The methods used in these experiments will be described in this section. Since 6-OHDA lesioned animals were used for some recording experiments the methods used in producing and evaluating the lesions are described here as well. The last experimental section utilizes the anatomical techniques of electron-microscopic autoradiography which will be described separately in the next section.

2. Single-Unit Recording

This section describes the methods used to record the action potentials of individual neurons by an electrode placed extracellularly in the vicinity of the cell body. Single-barrel glass micropipettes were pulled to a fine tip, broken back to a diameter of less than 1 micron, and filled with a conducting electrolyte solution of 2 M NaCl, which was saturated with Fast Green FCF (Fisher) dye. The impedances of the electrodes that were used fell in the range of 3-6 MΩ when measured at 60 Hz in vitro.

Male Sprague-Dawley albino rats (Charles River) 200-300 g were anesthetized with chloral hydrate, 400 mg/kg i.p.; additional injections were given as needed. Rats were mounted in a stereo-

taxic instrument; a burr hole was made over the raphe area whose center was in the midline approximately 0.5 mm anterior to lambda. Electrode signals were passed through a high-input impedance amplifier and monitored on an oscilloscope. Integrated firing rate was recorded in consecutive 10 sec samples from the rate meter. The body temperature was kept at 36-37^{o}C. Units displaying a characteristic slow firing rate (approximately 1 spike/sec), regular rhythm and wide duration action potential (1-2 msec) typical of histochemically and antidromically identified 5-HT cells were tested (Aghajanian and Haigler, 1974; Wang and Aghajanian, 1977b). Recording sites were marked at the end of each experiment by passing 20 μA of cathodal current through the recording barrel for 15 min, depositing a green spot at the electrode tip (Thomas and Wilson, 1965). Rats were perfused with 10% formalin. The location of the electrode tip was confirmed by histological examination to correspond to the location of 5-HT-containing neurons in the dorsal raphe nucleus.

3. Microiontophoresis

In order to apply drugs to a limited area in the vicinity of the recording electrode, the microiontophoretic technique, in which drugs present in ionized form are ejected from micropipettes by ionic currents was used (Curtis, 1964). The amount of drug ejected in this manner is approximately proportional to both the magnitude of the applied current and the duration of the ejection period. In the interval in between drug ejections, a small retaining current, usually 10 nanoamperes, of appropriate polarity was applied in order to prevent diffusion of the drug out of the pipette.

Either double-barrel, 5-barrel or 6-barrel arrays of micropipettes

were employed in order to combine a recording electrode with drug delivery channels. Double barrel electrodes were constructed from a recording barrel prepared as described above and a second micropipette broken back to a tip diameter of 2-5 μm. The recording barrel was positioned 15-30 μm ahead of the drug barrel and the two were cemented together (Wang and Aghajanian, 1977a). 5-barrel micropipettes were prepared in which a central barrel was fused with four surrounding side barrels. The 5-barrel assembly was then pulled to a fine tip, and broken back slightly (Haigler and Aghajanian, 1974a). In this way, the central recording barrel and each of the side barrels were separated by only a thin glass wall. This arrangement allowed the ejection of drugs in the immediate vicinity of the recording electrode. Impedances were 3.5-4.5 MΩ in the central recording barrel. The drug solutions were directly injected into side barrels which contained a few strands of fiberglass to allow rapid filling of tips by capillary action (Tasaki et al., 1968). The central recording barrel was filled with 2 M NaCl solution saturated with Fast Green dye. One side barrel, the balance channel, was filled with 4 M NaCl. This channel was used to pass a current which was equal in magnitude to the sum of the currents in the other channels, but of opposite polarity, in order to eliminate any possible artefacts produced by net current flow away from the micropipette assembly. Circuits for drug ejection and current balance were similar to those described by Salmoiraghi and Weight (1967). 6-barrel electrodes were constructed by positioning a single-barrel recording electrode 20-30 μm ahead of a 5-barrel micropipette that had been broken back to a tip diameter of 10-20 μm.

4. Drugs

The drugs administered during each of the first three sections of the Experimental Studies are listed below.

a. Section I

Reserpine powder (RES, Regis) for i.v. administration and iontophoresis was dissolved by addition of a few drops of 10% phosphoric acid. The solution was titrated to pH 4.2 by addition of NaOH solution. The reserpine solution was freshly prepared for each recording session. Parachlorphenylalanine methylester HCl (PCPA, Regis) 400 mg/kg i.p. was injected 40 hr prior to recording experiments.

The following drugs were also administered systemically: l-amphetamine sulfate (A); d-lysergic acid diethylamide bitartrate (LSD); d,l-p-chloroamphetamine hydrochloride (PCA, Regis); l-norepinephrine bitartrate (NE, Regis); clonidine hydrochloride (CLON, Boehringer Ingelheim).

The following drug solutions were prepared for microiontophoresis: 5-HT creatinine sulfate pH 5.0, 0.04 M (5-HT, Regis); l-norepinephrine bitartrate 0.1 M, pH 4.0 (NE); l-glutamate monosodium pH 8.0, 0.1 M (G, Mann Labs).

b. Section II

The following drugs were administered intravenously: WB-4101 (2-2',6'-dimethoxy (phenoxyethylamino) methyl benzodioxan HCl) (WB, WB Pharmaceuticals), piperoxane chlorhydrate (PIP, Rhone-Poulenc), thymoxamine HCl (TMX, Wm. Warner, Ltd.), phenoxybenzamine HCl (PBZ, SK & F), dihydroergocryptine mesylate (DHK, Sandoz), phentolamine mesylate (PHE, Ciba) d-lysergic acid diethylamide

bitartrate (LSD), l-amphetamine sulfate (A, Aldrich), reserpine (RES, Regis).

Solutions for microiontophoresis were as follows: WB (0.1 M) pH 5.0; PIP (0.1 M) pH 4.0; TMX (0.1 M) pH 4.0; PBZ (2.5 mg/ml) pH 3.6; DHK (3.0 mg/ml) pH 5.0; PHE (20 mg/ml) pH 5.5; serotonin creatine sulfate (0.05 M) pH 5.0 (5-HT, Regis); l-norepinephrine bitartrate (0.1 M) pH 4.0 (Regis, NE); monosodium glutamate (0.1 M) pH 8.0 (G, Mann labs); Sotalol HCl (0.1 M) pH 4.0 (SOT, Regis); l-isoproterenol-d-bitartrate (0.1 M) pH 4.0 (ISO, Regis); l-phenylephrine HCl (0.1 M) pH 4.0 (PE, Sigma); salbutamol sulfate (0.1 M) pH 4.0 (SALB, Schering); l-α-methylnorepinephrine (0.1 M) pH 4.0 (α-MNE, Sterling-Winthrop). The following drugs were also used in ionically diluted solutions when iontophoresed from 5-barrel electrodes (section h of Results): WB-4101 (0.01 M) in 0.1 M NaCl, pH 5.0; glutamate (0.01 M) in 0.1 M NaCl, pH 8.0.

c. Section III

The drug solutions used for microiontophoresis were prepared as follows: WB-4101 (2-2',6'-dimethoxy(phenoxyethylamino) methyl benzodioxan HCl) 0.01 M in 0.1 M NaCl pH 5.0 (WB, WB Pharmaceuticals); phentolamine mesylate 20 mg/ml pH 5.5 (PHE, Ciba) l-norepinephrine-D-bitartrate, 0.1 M pH 4.0 (NE, Regis); l-glutamate monosodium, 0.1 M in 0.1 M NaCl, pH 8.0 (G, Mann Labs); γ-amino-butryic acid, 0.1 M or 0.002 M in 0.1 M NaCl (GABA, Calbiochem); 5-HT creatinine sulfate 0.05 M, pH 4.0 (5-HT, Sigma); glycine HCl, 0.1 M in 0.1 M NaCl, pH 4.0 (GLY, Sigma); strychnine, 10 mg/ml, pH 4.0 (STR, Sigma); picrotoxin, saturated solution, pH 4.0 (PIC,

K & K Laboratories); (+)N-methyl-bicuculline iodide, 10 mg/ml, pH 4.0 (BIC, Pierce).

5. Data Analysis

The ED50 of systemically administered drugs was determined by log-probit analysis from the cumulative dose-response curves. The IT50 which is a measure of the dose of iontophoretically applied drugs required to produce a 50% depression of firing rate was calculated as the product of I, the current in nanoamperes, and T50, the time in seconds required to obtain a 50% depression (deMontigny and Aghajanian, 1977).

6. Intraventricular 6-hydroxydopamine Injections

In some rats, intraventricular injections of 6-OHDA, a selective catecholamine neurotoxin (Malmfors and Thorenen, 1971) were administered in order to damage central NE systems. Twenty minutes prior to intraventricular injections, animals were pre-treated with chlorimipramine, 20 mg/kg, i.p. (Ciba) a 5-HT uptake blocker, in order to decrease the possibility of accumulation of 6-OHDA into and subsequent damage of 5-HT neurons. Rats were anesthetized with chloral hydrate, 400 mg/kg, i.p., and mounted in a stereotaxic apparatus. 6-OHDA (Regis) was prepared in concentrations of either 5 or 7.5 mg/ml (free base) in a normal saline solution, containing 1 mg/ml ascorbic acid. Twenty microliters of this solution, containing a total of either 100 or 150 µg of 6-OHDA was injected into the lateral ventricle according to the technique described by Svensson et al. (1975). Briefly, a 22 gauge Hamilton syringe, containing the 6-OHDA solution was lowered into

the brain through a burr hole placed just anterior to bregma and approximately 1 mm lateral to the midline. The syringe was lowered approximately 2.5-3.5 mm below the dura.

Prior to injection, the placement of the tip in the lateral ventricle was verified by aspirating a small amount of cerebrospinal fluid into the syringe. The injected animals were used for either recording or histofluorescence experiments 4-7 days later.

7. Histofluorescence

The formaldehyde-condensation histofluorescence technique for visualization of monoamines (Falck et al., 1962) was used to determine the extent of damage produced by 6-OHDA injection. Both vehicle control and 6-OHDA pretreated animals were given the monoamine oxidase inhibitor pargyline (Regis) 100 mg/kg, i.p., 7 hrs before sacrifice, in order to increase the brain levels of monoamines. Following chloral hydrate anesthesia, animals were decapitated and the brains processed for histofluorescence by the Falck-Hillarp procedure (Falck et al., 1962). Briefly, frontal slabs of brain tissue were frozen rapidly by immersion in a propane-propylene oxide mixture cooled by liquid nitrogen. The tissue pieces were freeze-dried at -35^{o}C for 4 days, and then exposed to formaldehyde vapors in a sealed chamber. Following embedding of the tissue pieces in paraffin, 10 micron sections were examined by fluorescence microscopy.

II. Electron-Microscopic Autoradiography

1. Overview

An overview of the autoradiographic method for identifying

NE or 5-HT neuronal processes is presented here prior to the more detailed description given below. Small amounts of either tritiated NE or 5-HT were placed in the cerebrospinal fluid by intraventricular injection. Following diffusion into the brain parenchyma, the tritiated compound was selectively accumulated by neuronal processes which possess high-affinity uptake systems for the substance. The animal was then perfused with fixative which 'binds' the labelled compound in situ to the tissue preventing its removal by solvents during further processing of the tissue. Tissue sections for either light or electron microscopy were then cut, placed on slides and coated with photographic emulsion. In the process of autoradiography, the beta particles emitted by the tritium result in the production of silver photographic grains. The grains are opaque and electron dense and when developed can be visualized by either light or electron-microscopy.

The combination of Ilford emulsion (with photographic crystals 1000-1600 A in size) and thin sections of tissue (< 1000 A) permits a theoretical distance between the location of the beta emitter bound to the tissue, and the position of the developed grains of about 1850 A (Bachman and Salpeter, 1965).

2. Intraventricular Injections of Tritiated NE or 5-HT

Male albino rats (Charles River, Inc.) weighing 225-275 g were used. Just prior to injection with tritiated NE or 5-HT, the rats (n=16) were pretreated with the monoamine oxidase inhibitor, pargyline, 100 mg/kg, i.p., to prevent the breakdown of these amines. Fluoxetine, a specific 5-HT uptake inhibitor (Wong et al., 1975) 10 mg/kg,

i.p. was also given at this time to rats receiving ^{3}H-NE in order to prevent uptake of ^{3}H-NE into 5-HT neurons. Alternatively, desipramine (25 mg/kg, i.p.), a NE uptake blocker (Glowinski and Axelrod, 1964) was given to rats receiving ^{3}H-5-HT.

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. Two hundred μCi of either tritiated NE or 5-HT was injected (25-43 Ci/mM New England Nuclear or Amersham) intraventricularly, according to the method described by Svensson et al. (1975) in volume of 50 microliters at a rate of 1 microliter per minute. Prior to use, the purity (> 90%) of the tritiated compounds was confirmed by thin layer chromatography.

Ninety minutes following completion of the injection, the rats were perfused transcardially first with a 4% dextran-saline flush and then for 1 hour with cold fixative consisting of paraformaldehyde (1%), glutaraldehyde (2%) (Fisher) in 0.15 M sodium phosphate buffer (0.1 M, pH 7.2). Tannic acid (2%) (Fisher) was included as a mordant to improve final contrast.

In some cases, rats receiving tritiated NE had been pretreated 48 or 72 hours earlier with an intraventricular injection of 5,7-dihydroxytryptamine (5,7-DHT) or 6-hydroxydopamine (6-OHDA) respectively. Rats given intraventricular injections of the catecholamine neurotoxin, 6-OHDA (100 μg, free base) were pretreated with chlorimipramine, 20 mg/kg, i.p. twenty minutes prior to the intraventricular injection in order to protect 5-HT neurons. Prior to intraventricular injection of the selective serotonin neurotoxin, 5,7-DHT (200 μg, free base), the animals were pretreated

with DMI 25 mg/kg, i.p. This treatment has been shown to enhance the selectivity of 5,7-DHT's action (Bjorklund et al., 1975). Previous studies have shown that this procedure selectively destroys the 5-HT neurons throughout the dorsal raphe (Aghajanian et al., 1978).

3. Preparation of Tissue for Microscopy and Autoradiography

Following perfusion, brains were removed and frontal slabs through the area of the dorsal raphe nucleus were blocked and wahsed 30 minutes in phosphate buffer. Blocks were then immersed in 1% cold osmium tetroxide in phosphate buffer for 2 hours, washed overnight in phosphate buffer, rinsed in 0.1 M sodium acetate (pH 7.2) and stained en bloc with cold 1% uranyl acetate for 2 hours. Blocks were then rewashed in sodium acetate, dehydrated, and embedded in Maraglas.

Light autoradiography was carried out on thick sections (2 μ) using Ilford G-5 emulsion and D-19 (Kodak) developer. Electron-microscopic autoradiography was performed on thick (silver to pale gold) sections. The thin sections were placed on celloidin coated slides (2% in amyl acetate) by the loop method. They were then lead-citrate stained, carbon-coated in a vacuum evaporator, dipped in Ilford L-4 emulsion at a dilution prechecked to give a monolayer of silver halide crystals and exposed in the dark for 1-6 months. Phenidone A (Ciba-Geigy) fine grain developer was used. After development, the celloidin membranes were floated onto a clean surface of water and 200 mesh copper grids were placed over the sections. The membranes were picked up on filter paper discs using a suction device. After drying, the grids were lifted from the

membrane and the celloidin was thinned by dipping in amyl acetate. Sections were examined in a Zeiss EM9S electron microscope.

Tissue from one group of animals (n=5) was processed for glyoxylic acid-induced histofluoresence as described by de la Torre (1979), in order to monitor the adequacy of depletion of NE terminals induced by 6-OHDA. Another group (n=6) were processed for formaldehyde-induced histofluorescence to confirm the degeneration of 5-HT cell bodies following 5,7-DHT treatment.

4. Data Analysis

The density of labelled terminals was determined by the following method. Sections were developed at regular intervals until clusters of 7 or more developed grains were observed. In successive weeks, the number of clusters observed per grid square was monitored until a plateau level was reached, suggesting that a saturation of NE-labelled terminals had occurred. The number of grains per grid square (2,025 square microns) was determined in sections developed during this plateau phase.

EXPERIMENTAL STUDIES

I. Reserpine Suppression of Dorsal Raphe Neuronal Firing: Reversal by Local NE Iontophoresis

1. Background

The activity of the serotonin (5-HT)-containing cells of the nucleus raphe dorsalis is sensitive to both serotonergic and adrenergic influences. Increased levels of 5-HT produced either by tryptophan loading or monoamine oxidase inhibition decreases their firing rate (Aghajanian et al., 1970; Trulson and Jacobs, 1975; Gallager and Aghajanian, 1976c). Inhibition of 5-HT reuptake also diminishes the activity of 5-HT cells (Sheard et al., 1972; Bramwell, 1974; Mosko and Jacobs, 1977; Gallager and Aghajanian, 1975). These findings have suggested the concept that 5-HT cell activity is inversely related to 5-HT "availability" (Aghajanian, 1972b).

Pharmacological evidence also suggests the presence of an adrenergic influence on the dorsal raphe. Reduction of adrenergic tone either by blockade of postsynaptic α-receptors, inhibition of catecholamine synthesis, or cessation of adrenergic neuronal firing leads to a depression of 5-HT cell firing (Svensson et al., 1975; Gallager and Aghajanian, 1976a). Furthermore, the amphetamines, which release catecholamines (Glowinski and Axelord, 1965; Stein and Wise, 1969; Carr and Moore, 1970), reverse this suppression.

Reserpine suppresses the firing of 5-HT neurons in the dorsal raphe (Aghajanian and Haigler, 1972). Since reserpine markedly

depletes brain concentrations of both 5-HT (Brodie et al., 1955) and catecholamines, (Holzbauer and Vogt, 1956; Bertler et al., 1956) either of these actions might be responsible for the effect of reserpine on raphe activity. By disrupting the normal storage mechanisms, reserpine could cause an overflow of 5-HT. This increased availability of 5-HT might then produce the depression of raphe cell firing. Alternatively, reserpine's action could stem from its interactions with the adrenergic system. By interfering with adrenergic transmission reserpine could secondarily cause a depression in 5-HT cell firing.

In an attempt to further elucidate the role of serotonergic and adrenergic influences on the dorsal nucleus, experiments aimed at defining the mechanisms underlying reserpine's action on 5-HT cell activity were conducted.

2. Results

a. Time Course of Effects of I.V. Administered Reserpine on 5-HT Cell Activity

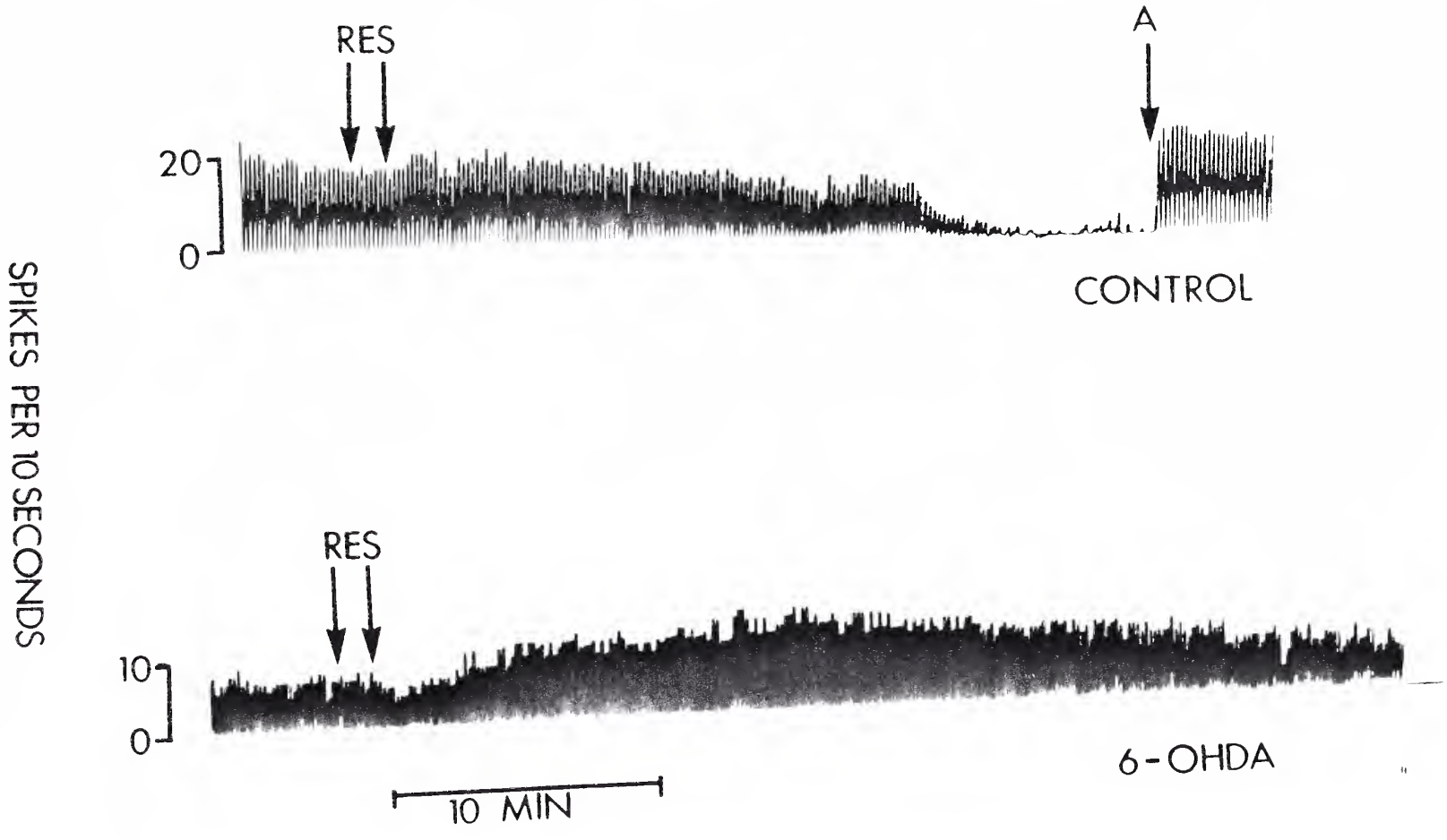
The i.v. administration of reserpine, 5 mg/kg, usually caused an initial transient increase in firing rate. Within 20-30 min, this was succeeded in all cells by a marked depression of firing rate (n=23). Recovery to normal firing pattern was gradual, beginning at 18-24 h and approaching completion between 24-48 h, in all 8 animals studied.

b. Microiontophoresis of Reserpine

The iontophoretic application of reserpine (10 nA, 4-10 min) gradually depressed firing rate by an average of 15%

Figure 1:

Suppression of 5-HT cell firing by reserpine: reversal by l-amphetamine, blockade by intraventricular 6-OHDA injection. In control animals (top trace) i.v. injection of 5 mg/kg reserpine suppresses firing within 30 min. l-Amphetamine (A) i.v. 0.5 mg/kg completely reverses reserpine effect. 5 days after intraventricular injection of 6-OHDA (100 µg), reserpine's depressant effect is blocked (bottom trace).


RES
A
20
0
CONTROL
SPIKES PER 10 SECONDS
RES
10
0
6-OHDA
10 MIN

(n=9). It did not alter the rapid inhibition of cells produced by iontophoretically applied 5-HT (Aghajanian et al., 1972b).

c. 6-OHDA Lesions: Effects on the Reserpine Induced Depression of 5-HT Cells

To ascertain the role of the adrenergic system in reserpine's action, the effect of reserpine was tested in animals pretreated with intraventricular injections of 6-OHDA (Ungerstedt, 1971). Consistent with a reduction of adrenergic tone, Svensson et al. (1975) reported an erratic and abnormally slow firing pattern of the 5-HT cells for two days following injection. However, in animals tested 4-7 days after injection, the rate and pattern of firing of cells was indistinguishable from that in untreated animals, suggesting that 5-HT cells may adapt to a severely compromised adrenergic system. During this period, reserpine's usual depressant effect was prevented in all 4 or the animals receiving 150 μg (free base) and in 9 out of 10 animals receiving 100 μg (Fig. 1). Examination of tissue sections processed for histofluoresence revealed, inter alia, a loss of catecholamine fluorescence in terminals near the dorsal raphe; 5-HT fluoresence in 6-OHDA pretreated animals did not differ from controls injected only with the ascorbic acid vehicle.

d. Reversal of Depressant Effect of Reserpine on 5-HT Cells

If reserpine's effect is mediated by its interaction with the adrenergic system, then restoration of adrenergic tone

should reverse reserpine's depressant effect. Amphetamine is capable of releasing NE following reserpine administration (Kalisker et al., 1975). Indeed, small doses of l-amphetamine (0.5 mg/kg) promptly restored 5-HT cell firing (n=6). In addition, clonidine which at high doses is a directly acting central postsynaptic α-agonist (200-400 μg) (Anden et al., 1970) sharply reversed reserpine's action (n=5) (fig. 2).

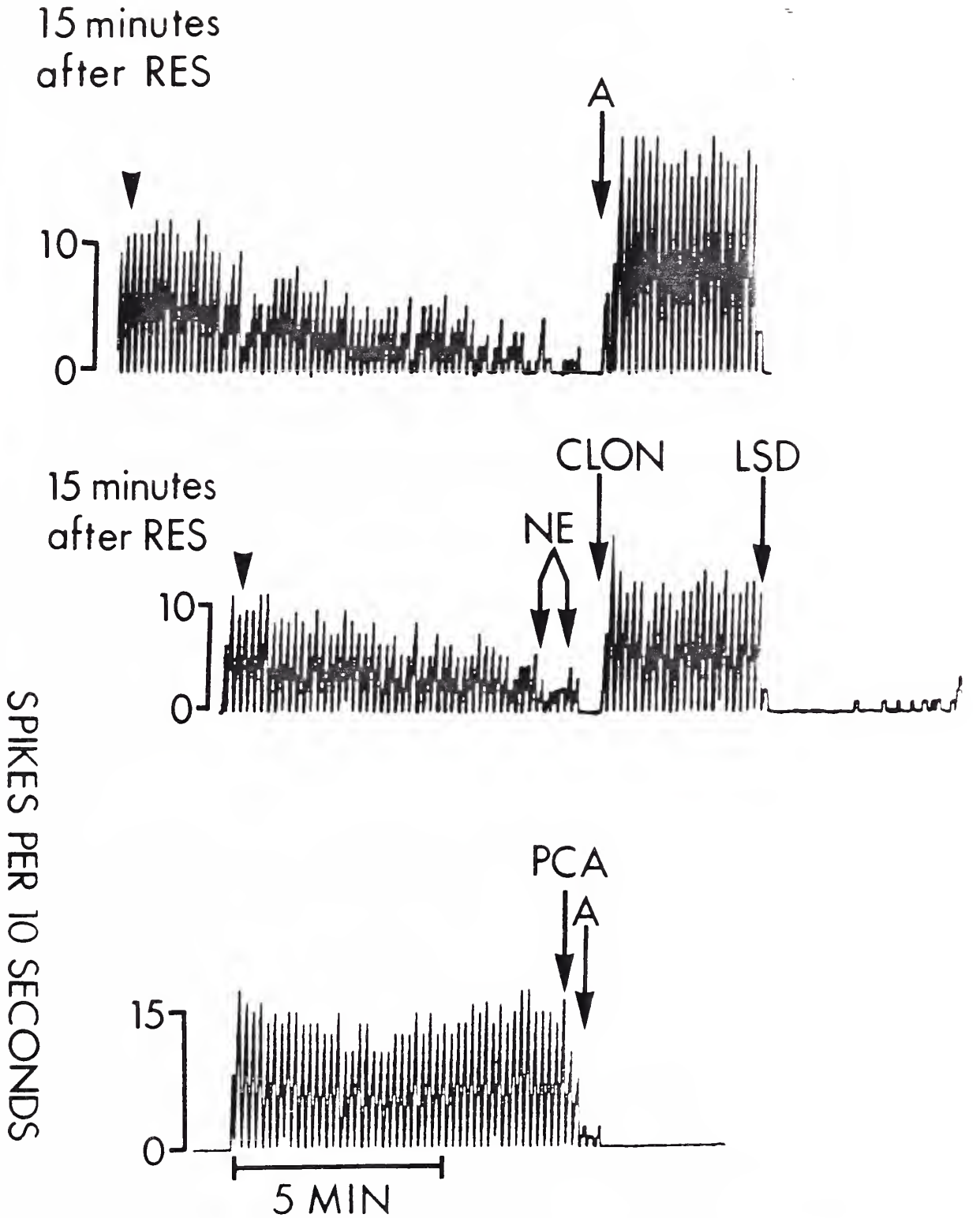
Since clonidine, in this dose range, has a direct action on peripheral post-synaptic α-receptors (Kobinger and Walland, 1967; Boissier et al., 1968; Rand and Wilson, 1968) control experiments were performed to determine the effect on raphe firing of the increase in blood pressure produced by its potent, vasoconstrictive action. Following reserpine administration, NE in doses up to 15 μg/kg (n=3) i.v. did not affect the firing rate, indicating that a peripheral α-adrenergic action is not sufficient to account for the restoration of raphe firing produced by amphetamine and clonidine.

e. Effect of NE Iontophoresis on 5-HT Cell Firing

In an attempt to determine whether the effects of adrenergic drugs could be related to catecholamine terminals present in the vicinity of the 5-HT neurons, the effect of iontophoretically applied NE on raphe cells was tested. In untreated animals, the effect produced by iontophoresis of NE from double-barrel electrodes on 5-HT cell firing varied with firing rate. Of 30 cells tested, with currents in the range of 5-15 nA, three-fifths showed no change in firing rate, one-fifth showed moderate

Figure 2:

Reversal of reserpine's depressant effect by l-amphetamine or clonidine; inability of l-amphetamine to reverse PCA. Reserpine 5 mg/kg, i.v. was administered 15 min prior to time indicated by arrowhead. Both l-amphetamine 0.5 mg/kg and clonidine 200 µg/kg reverse reserpine's inhibition of firing. The inability of i.v. NE (15 µg/kg) to achieve reversal is demonstrated in middle trace. The same cell is then inhibited by the usual dose of LSD (10 µg/kg). The inhibition of 5-HT cells produced by PCA (0.75 mg/kg) is not reversed by 1.5 mg/kg of A (bottom trace).


15 minutes
after RES
A
10
0
15 minutes
after RES
CLON
LSD
NE
10
0
PCA
A
15
0
5 MIN
SPIKES PER 10 SECONDS

increases of less than 50% in rate, while the remaining fifth all of which had the slowest firing rates (0.1-0.4 spikes/sec) had sharp increases more than doubling their rate. However, following the systemic administration of reserpine, low currents of NE 2-10 nA, dramatically reversed the profoundly depressed firing rate in all cells (n=16) regardless of original baseline rate (fig. 3).

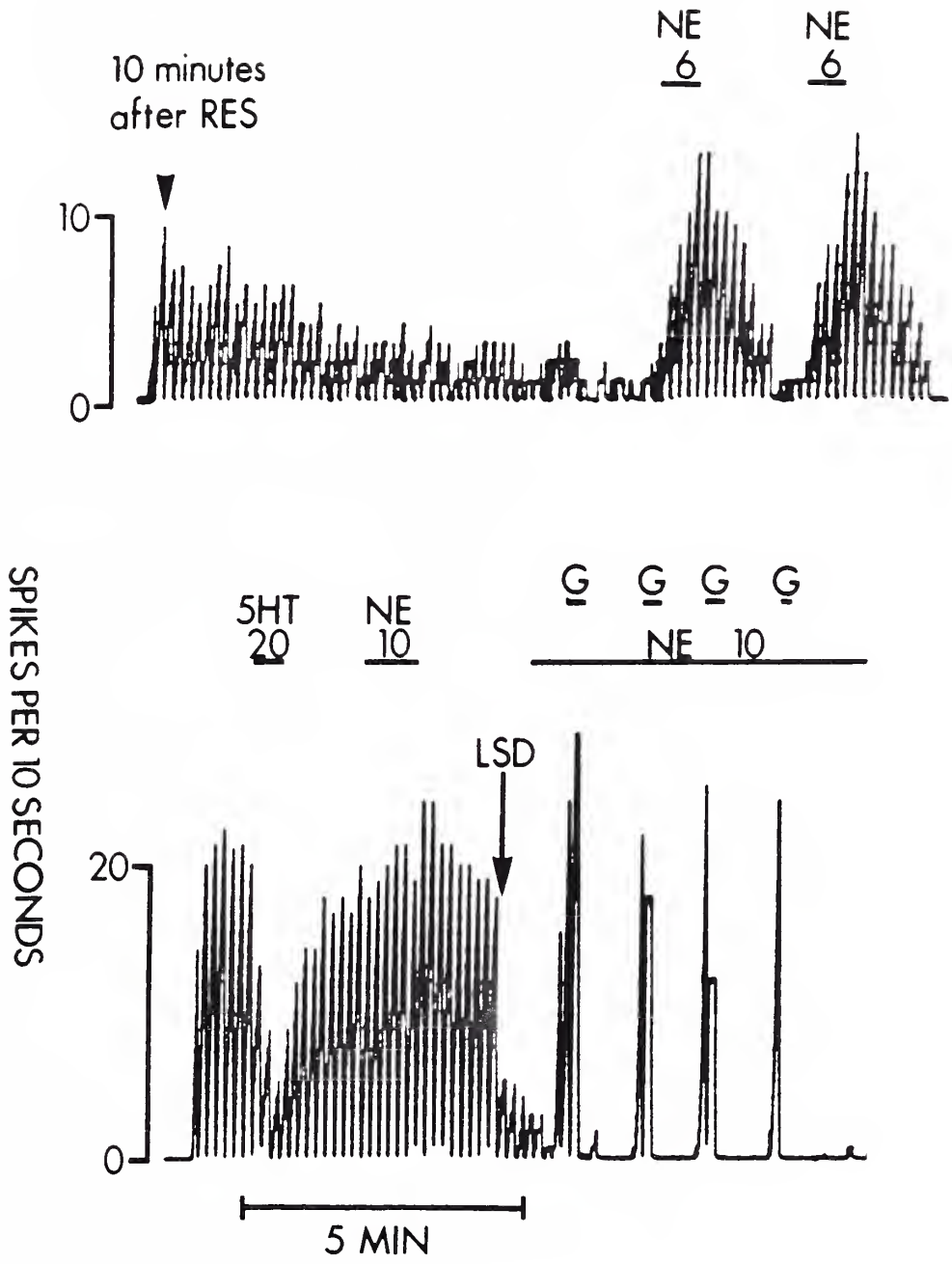
LSD (10-20 μg) administered i.v. inhibits the firing of 5-HT cells. Since this action is not readily reversed by amphetamine (Foote et al., 1969) or blocked by intraventricular injections of 6-OHDA (Svensson et al., 1975; Gallager and Aghajanian, 1976a), it is unlikely that any possible interactions of LSD with adrenergic systems contribute significantly to its ability to inhibit 5-HT cell firing. Thus, the inability of iontophoretically administered NE to reverse the depression of 5-HT cells produced by LSD, while glutamate retains its excitatory action (n=5) (fig. 3), argues against the possibility that general excitatory effects of NE, are responsible for its reversal of reserpine's suppression of 5-HT cell firing.

f. Lack of Effect of PCPA Pretreatment on Reserpine Induced Depression of 5-HT Cells

Reserpine's action might stem from its interaction with 5-HT neurons. By disrupting the storage mechanism of 5-HT, it could increase the availability of 5-HT which could then depress the firing of 5-HT cells. However, PCPA, a potent, specific inhibitor of tryptophan hydroxylase which depletes 5-HT (Koe and Weissman, 1966; Jequier et al., 1967) did not alter the effects

Figure 3:

Local iontophoresis of NE reverses inhibition of 5-HT cell activity produced by reserpine but not LSD. Reserpine (5 mg/kg) was given i.v. 10 min prior to time indicated by arrowhead (top trace). 6 nA of NE applied iontophoretically reverses reserpine's effect. In bottom trace, iontophoretically applied 5-HT (20 nA) rapidly and reversibly inhibits 5-HT cell firing while NE (10 nA) has little effect on firing. LSD (15 µg/kg) i.v. inhibits firing. 10 nA of NE which reverses depressed firing rate produced by reserpine, is ineffective in reversing LSD-induced inhibition. However, pulses of glutamate (10 nA, G) still excite the cell demonstrating its continued presence in the recording field of the electrode.


10 minutes
after RES
NE
6
NE
6
10
0
SPIKES PER 10 SECONDS
5HT
20
NE
10
G G G G
NE 10
LSD
20
0
5 MIN

of reserpine administration (n=6) or its reversal by l-amphetamine (n=2).

g. PCA Depression of 5-HT Cell Firing Not Reversed By l-Amphetamine

I.v. administration of parachloramphetamine, PCA inhibits raphe cell firing (Sheard, 1974; Mosko and Jacobs, 1977) probably through its ability both to release 5-HT and inhibit it's reuptake (Sanders-Bush et al., 1974). If reserpine were acting in a similar manner, then we would expect similar responses to amphetamine administration. Yet l-amphetamine (1-2 mg/kg) in contrast to its abrupt reversal of the reserpine-induced cessation of firing, did not reverse PCA induced depression of firing (n=6) (Fig. 2).

3. Discussion

The results of this section suggest that reserpine's suppression of firing of raphe neurons is mediated through its effect on the adrenergic system. The ability of the catecholamine terminal destruction produced by the specific neurotoxin, 6-OHDA, to prevent reserpine's effect argues strongly that reserpine's interaction with the adrenergic input underlies the depression of activity witnessed. Furthermore, in non-lesioned animals, the effect of reserpine can be reversed by the restoration of adrenergic tone. Evidence has been accumulating that l- and d-amphetamine are nearly equipotent in affecting the function of NE neurons, while the d-isomer of amphetamine is much more potent than the l-isomer in its effect of dopamine neurons (Svensson, 1971; Ferris et al., 1972; Harris and Baldessarini, 1973; Thornburg

and Moore, 1973; Bunney et al., 1975; Holmes and Rutledge, 1976). Therefore, the l-isomer was administered because of its more selective action on NE neurons. Small doses of l-amphetamine caused a dramatic, prompt reinstatement of 5-HT cell activity which had been depressed by reserpine. The administration of a post-synaptic α-agonist, clonidine, achieved a similar effect.

These results are consistent with those observed following a reduction of adrenergic activity produced in other ways. Extensive destruction of the adrenergic system produced by 6-OHDA slows 5-HT cells for several days. In addition, the inhibition of adrenergic neuronal firing caused by low doses of clonidine silences 5-HT cell activity. Furthermore, clonidine's inhibitory action on 5-HT cells is analogous to reserpine's in being prevented by 6-OHDA and reversed by amphetamine, as well as by higher doses of clonidine itself (Svensson et al., 1975).

Reserpine has been shown directly to block adrenergic transmission in the peripheral autonomic system (Von Euler, 1969: Sedvall and Thornson, 1965). This may be the result of depletion of a small functional NE pool directly involved in transmission (Haggendal and Lindqvist, 1964). Thus, blockade of adrenergic transmission by reserpine could account for the silencing of raphe cell firing.

On the other hand, certain behavioral effects of reserpine have been proposed to stem from its ability to "release" 5-HT, resulting presumably from interference with the normal storage mechanisms (Shore et al., 1957). Conceivably, this increased

availability of 5-HT might then explain the depression of 5-HT cell activity observed. However, several studies have suggested that transmitter synthesized during this time is subjected to intraneuronal degradation (Kopin and Gordon, 1962, 1963; Stjarne, 1964; Iversen et al., 1965; Glowinski et al., 1966). In addition, the fact that a blockade of 5-HT synthesis by PCPA failed to alter reserpine's action does not support the view that reserpine's questionable ability to release 5-HT extracellularly mediates its suppression of 5-HT cell firing. However, since PCPA does not deplete 5-HT entirely from cell bodies in the dorsal raphe (Aghajanian et al., 1973) we cannot exclude serotonergic involvement in reserpine's action on this basis. Therefore, additional evidence was sought by experiments employing PCA. This compound, which releases 5-HT, inhibits 5-HT cell firing. Its depressant action on raphe units is effectively blocked by PCPA pretreatment (Mosko and Jacobs, 1977). This strongly suggests that the inhibition of 5-HT cells produced by PCA, stems from its ability to increase 5-HT "availability". If indeed, reserpine were acting in a similar fashion, we would predict that PCA's inhibition of raphe firing would be reversed by l-amphetamine. This was not the case.

Perhaps the most surprising result presented above is the ability of locally iontophoresed NE to reverse reserpine's depression of raphe firing. The presence of a dense catecholamine innervation of the dorsal raphe has been established by biochemical, histofluoresence, and immunocytochemical techniques (Fuxe, 1965; Roizen and Jacobwitz, 1976; Swanson and Hartman, 1975). The

ability of local NE iontophoresis to mimic the actions of amphetamine and clonidine in reversing reserpine's inhibition of single unit activity suggests the possibility that these terminals provide the anatomical basis of the adrenergic drug effects observed on the 5-HT cells of the dorsal raphe.

II. Suppression of Firing Activity of 5-HT Neurons in the Dorsal Raphe by Alpha-Adrenoceptor Antagonists

1. Background

Pharmacological evidence suggests that the firing activity of serotonin-containing (5-HT) cells of the dorsal raphe nucleus is dependent on a tonically active adrenergic system. Administration of either a low dose of clonidine (Svensson et al., 1975), which acts presynaptically to inhibit norepinephrine (NE) cell firing, or reserpine, which impairs noradrenergic transmission, suppresses 5-HT cell firing (Section I of Experiments). Furthermore, amphetamine, which releases catecholamines (Glowinski and Axelrod, 1965; Stein and Weiss, 1969; Carr and Moore, 1970) rapidly restores firing after either of these treatments.

Antipsychotic drugs which are thought to possess adrenergic blocking activity (Keller et al., 1973) reduce 5-HT cell firing when administered systemically (Gallager and Aghajanian, 1976a). An alpha-adrenoceptor is thought to mediate these effects since piperoxane, an α-antagonist, but neither sotalol, a β-adrenoceptor antagonist, nor haloperidol, a dopamine antagonist, is effective in reducing 5-HT cell firing (Gallager and Aghajanian, 1976a). In addition, clonidine which in high doses acts centrally as a postsynaptic alpha agonist (Anden et al., 1970), can restore activity after the suppression of firing produced by either reserpine or small doses of clonidine (Svensson et al., 1975).

The dorsal raphe receives a prominent adrenergic input as

demonstrated by histofluorescence (Fuxe, 1965), biochemical (Roizen and Jacobowitz, 1976) and histochemical techniques (Swanson and Hartman, 1975). Thus, the adrenergic terminals mediating the adrenergic-serotonergic interaction could be located in the dorsal raphe itself. This suggestion is supported by the ability of NE to reverse the reduction of firing produced by reserpine when NE is applied iontophoretically in the vicinity of 5-HT cells (Section I of Experiments).

To determine whether alpha-adrenoceptor blockers, in general, can suppress 5-HT cell firing, several different types of alpha-adrenoceptor antagonists were tested in the present study. These agents represent a variety of chemical classes: a haloalkylamine, phenoxybenzamine (Nickerson and Nomaguchi, 1951), an ergot alkaloid, dihydroergocryptine (Rothlin, 1946), an imidazoline, phentolamine (Meier and Yonkman, 1949), two benzodioxans, WB-4101 and piperoxan (Mottram and Kapur, 1975; Bacq and Fredericq, 1934), and a thymoxy-alkylamine, thymoxamine (Greeff and Schumann, 1953). The effects of these drugs on 5-HT cell firing were determined after their systemic administration. Furthermore, in the light of the possibility that alpha-adrenoceptor antagonists block the effects of NE released from adrenergic terminals located within the dorsal raphe, the effects of the local, iontophoretic application of these drugs on single unit activity of 5-HT cells in the dorsal raphe were also investigated.

2. Results

a. Effects of Systemic Administration of Alpha-

Adrenoceptor Antagonists on 5-HT Cell Activity

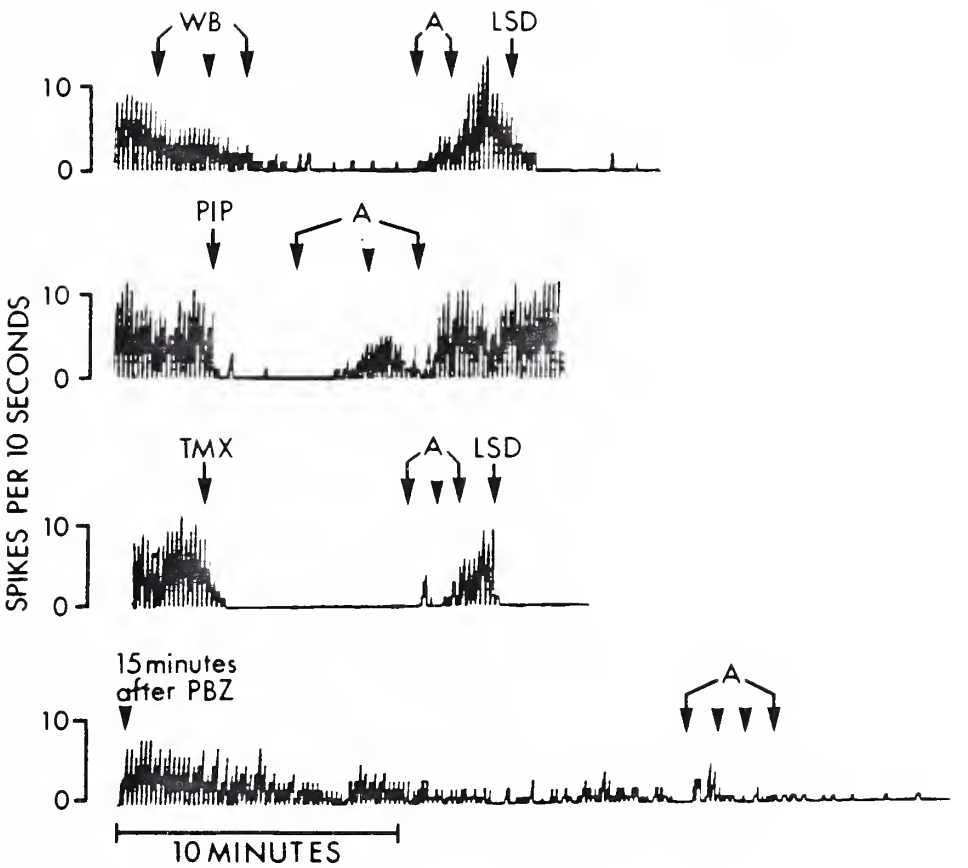
Intravenous administration of the alpha-adrenoceptor antagonists WB-4101 (n=27), piperoxan (n=8) or thymoxamine (n=9) rapidly and totally suppressed 5-HT cell firing. The ED50 for suppression of firing for these drugs was as follows: WB-4101, 41 ± 20 µg/kg (ED50 ± SD); piperoxan 0.64 ± 0.20 mg/kg; thymoxamine, 0.42 ± 0.31 mg/kg. Phenoxybenzamine (3.0 mg/kg, n=6) also suppressed firing but this occurred gradually over 30 min (fig. 4). In animals tested after previous injections of PBZ (25 mg/kg, i.p.), recovery of activity had commenced by 15 hr (n=4) and gradually neared completion at 36 hr (n=3). In contrast to the above drugs, large doses of dihydroergocryptine (6.0 mg/kg, i.v. n=4) only partially suppressed firing (10-50%). Phentolamine, at doses up to 10.0 mg/kg (i.v.) (n=4) did not suppress firing rate by more than 20%. Perhaps the weak activity of these two drugs when given by the systemic route stems from their limited ability to enter the brain (see Discussion).

b. Effects of 6-OHDA Lesions on WB-4101 Suppression of 5-HT Cell Firing

The destruction of adrenergic terminals by 6-OHDA has been shown to block the ability of the pre-synaptically acting drugs, reserpine and low doses of clonidine (Section I of Experiments) (Svensson et al., 1975) to suppress the firing activity of 5-HT cells. Therefore, the effect of 6-OHDA pretreatment on the ability of an alpha-adrenoceptor antagonist, WB-4101, to suppress 5-HT cell firing was examined. Animals were given

Figure 4:

Suppression of 5-HT cell firing by alpha-adrenoceptor antagonists: reversal of competitive blockade by l-amphetamine. Intravenous administration of WB-4101 (WB: successive doses of 30, 15, 15 µg/kg given at times indicated by arrows), piperoxan (PIP: 1 mg/kg), or thymoxamine (TMX: 1.5 mg/kg) rapidly suppressed firing. Phenoxybenzamine (PBZ: 3.0 mg/kg, i.v.) gradually suppressed firing within 30 min. l-Amphetamine (l-A: cumulative doses of 2 mg/kg, i.v.) restored activity following suppression of firing caused by the three competitive antagonists. However, l-A (5 mg/kg, i.v.) was ineffective in overcoming the non-equilibrium blockade produced by phenoxybenzamine. The ability of LSD (10-15 µg/kg, i.v.) to inhibit 5-HT cell firing remained unimpaired.


WB
A
LSD
10
0
PIP
A
10
0
SPIKES PER 10 SECONDS
TMX
A
LSD
10
0
15 minutes
after PBZ
A
10
0
10 MINUTES

intraventricular injections of 6-OHDA (150 µg, free base). For the first few days, 5-HT cells fired slowly and erratically, consistent with the loss of adrenergic tone. However, in animals tested 4-7 days after injection, the normal rate and pattern of firing resumed (Svensson et al., 1975), suggesting that 5-HT cells may adapt in some manner to a severely compromised adrenergic system. During this period, the ability of WB-4101 to suppress firing activity was tested. The ED50 for suppression of firing by WB-4101 was found to be 91 $\pm$ 45 µg/kg (ED50 $\pm$ SD) (n=9). This value was significantly greater than that obtained in unlesioned animals, 41 $\pm$ 20 µg/kg ($p < 0.02$).

c. Effect of l-Amphetamine on Suppression of 5-HT Cell Firing Produced by Alpha-Adrenoceptor Antagonists

As the administration of a variety of alpha-adrenoceptor antagonists suppressed 5-HT cell firing, it was of interest to observe the effects of an increase in adrenergic tone produced by amphetamine on this suppression. Evidence has been accumulating that l- and d-amphetamine are nearly equipotent in affecting the function of NE neurons, while the l-isomer of amphetamine is much less potent than the d-isomer in its action on dopaminergic neurons (Svensson, 1971; Ferris et al., 1972; Harris and Baldessarini, 1973; Thornburg and Moore, 1973; Bunney et al., 1975; Holmes and Rutledge, 1976). Therefore, the l-isomer was selected for use because of its more selective action on NE neurons. First, doses of WB-4101 or thymoxamine causing greater than 80% suppression of firing were administered. Following the administration of WB-4101, l-amphetamine

(2.0-5.0 mg/kg, i.v.) was administered. When l-amphetamine was given during a period of total suppression of firing, it restored activity in only 2 out of 5 animals, suggesting that the limited catecholamine supply released by amphetamine might be unable always to overcome the high degree of competitive blockade produced by WB-4101. However, when l-amphetamine was given after recovery to a low level of firing, it consistently restored cell firing close to baseline rate (n=5). Following thymoxamine administration, l-amphetamine was always able to restore firing (n=5). As previously reported (Gallager and Aghajanian, 1976a), l-amphetamine also restored activity when administered during the suppression of firing caused by piperoxan (n=3). If the NE releasing action of amphetamine does underly the observed restoration of firing activity, then amphetamine might be effective only during the suppression of 5-HT cell firing produced by competitive antagonists. Therefore, the response to l-amphetamine was examined when administered following phenoxybenzamine, which produces a non-equilibrium blockade (Nickerson, 1957). After phenoxybenzamine, doses of l-amphetamine up to 5.0 mg/kg (n=4) were ineffective in restoring activity (fig. 4).

d. Effect of NE Iontophoresis on 5-HT Cell Firing

Previous studies have examined the effect of NE iontophoresis on the spontaneous firing activity of 5-HT cells (Svensson et al., 1975; Gallager and Aghajanian, 1976a; Aghajanian et al., 1972b; Couch, 1970; Haigler and Aghajanian, 1973). These investigators, using 5-barrel electrodes, reported variable

effects in the dorsal raphe. These observations differ from the absence of depressant responses noted when NE is iontophoresed from 2- or 6-barrel electrodes in which the drug ejection barrel is separated by 20-30 μ from the recording electrode (Section I of Experiments). This difference between the effect of NE when iontophoresed in the vicinity of the soma with 5-barrel electrodes and when applied at a distance with 6-barrel electrodes suggests that the depressant response observed with the former may be related to high concentrations of NE deposited close to the cell body. Therefore, a more detailed investigation of NE's effect was undertaken in the present study. When low currents of NE were tested from 5-barrel electrodes, activation of 5-HT cell firing prevailed; however, at higher currents an increasing proportion of depressant responses was observed (Table 1).

e. Reversal of Effects of Competitive Alpha-Adrenoceptor Blockade by NE Iontophoresis

Following systemic administration of several competitive alpha-adrenoceptor blocking agents, the ability of NE iontophoresis to restore firing activity was tested. Six-barrel electrodes were used in order to avoid the direct depressant effects of NE, which occur with 5-barrel electrodes. As illustrated in Figure 5, the iontophoresis of NE ($\leq$ 15 nA) reversed the diminished activity caused by the competitive alpha-adrenoceptor blocking agents, WB-4101 (7 of 8 trials), piperoxane (3 of 4 trials), and thymoxamine (5 of 5 trials). In contrast, following phenoxybenzamine, which produced a non-equilibrium blockade, NE, at currents up to

Table 1. Response of 5-HT Cells to NE Iontophoresis

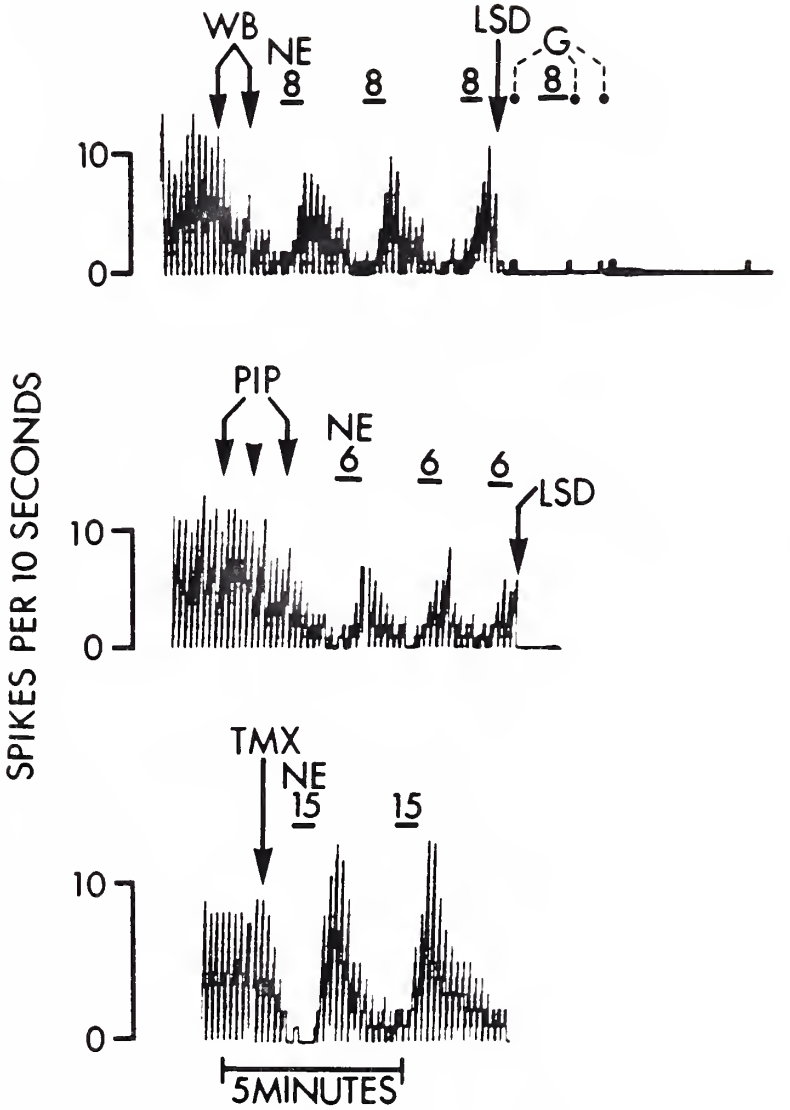
Current (nA)	Number of cells (Avg. rate) (spikes/10 sec)	Activated (%)	No change (%)	Inhibited (%)	Mean % response
2.5	10 (8.0)	80	20	0	152 ± 11
5	18 (9.2)	61	33	6	144 ± 13
10	8 (12.8)	25	50	25	93 ± 10
20	8 (11.2)	12	0	88	59 ± 14*

The response of 5-HT cells in the dorsal raphe to several doses of NE, iontophoretically applied from 5-barrel electrodes; NE was iontophoresed for 1 min at the currents shown. The mean baseline rate for each cell group is shown in parenthesis. Response is measured as the average rate of cell firing during 1 min interval 30-90 sec following onset of NE iontophoresis compared to baseline rate. Activation is defined as a response of greater than 120%. Cells which did not alter their rate by more than 20% are labelled as unchanged. Inhibition is defined as a reduction in rate of greater than 20%. Mean response and standard error of mean for each group are shown in right hand column.

* Differs from both 2.5 and 5 nA groups by one-way analysis of variance ($p < 0.01$).

Figure 5:

Reversal by NE iontophoresis of suppression of 5-HT cell firing produced by systemic administration of alpha-adrenoceptor antagonists. Intravenous administration of the competitive antagonist WB-4101 (two doses of 25 µg/kg) piperoxan (3 doses of 0.4 mg/kg) or thymoxamine (0.8 mg/kg) caused a reduction of firing rate. Iontophoresis of NE in the vicinity of 5-HT cells promptly restored activity. As shown in the top panel, the inhibitory action of LSD (10 µg/kg) was unaffected by NE iontophoresis. Pulses of glutamate (G) elicited spikes demonstrating the continued presence of the 5-HT cell in the recording field.


WB
NE
8
8
8
LSD
G
8
10
0
PIP
NE
6
6
6
LSD
10
0
TMX
NE
15
15
10
0
5MINUTES
SPIKES PER 10 SECONDS

15 nA, was ineffective in restoring activity even though activation by glutamate iontophoresis could still be elicited (n=4). At currents up to 15 nA, NE was also unable to reverse the inhibition produced by intravenously administered LSD (10-15 μg/kg, n=6), which is thought to inhibit 5-HT cell firing by stimulating 5-HT autoreceptors (Aghajanian and Wang, 1978).

f. Effects of Iontophoresis of Alpha-Adrenoceptor Antagonists on 5-HT Cell Activity

Adrenergic terminals have been demonstrated within the dorsal raphe. Thus, the suppressant effects produced by the systemic administration alpha-adrenoceptor antagonists could result from the blockade of NE released from these terminals. Therefore the effects of alpha-adrenoceptor antagonists were examined when they were iontophoretically applied in the vicinity of 5-HT cells. Iontophoresis of alpha-antagonists, but not sotalol, a β-adrenoceptor antagonist (Lish et al., 1965), gradually, totally, and reversibly suppressed 5-HT cell firing (Table 2). Iontophoretic application of NE from 6-barrel electrodes effectively restored 5-HT cell firing during a period of suppression produced by alpha-antagonists (fig. 6). Since 5-HT cells have been found to be inhibited by 5-HT (Aghajanian et al., 1972b), it was of interest to determine whether these agents might be stimulating 5-HT autoreceptors and thereby reducing 5-HT cell firing. However, in contrast to the ability of NE to reverse the effects of alpha-adrenoceptor antagonists. NE (10-15 nA) did not diminish the inhibition produced by 5-HT iontophoresis (n=6). This observation supports the contention

Table 2. Response of 5-HT Neurons in the Dorsal Raphe to Iontophoretically Applied Adrenergic Antagonists

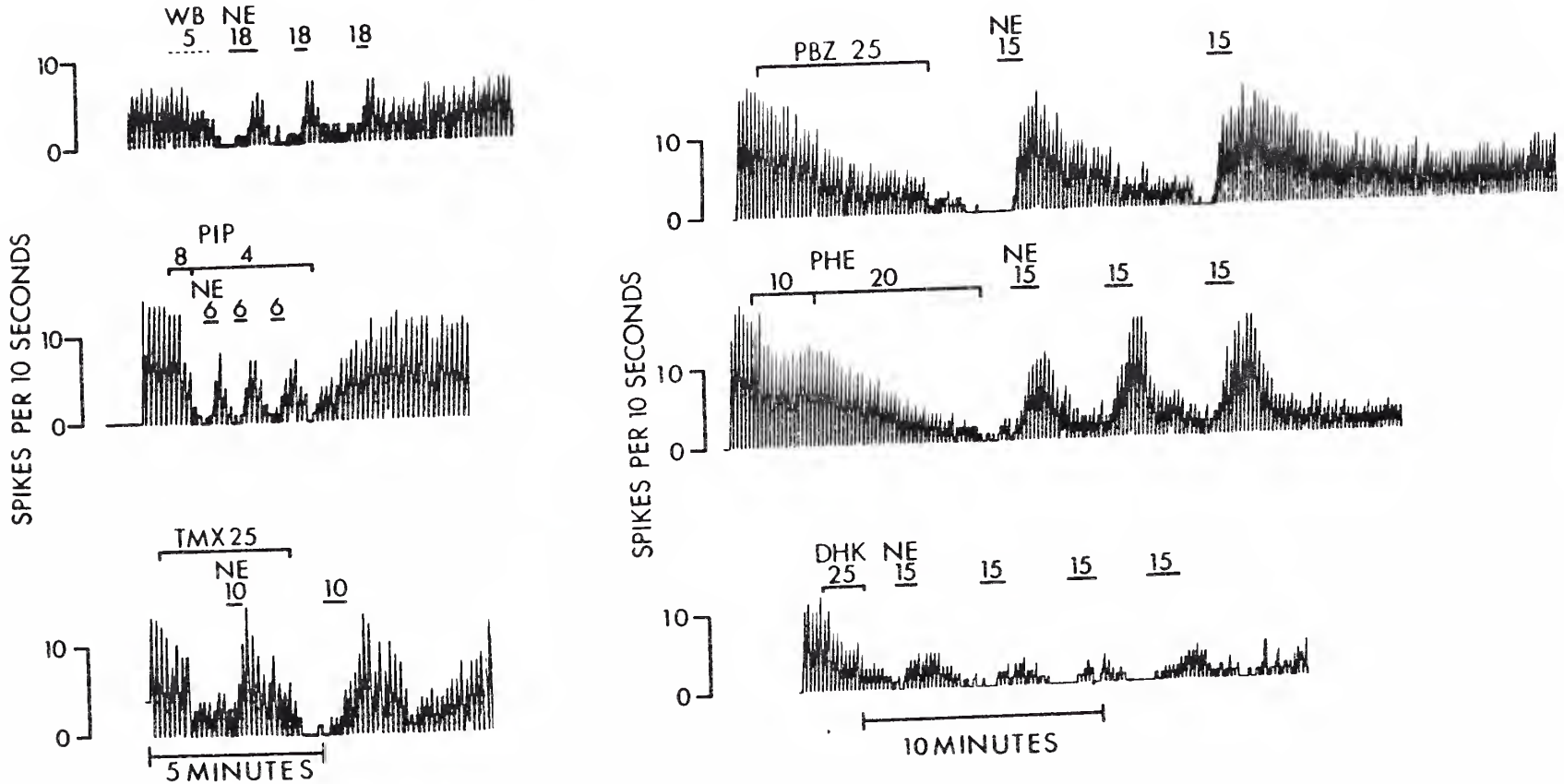
Drug	Current (nA)	Duration (min)	$IT_{50} \times 10^{-3}$	Number of Cells Suppressed	No Change	NE reversal
WB-4101	0.5-10	1-2	0.240 (0.210-0.278)	42	-	14/15
PIP	10-30	2-3	0.883 (0.664-1.17)	13	-	4/4
TMX	20-35	2-4	2.05 (1.56-2.68)	8	-	4/4
PBZ	25	2-3	2.46 (2.07-2.92)	7	-	6/6
PHE	25	2-3	1.29 (0.88-1.87)	9	-	6/6
DHK	10-25	1-3	1.79 (1.50-2.13)	11	-	2/6
SOT	25	3-10		-	6	

The effect of alpha-adrenoceptor antagonists was tested from 6-barrel electrodes. The range of current and duration parameters used to obtain total suppression of activity for at least 30 sec are listed. The IT_{50} is calculated as the product of the time (sec) and current (nA) which produced 50% suppression of firing. The geometric mean is listed because it simplifies subsequent statistical analysis (Fleming et al., 1972). The values in parentheses are the geometric mean + SEM. The β-adrenoceptor antagonist sotalol did not suppress firing rate by more than 20%. Reversal of suppression by NE is defined as restoration of firing rate to greater than one-half of baseline rate. An increase in firing rate was never observed for any of the drugs tested.

* Differs from other groups by one-way analysis of variance ($p < 0.01$).

Figure 6:

Suppression of 5-HT cell firing by iontophoresis of alpha-adrenoceptor antagonists. Iontophoretic application of piperoxan, thymoxamine, WB-4101, phenoxybenzamine, phentolamine (PHE), and dihydroergocryptine (DHK) produced complete suppression of 5-HT cell firing. Iontophoretic application of NE surmounted this competitive blockade. However, NE was less effective following iontophoresis of dihydroergocryptine than after the application of the other alpha-adrenergic antagonists tested.


WB NE
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PIP
8 4
NE
6 6 6
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TMX 25
NE
10 10
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5 MINUTES
SPIKES PER 10 SECONDS
PBZ 25
NE
15 15
10
0
PHE
10 20
NE
15 15 15
10
0
DHK NE
25 15 15 15 15
10
0
10 MINUTES
SPIKES PER 10 SECONDS

that alpha-adrenoceptor antagonists act independently of the 5-HT autoreceptor. Of note was the observation that the reversal of dihydroergocryptine by NE was less complete than that produced with other alpha-adrenoceptor antagonists tested. It is possible that dihydroergocryptine may share agonist activity at the 5-HT autoreceptor with another ergot alkaloid, LSD.

g. Ability of Adrenergic Agonists to Restore 5-HT Cell Activity During Alpha-Adrenoceptor Blockade

The ability of adrenergic agonists to reverse the suppressant effects of iontophoretically applied WB-4101 was tested. Each agonist was compared with NE on the same cell. The response to each drug was defined as the highest average firing rate observed during three consecutive 10 sec intervals. Phenylephrine (PE), a selective α-agonist (Furchgott, 1972) was nearly equipotent to NE when applied at the same current and duration. The ratio of the PE response to that of NE was 0.85 ± 0.16 (mean $\pm$ SEM, n=6). Under these conditions, phenylephrine's action lasted more than twice as long as that of NE, possibly due to less efficient inactivation mechanisms (Trendelenburg, 1972; Tye et al., 1967). α-Methylnorepinephrine was less than one-fourth as potent as NE when tested at the same current and duration (n=5). Isoproterenol, a strong β-agonist which possesses activity at alpha-adrenergic receptors at high doses (Trendelenburg, 1974; Bevan et al., 1977) produced only a weak activation even when administered for twice as long at twice the current used for NE (n=5). Salbutamol, another selective β-agonist (Cullum et al., 1969; Brittain et al., 1970) was unable

to produce activation under the same conditions used for isoproterenol (n=7) (fig. 7).

h. Blockade of NE Activation of 5-HT Cells by Alpha-Adrenoceptor Antagonists

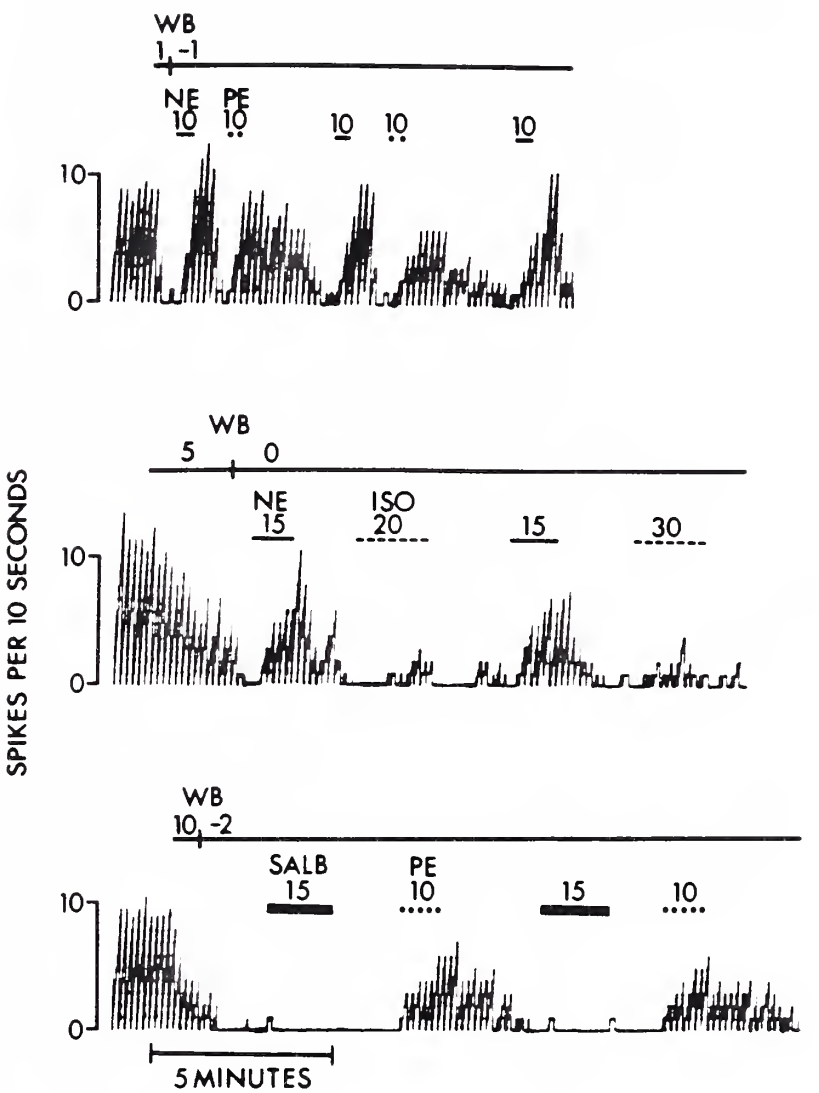
Systemic administration of reserpine has been found to suppress 5-HT cell firing (Aghajanian and Haigler, 1972). Following cessation of activity, NE iontophoresis restored 5-HT cell firing (Section I of Experimental Studies). Since spontaneous 5-HT cell firing may be dependent on NE released from terminals present within the dorsal raphe, the effects of α-adrenoceptor antagonits was examined on 5-HT cell activity which is clearly dependent on NE applied iontophoretically in the vicinity of 5-HT cells. Reserpine (5 mg/kg, i.v.) was administered causing a suppression of 5-HT cell firing activity. Then, 5-HT cells were located by intermittent iontophoresis of glutamate from 5-barrel electrodes. This procedure caused a transient train of spikes. Iontophoresis of NE ($<$ 10 nA for 1 min) produced a resumption of firing activity in all 5-HT cells located in this manner (n=15). Low doses of WB-4101 (1-5 nA for 1-5 min, n=5) and phentolamine (1-2 nA for 1-3 min, n=6) blocked the activation of 5-HT cell firing by NE by more than 50% but did not alter the ability of glutamate to excite these cells (fig. 8).

3. Discussion

The results of this section support the hypothesis that the firing activity of 5-HT neurons in the dorsal raphe is dependent on the tonic activation of alpha-adrenoceptors. A chemically

Figure 7:

Reversal of suppression of 5-HT cell firing by adrenergic agonists. Iontophoresis of WB-4101 totally inhibited 5-HT cell firing. A nearly constant degree of blockade was maintained by the use of low retaining currents. Phenylephrine (PE) was almost as potent as NE in restoring activity (top panel) whereas isoproterenol (ISO) was only weakly active (middle panel). Furthermore, salbutamol (SALB) was totally ineffective in restoring activity (bottom record).


WB
1 -1
NE
10
PE
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10
0
WB
5
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NE
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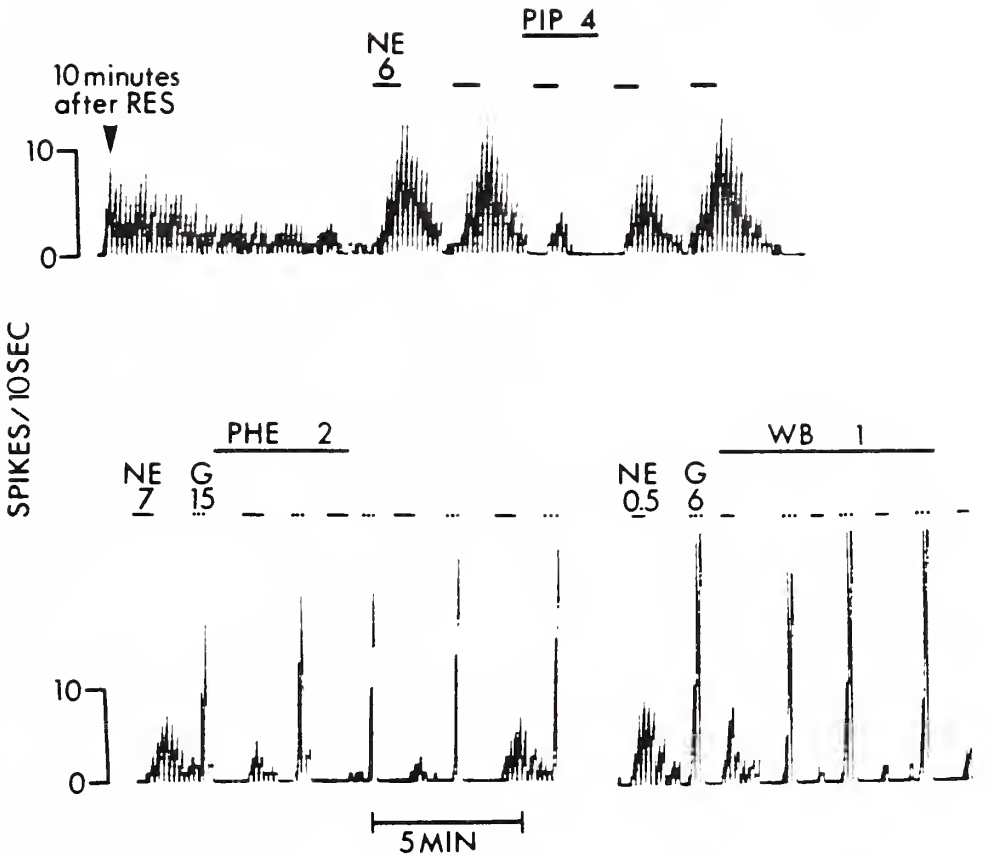
ISO
20
15
30
10
0
WB
10 -2
SALB
15
PE
10
15
10
10
0
5 MINUTES
SPIKES PER 10 SECONDS

Figure 8:

Blockade of NE activation of 5-HT cell firing by alpha-adrenoceptor antagonists. Following suppression of 5-HT cell firing by reserpine (RES: 5 mg/kg, i.v.) iontophoresis of NE restored firing. The alpha-adrenoceptor antagonist piperoxan (PIP: 4 nA) blocked NE activation of 5-HT cell firing (top panel). Blockade of NE activation following reserpine (5 mg/kg, i.v., not shown) is also demonstrated for the alpha-antagonists phentolamine (PHE, 2 nA) and WB-4101 (WB, 1 nA) (bottom panel). Furthermore, the excitation produced by glutamate (G) was unaltered by these drugs, demonstrating the specificity of their blockade.


PIP 4
NE
6
10 minutes
after RES
10
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SPIKES/10SEC
PHE 2
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G
15
WB 1
NE
0.5
G
6
10
0
5 MIN

diverse group of alpha-adrenoceptor antagonists suppressed the firing of 5-HT cells in the dorsal raphe when administered either systemically, or iontophoretically in the vicinity of 5-HT cells. Previous anatomical studies have demonstrated that the dorsal raphe receives an adrenergic input. Together, these observations suggest that NE terminals present within the dorsal raphe could mediate a tonically active adrenergic influence on 5-HT cells located there.

The potency of systemically applied drugs in suppressing 5-HT cell firing is WB-4101 > piperoxan ≈ thymoxamine. This ranking correlates well-with other measures of alpha-adrenoceptor blocking activity. In the vas deferens, WB-4101 is much more potent than thymoxamine (Mottram and Kapur, 1975), and piperoxan and thymoxamine are of roughly similar potency at post-synaptic sites in the periphery (Drew, 1976). Upon iontophoretic application of these drugs in the dorsal raphe, WB-4101 retained a much greater potency than either piperoxan or thymoxamine. Interestingly, systemically applied dihydroergocryptine and phentolamine failed to elicit significant changes in firing at doses which cause significant peripheral alpha-receptor blockade. However, the dramatic increase in effects displayed by phentolamine and dihydroergocryptine, when they were applied iontophoretically, suggests that they possess activity at this central site, but penetrate the brain poorly. This interpretation is supported by the behavioral studies of Rothlin (1946) and the biochemical studies of Anden and Strombom (1974).

The possibility that the effects of systemically administered agents stem from peripheral alpha-adrenergic agents stem from

peripheral alpha-adrenergic blockade is unlikely, because (1) when these agents were administered iontophoreticaly they had similar effects, and (2) phentolamine at doses which produce alpha-adrenoceptor blockade peripherally but apparently penetrate the brain poorly was devoid of effects on 5-HT cell firing.

The delayed onset of action of phenoxybenzamine and prolonged effect in the dorsal raphe following systemic administration is consistent with a similar pattern observed in the periphery (Nickerson, 1962). The reduction in 5-HT cell firing produced was not reversible by either amphetamine or NE as would be expected for a nonequilibrium blockade (Nickerson, 1957). However, following short term iontophoretic application the inhibition produced was of a reversible nature, as reported by Bevan et al. (1977) in another area.

The results of this section suggest that the release of NE from adrenergic terminals present within the dorsal raphe maintains 5-HT cell firing activity. However, iontophoresis of NE in the vicinity of these 5-HT neurons has been previously reported to produce variable effects on their firing rate. At first glance, these findings appear to be inconsistent with the suggestion that the adrenergic input to the dorsal raphe activates 5-HT cells. However, the results of this study reveal that at low currents, NE exerts predominantly an activating influence on their spontaneous firing activity. In addition, NE can reverse the suppression of 5-HT cell firing produced by reserpine. Several lines of evidence indicate that the activation of 5-HT cells by NE is

mediated by alpha-adrenoceptors: (1) NE reverses the suppression produced by alpha-adrenoceptor antagonists when they are administered either systemically or iontophoretically; (2) phenylephrine, another alpha-adrenoceptor agonists, shares ability of NE to reverse the suppression produced by an alpha-adrenoceptor antagonist, WB-4101; (3) alpha-adrenoceptor antagonists block the activation of 5-HT cells by NE following the administration of reserpine. This demonstration that alpha-adrenoceptors mediate the activation by NE further strengthens the hypothesis that the adrenergic system exerts an activating influence on 5-HT cells in the dorsal raphe. It is unclear whether the depressant effect observed at higher levels of NE has any physiological significance.

Since the 5-HT cells of the dorsal raphe respond to alpha-adrenoceptor blockade, both locally and systemically, they provide a potentially useful means of evaluating physiologically the central alpha-adrenoceptor antagonist properties of drugs. The pharmacology of this receptor resembles that of the classical post-synaptic α-adrenergic receptor found in the periphery. Alpha-adrenergic receptors located in the locus coeruleus have been classified in the $alpha_2$-category (Cedarbaum and Aghajanian, 1977) proposed by Langer (1974) and Starke et al. (1975). In the locus coeruleus α-MNE is more potent than NE, while PE is only weakly active. In contrast, in the dorsal raphe a reversal of this rank ordering was observed: PE is nearly equipotent to NE, while α-MNE is only weakly active. This suggests that this receptor may be of the alpha-1 type. In addition, WB-4101 and

thymoxamine exhibit preferential blockade of α_1-receptors (Kapur and Mottram, 1978; Drew, 1977; Marshall et al., 1978).

If 5-HT cell firing is dependent on adrenergic tone, then destruction of the adrenergic input to the dorsal raphe by 6-OHDA should and does suppress firing. However, the ability of 5-HT cells to adapt to the chronic loss of adrenergic tone and resume an apparently normal pattern of firing remains unexplained. The inability of drugs which act pre-synaptically i.e., reserpine and low doses of clonidine, to suppress firing following 6-OHDA pre-treatment (Section I of Experiments) (Svensson et al., 1975), suggests that 5-HT cell firing has become independent of adrenergic tone. However, the partially retained ability of alpha-adrenoceptor antagonists which act postsynaptically to suppress firing activity favors the notion that some adrenergic component still contributes to maintaining 5-HT cell firing. Further study of the manner in which 5-HT cells adapt to the chronic loss of adrenergic tone is needed to explain these pharmacological responses.

III. Suppression of Serotonergic Neuronal Firing by Alpha-Adrenoceptor Antagonists: Evidence Against GABA Mediation

1. Introduction

The pharmacological studies presented above suggest that the noradrenergic (NE) innervation of the dorsal raphe (Fuxe, 1965) plays an essential role in maintaining the tonic activity of the serotonin (5-HT)-containing cells located there. The demonstration that a variety of alpha-adrenoceptor antagonists, when applied iontophoretically in the vicinity of 5-HT cells, completely suppress their spontaneous firing activity supports this hypothesis. Also, iontophoretic application of NE or the alpha-adrenoceptor agonist, phenylephrine, readily reverses the suppression of 5-HT cell firing produced by WB-4101, an alpha-adrenoceptor antagonist. These physiological findings imply that local changes in noradrenergic transmission within the dorsal raphe can strongly influence the tonic firing of 5-HT cells.

As the dorsal raphe contains non-serotonergic, as well as serotonergic neurons (Aghajanian et al., 1978) the tonic activation of 5-HT cells by the NE innervation could stem from either a direct action of NE on 5-HT cells themselves or from an indirect action involving interposed non-serotonergic neuronal elements. The systemic administration of picrotoxin, a GABA antagonist, has been reported to partially reverse the suppression of 5-HT cell firing produced by systemically applied alpha-adrenoceptor antagonists; this finding led to the proposal that GABA might mediate the suppression of 5-HT cell firing produced by alpha-adrenoceptor

blockade (Gallager and Aghajanian, 1976b). This model postulates that alpha-adrenoceptor antagonists act by blocking NE's tonic inhibition of GABA-ergic interneurons within the dorsal raphe, allowing them to fire; these disinhibited interneurons then directly inhibit the 5-HT cells. Recent biochemical and anatomical studies which have demonstrated the presence of GABA-ergic neurons with the dorsal raphe provide a basis for this scheme of NE's action (McGeer et al., 1979; Belin et al., 1979; Gamrani et al., 1980). Also, the population of non-serotonergic neurons in the dorsal raphe which displays a firing pattern reciprocal to that of 5-HT cells (Aghajanian et al., 1978) could fill the role assigned to the postulated interneuron.

The interneuron model predicts that blockade of GABA receptors present on 5-HT cells should reverse the suppression of 5-HT cells produced by alpha-adrenoceptor antagonists. Alternatively, if NE regulates firing by a mechanism which is independent of GABA-ergic transmission, then blockade of GABA receptors would not be expected to alter the suppression of 5-HT cells produced by alpha-adrenoceptor antagonists. The previous physiological studies would seem to favor the first alternative as the GABA antagonist, picrotoxin, at least partially reversed the suppression of 5-HT cell firing induced by alpha-adrenoceptor antagonists (Gallager and Aghajanian, 1976b). However, these results are not conclusive as picrotoxin was given systemically and could have acted at sites ouside the dorsal raphe to restore 5-HT cell firing. In order to distinguish more precisely between these alternatives, we have

examined the effect of iontophoretically applied GABA antagonists on the suppression of 5-HT cell firing produced by alpha-adrenoceptor antagonists.

2. Results

a. Suppression of 5-HT Cell Firing by Alpha-Adrenoceptor Antagonists: Specificity of NE Reversal

Consistent with previous reported results, microiontophoretic application of the alpha-adrenoceptor antagonists, phentolamine and WB-4101, rapidly and totally suppressed 5-HT cell firing (Section II of Experiments). Although these drugs share potent alpha-adrenoceptor antagonist activity, it was of interest to provide experimental evidence that they suppress 5-HT cell firing by blocking alpha-adrenoceptors and not by exerting a general depressant action. For example, phentolamine has been reported to produce local anesthetic effects (Curtis, 1968). However, at currents used in this study (Table 3), a decrease in spike height, indicative of a local anesthetic effect was not observed during application of either drug. On the other hand, if the action of these drugs stems from alpha-adrenoceptor blockade, then NE should be able to reverse the competitive blockade produced by these drugs. In agreement with previous results (Section II of Experiments), iontophoretic application of NE ($\leq$ 10 nA), during the suppression of 5-HT cell activity produced by phentolamine or WB-4101, rapidly restored firing to a level close to their initial rates (Table 3, Fig. 9, top panel).

The assumption that NE restored firing by its action at

alpha-adrenoceptors and not by a general excitatory action was also examined. NE was not able to reverse the effects of 5-HT or GABA (Table 3, Fig. 9), two substances also known to suppress the firing of 5-HT cells (Aghajanian et al., 1972b; Gallager and Aghajanian, 1976b). In addition, the effects of NE were found to differ from those of glutamate, an excitatory transmitter. While low doses of NE produced modest elevations in the baseline firing rate of 5-HT cells, NE's action on these cells became more pronounced during the suppression of firing produced by alpha-adrenoceptor blockade (Section II of Experiments). In contrast, glutamate's excitatory action was reduced during the inhibition produced by either GABA (n=3), 5-HT (n=2) or the alpha-adrenoceptor antagonists WB-4101 (n=6) and phentolamine (n=5) (Fig. 9). As shown in the top panel of Figure 1, NE restored the excitatory effects of glutamate as well as the baseline firing rate in the presence of phentolamine.

b. Effect of GABA and Glycine Antagonists on the Suppression of 5-HT Cell Firing Produced by Alpha-Adrenoceptor Antagonists

If the release of GABA mediates the suppression of firing produced by alpha-adrenoceptor antagonists, then iontophoretic application of GABA antagonists would be expected to restore firing. Therefore, the ability of the GABA antagonists, picrotoxin (Galindo, 1969) and N-methyl bicuculline (Pong and Graham, 1972; Johnston et al., 1972) to reverse the suppression of firing produced by WB-4101 and phentolamine was examined.

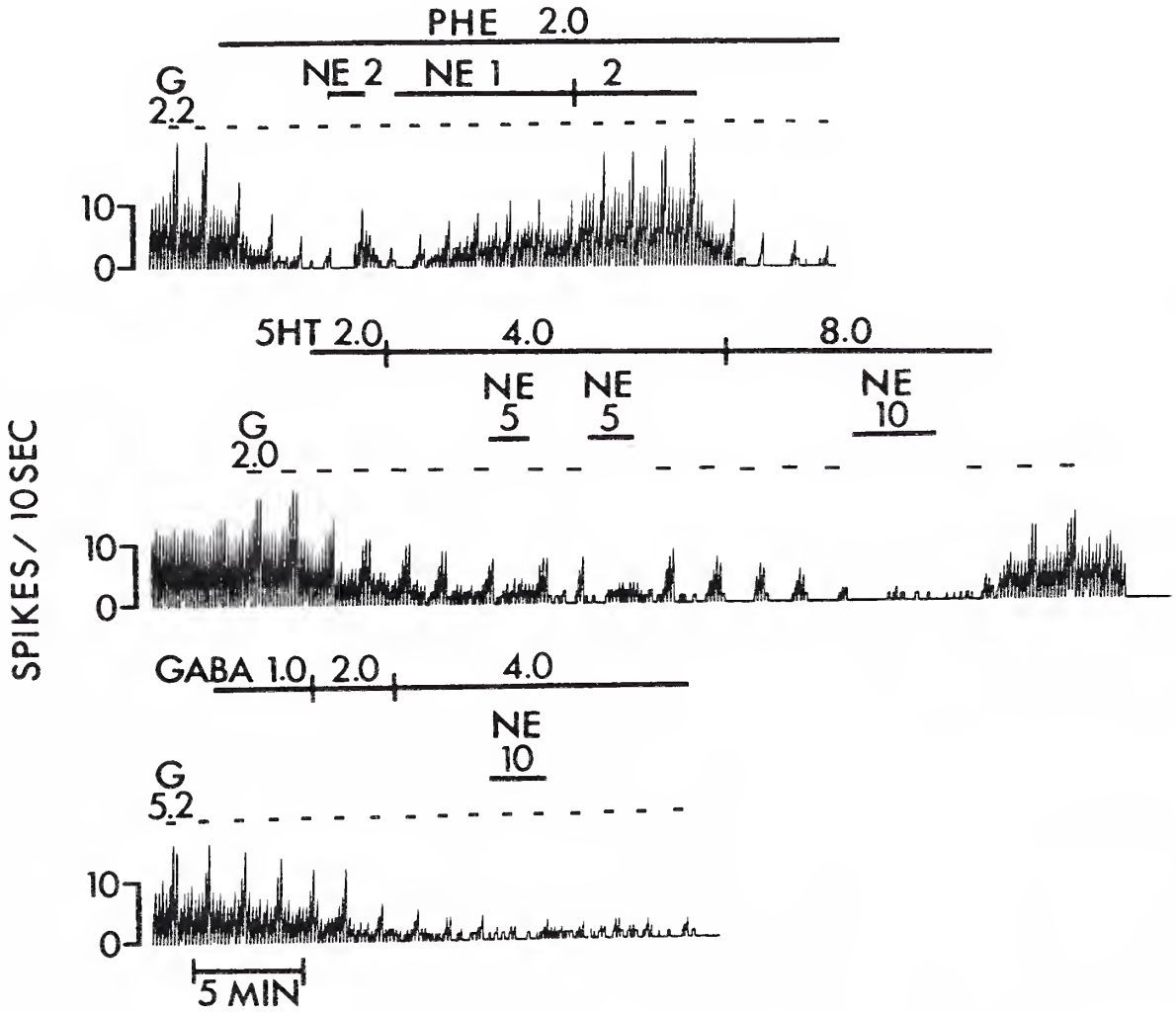
Table 3: Suppression of 5-HT Cell Firing by Alpha-Adrenoceptor Antagonists, 5-HT, and GABA: Selectivity of NE Reversal

Drug	Current (nA)	Duration (min)	NE Reversal
WB-4101	1-15	1-3	13/13
Phentolamine	0.2-5	1-5	12/12
5-HT	1-10	0.25-2	0/5
GABA	0.3-5	0.5-5	0/14

Table 3: The ranges of current and duration parameters used for several drugs which suppress 5-HT cell firing are noted. NE ($\leq$ 10 nA, 1-2 minutes) was applied during the suppressed activity produced by the drugs listed. NE reversal of 5-HT cell suppression was defined as the restoration of firing rates to greater than one-half of baseline rate.

Figure 9:

Suppression of 5-HT cell firing by phentolamine, 5-HT, or GABA: selectivity of NE reversal. Iontophoretic application of either phentolamine (PHE, 2.0 nA) 5-HT (2, 4, and 8 nA) or GABA (1, 2, and 4 nA) suppressed 5-HT cell firing. NE reversed the suppression produced by phentolamine but not by 5-HT or GABA (0.002 M in 0.1 M NaCl), demonstrating the selectivity of NE's reversal. In addition, the effect of suppression of firing on the response to glutamate is demonstrated. In each record, pulses of glutamate (G) which elicited a moderate excitation were applied at regular intervals. The absolute increase in rate produced by glutamate was reduced during suppression of firing. Note that glutamate's effect returned to its initial level when the firing rate was restored by NE (top trace) suggesting that the decrease in gluatamate's response was not due to a general depressant action of phentolamine.


PHE 2.0
NE 2
NE 1
2
G
2.2
10
0
5HT 2.0
4.0
8.0
NE
5
NE
5
NE
10
G
2.0
10
0
SPIKES/ 10SEC
GABA 1.0
2.0
4.0
NE
10
G
5.2
10
0
5 MIN

Picrotoxin (2.5-5 nA; 2 min) selectively antagonized GABA's inhibitory action (Fig. 10). It produced nearly total blockade of GABA's action without decreasing the inhibitory response to 5-HT (n=6) consistent with previously reported results (Gallager and Aghajanian, 1976b). However, even doses of picrotoxin which exceeded those necessary to block GABA (5-10 nA, 3-10 minutes) failed to reverse the suppression of firing produced by either WB-4101 (n=7) or phentolamine (n=5) (Fig. 11). Higher currents of picrotoxin have been reported to interfere with the inhibitory response to 5-HT and to exert some depressant effects on baseline firing (Gallager and Aghajanian, 1976b). Therefore, higher currents were not tested extensively. However, in two cells studied, even a high dose of picrotoxin (20 nA, 5 minutes) failed to reverse the suppression produced by WB-4101.

N-methyl bicuculline (1-5 nA, 1 minute) also produced nearly complete blockade of GABA's action on 5-HT cells without altering their inhibitory response to 5-HT (n=10) (fig. 10). It was observed that application of higher doses of N-methyl bicuculline (5 nA, 1-5 minutes) (n=19) produced 20-100% increases in firing rate in 11 cells and less than 20% change in eight. Following extended periods of ejection (> 5 minutes) N-methyl bicuculline (5 nA) tended to disrupt the normal, regular firing pattern of 5-HT cells. Since N-methyl bicuculline increased baseline firing rate, any reversal of the suppression produced by α-adrenoceptor blockade would have to be evaluated in light of its activating effect. In an attempt to detect a specific reversal of the effects of alpha-adrenoceptor

blockade by N-methyl bicuculline despite its more general excitatory action, the response of each cell to this drug was monitored before and during suppression of firing by either WB-4101 (n=9) or phentolamine (n=5). However, the absolute increase in firing rate produced by N-methyl bicuculline (5 nA, 2-4 minutes) during the suppression of firing was only equal to or less than that produced on baseline firing (Fig. 11, bottom panel). To further explore the possibility that N-methyl bicuculline's activating effect stems from an action independent of NE mechanisms, NE was applied in combination with N-methyl bicuculline during the suppression of firing. As shown in Figure 11 (bottom panel), during the suppression of firing produced by phentolamine, N-methyl bicuculline increased firing to a pleateau level, then NE produced a further activation which was nearly identical to its effect when applied alone. Application of NE and N-methyl bicuculline together produced an additive stimulatory effect equal to 94 $\pm$ 7% (mean $\pm$ S.E.M.) of the sum of the responses to the individually applied drugs (n=5).

Glycine potently inhibits 5-HT cell firing (Gallager and Aghajanian, 1976b). An alternative model for NE's regulation of 5-HT cells might substitute glycine for GABA. Therefore, the ability of the glycine antagonist, strychnine (Curtis et al., 1968) to reverse the suppression of 5-HT cell firing by WB-4101 was also examined. Strychnine has been demonstrated to potently and selectively antagonize glycine's inhibition of 5-HT cell firing (Gallager and Aghajanian, 1976b) (Fig. 10). Application of only

Figure 10:

Selective action of GABA and glycine antagonists. The selectivity of the GABA antagonists picrotoxin and N-methyl bicuculline are demonstrated in the top two traces. N-methyl bicuculline (BIC, 2.5 nA) totally blocked the inhibitory response to GABA (GA, 0.01 M in 0.1 M NaCl) without altering the response to 5-HT (top trace). Similarly, picrotoxin (PIC, 2.5 nA) did not reduce 5-HT's inhibition but totally blocked GABA's action (middle trace). Strychnine (STR, 2.5 nA) a glycine antagonist, selectively blocked glycine's (GLY) action without diminishing the response to GABA.

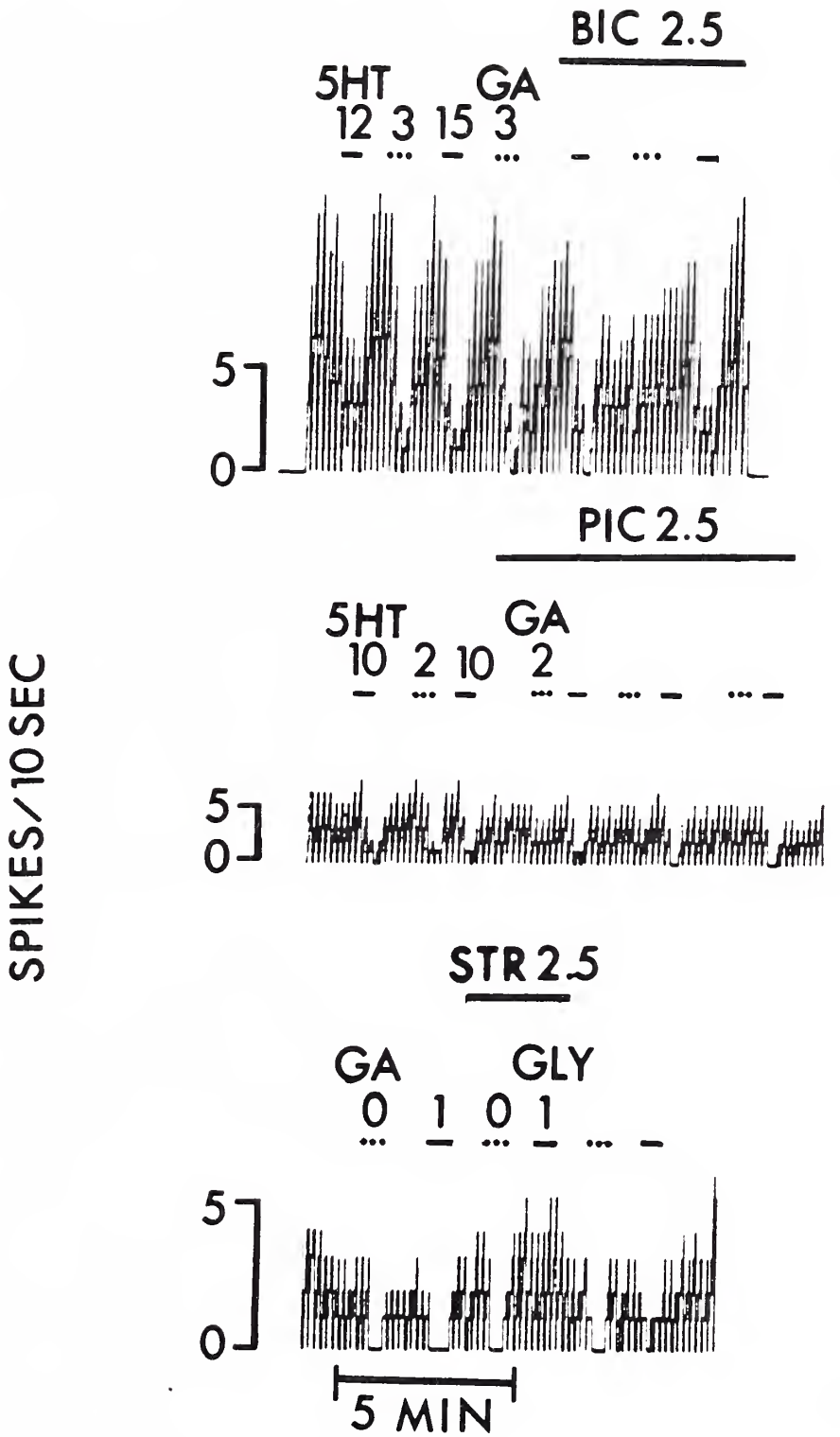
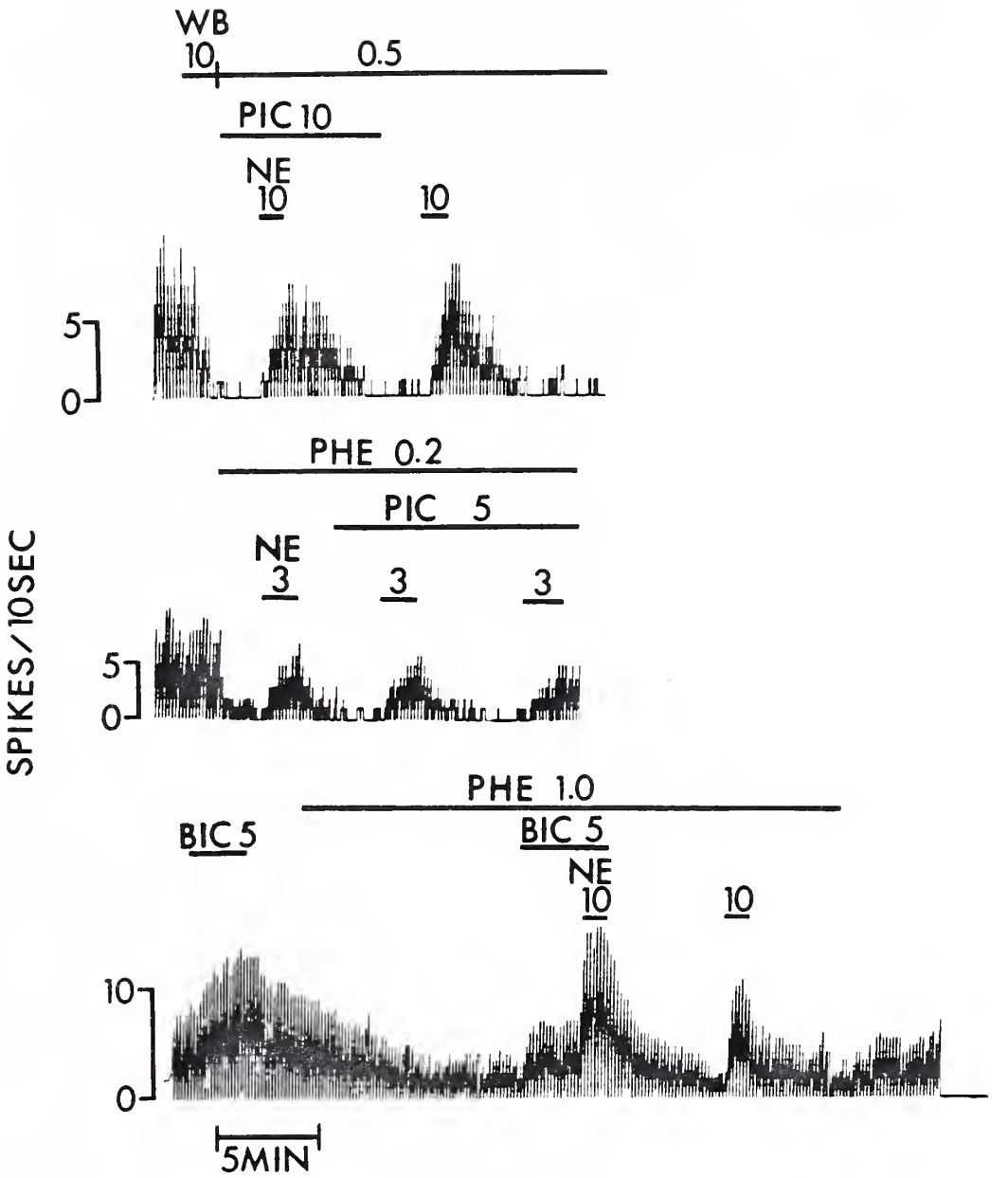

BIC 2.5
5HT
GA
12 3 15 3
5
0
PIC 2.5
5HT
GA
10 2 10 2
5
0
STR 2.5
GA
GLY
0 1 0 1
5
0
5 MIN
SPIKES/10 SEC

Figure 11:

Effect of GABA antagonists on suppression produced by alpha-adrenoceptor antagonists. WB-4101 (WB) and phentolamine (PHE) suppressed 5-HT cell firing. Picrotoxin (PIC) did not reverse the suppression while NE did, suggesting that GABA does not mediate the suppression (top and middle traces). In the bottom trace, N-methyl bicuculline (BIC) produced a similar excitation before and during the suppression of 5-HT cell firing caused by phentolamine. Furthermore, when NE and N-methyl bicuculline were applied together they produced an additive effect suggesting that NE and N-methyl bicuculline act by independent mechanisms.


WB
10
0.5
PIC 10
NE
10
10
5
0
PHE 0.2
PIC 5
NE
3
3
3
5
0
PHE 1.0
BIC 5
BIC 5
NE
10
10
10
0
5MIN
SPIKES/10SEC

1 nA of strychnine for 1 minute produced nearly complete blockade of glycine's inhibitory action (n=4). However, prolonged application of strychnine (5-10 nA; 3-5 minutes) did not reverse the suppression of 5-HT cell firing produced by WB-4101 (n=5).

3. Discussion

The physiological studies reported in this section provide evidence against the proposal that GABA mediates the suppression of 5-HT cells by alpha-adrenoceptor antagonists. According to this model, blockade of GABA receptors present on 5-HT cells should reverse this suppression of 5-HT cell firing. However, iontophoretic application of picrotoxin failed to interfere with the ability of alpha-adrenoceptor antagonists to suppress 5-HT cell activity. Similarly, N-methyl bicuculline produced no greater stimulation of firing during this suppression than before. Furthermore, the effects of N-methyl bicuculline and NE were additive suggesting that these agents act by independent mechanisms. These findings strongly suggest that GABA does not mediate the suppression of 5-HT cells by alpha-adrenoceptor antagonists. In addition, similar experiments with the glycine antagonist strychnine, suggest that glycine is not involved either.

The physiological evidence that GABA is not involved in NE's regulation of 5-HT cells is supported by the demonstration that doses of GABA antagonists tested completely block GABA's inhibitory action when applied iontophoretically. However, it could be argued that it may require higher concentrations of GABA antagonists to block synaptic GABA transmission than iontophoretically applied

GABA. For this reason, doses of GABA antagonists which greatly exceeded those necessary to effectively block iontophoretically applied GABA were used. Nevertheless, even excessive doses of picrotoxin did not produce any decrease in the effects of alpha-adrenoceptor blockade. Also, low currents of NE, applied during the suppression of firing produced by alpha-adrenoceptor antagonists were able to restore 5-HT cell firing. According to the model proposed, NE would be acting by inhibiting GABA-transmission. Therefore, it would be difficult to reconcile this model with the observation that NE, but not high doses of GABA antagonists, was able to reduce GABA transmission.

The observation that NE reversed the suppression of 5-HT cell firing produced by phentolamine and WB-4101 provides evidence that these drugs act by blocking alpha-adrenoceptors. However, NE's reversal could stem from a general excitatory action. The assumption that NE's reversal is specifically related to its action at alpha-adrenoceptors is supported by two observations. NE did not reverse the suppression produced by 5-HT or GABA. Second, NE's action differed from that of glutamate, an excitatory transmitter. During the suppression of firing produced by α-adrenoceptor antagonists, glutamate's prominent excitation decreased while NE's limited activation of baseline firing increased dramatically. These findings support the assertion that NE's reversal of alpha-adrenoceptor antagonists represents a specific interaction of NE with these drugs at alpha-adrenoceptors.

N-methyl bicuculline has been shown to inhibit brain acetyl-

cholinesterase (Svenneby and Roberts, 1973). The increase in firing observed in some 5-HT cells could stem from an enhancement of synaptically released acetylcholine. However, this explanation seems unlikely since 5-HT cells are not excited by iontophoretically applied acetylcholine (unpublished observations). An alternative explanation is based on previous studies which demonstrated that 5-HT cells receive GABA-ergic input (Wang et al., 1976; Wang and Aghajanian, 1977b). N-methyl bicuculline's activation of 5-HT cells could result from a reduction of background GABA-mediated inhibition. However, this action of N-methyl bicuculline is not shared by picrotoxin and therefore, it is uncertain whether this effect results from blockade of GABA receptors. Of note, the (-) optical isomer of N-methyl bicuculline, which does not share GABA antagonist properties with the commercially available (+) isomer used in this study, does possess similar excitatory properties (Collins and Hill, 1974). Therefore, N-methyl bicuculline's activation of 5-HT cells could stem from an action unrelated to GABA blockade.

As mentioned above, picrotoxin, when administered systemically, partially reverses the suppression of 5-HT cell firing produced by alpha-adrenoceptor antagonists. This observation led to the suggestion that GABA neurons mediated NE's action. However, the results of the present study favor a more direct action of NE on 5-HT cells. Certainly, picrotoxin administered systemically could affect 5-HT cells indirectly through an action on other neurons synaptically linked with the 5-HT cells. Although it is

not entirely clear which NE cell groups project to the dorsal raphe, it is noteworthy that systemically administered picrotoxin activates NE neurons in the locus coeruleus (unpublished observations). Thus, if picrotoxin activates NE systems which project to the dorsal raphe, the increased NE tone could overcome a competitive adrenergic blockade and restore 5-HT cell firing.

IV. Noradrenergic Innervation of Serotonergic Neurons in the Dorsal Raphe: Demonstration by Electron-Microscopic Autoradiography

1. Introduction

The dorsal raphe nucleus receives one of the heaviest noradrenergic (NE) innervations in the brain. This input has been demonstrated by histofluoresence (Fuxe, 1965), biochemical (Levitt and Moore, 1979; Saavedra et al., 1976; Versteeg et al., 1976), and immunocytochemical techniques (Grzanna and Molliver, 1980; Swanson and Hartman, 1975). The physiological studies presented above have suggested that the NE innervation plays an important role in maintaining the tonic firing activity of serotonin (5-HT) containing cells. For example, interruption of NE transmission by local, iontophoretic application of alpha-adrenoceptor antagonists totally suppresses 5-HT cell firing, and the iontophoresis of NE readily reverses this suppression (Section II of Experiments). Since the dorsal raphe contains both non-serotonergic, as well as serotonergic neurons (Aghajanian et al., 1978), the NE input may regulate 5-HT cells by a direct innervation of 5-HT cells themselves, or indirectly through an interposed non-serotonergic neuronal element. For example, the population of non-serotonergic neurons within the dorsal raphe which displays a firing pattern reciprocal to that of 5-HT cells (Aghajanian et al., 1978) might receive the NE innervation and mediate NE's activating effect on 5-HT cells.

In order to determine whether NE terminals innervate 5-HT cells, it was essential to identify both NE terminals and 5-HT neuronal elements simultaneously at the ultrastructural level.

NE terminals were labelled using previously developed techniques of electron microscopic autoradiography (Aghajanian and Bloom, 1966, 1967b; Descarries and Droz, 1968, 1970a, 1970b). The selectivity of this autoradiographic method was evaluated in two ways in this study: 1) by examining the effect of pretreatment with the selective catecholamine neurotoxin, 6-OHDA, on H^3-NE labelling and, 2) by comparing the H^3-5-HT autoradiographic pattern to that obtained with ^{3}H-NE. Postsynaptic neuronal elements were identified at an early stage of degeneration following treatment with a selective 5-HT neurotoxin, 5,7-dihydroxytryptamine (Baumgarten and Lachenmayer, 1972). Therefore, with this combination of techniques, both pre-synaptic NE terminals and post-synaptic 5-HT elements could be identified in the same tissue.

2. Results

a. H^3-NE Autoradiography of the Dorsal Raphe

Examination of sections of the dorsal raphe processed for light-microscopic H^3-NE autoradiography revealed small groupings of grains throughout the entire nucleus. Only a few labelled cell bodies were present consistent with the observation of Nowaczyk et al. (1978) (Figure 12, left panel). Examination of sections which were processed for EM autoradiography showed highly selective localization of grain clusters over unmyelinated axons, prejunctional varicosities and nerve terminals (Figure 13). These terminals contained both small agranular and larger dense core vesicles characteristic of adrenergic nerve terminals and formed symmetrical synaptic junctions. Post-synaptic elements were commonly small or

medium-sized dendritic profiles. Labelled-cell bodies were encountered only infrequently, as expected from their sparse presence in light autoradiograms.

b. H^3-5-HT Autoradiography of the Dorsal Raphe

As a means of evaluating the selectivity of NE-labelling, H^3-5-HT autoradiography was also performed. Sections of the dorsal raphe processed for light autoradiography showed accumulation of radioactivity by cell bodies throughout the entire nucleus. In addition, the supraependymal layer, which is known to contain 5-HT-accumulating terminals (Lorez and Richards, 1973; Richards et al., 1973; Richards and Tranzer, 1974) was heavily labelled (Figure 12, right panel). At the ultrastructural level, prominent labelling of cell bodies, dendrites, and unmyelinated axons was apparent. However, in contrast to the autoradiographic pattern of labelling obtained with H^3-NE, 5-HT-labelling nerve terminals forming specialized synaptic junctions were not observed, consistent with the report of Descarries et al. (1979). Of note, many small neuronal cell bodies did not show accumulation of radioactivity, in agreement with previous observations that non-serotonergic neurons are present within the dorsal raphe (Aghajanian et al., 1978; Descarries et al., 1979; Gamrani et al., 1980).

c. H^3-NE Autoradiography: Effect of 6-OHDA Pretreatment

In order to further evaluate the specificity of the labelling obtained by NE autoradiography, the effects of pretreatment with the selective catecholamine neurotoxin, 6-OHDA (100 µg, free base, 72 hr survival), on NE-labelling was examined. This

Figure 12

Light autoradiography of H^3-NE and H^3-5-HT. A H^3-NE light autoradiograph of the dorsal raphe (left panel) contains small clusters of grains throughout the section, while only one cell body is labelled (arrow). In contrast, H^3-5-HT light autoradiograph (left panel) displays prominent labelling of cell bodies. In addition, the supraependymal layer (arrow, right panel) is labelled by H^3-5-HT but not H^3-NE. Scale: 100 microns. The letter, A, denotes the aquaduct of Sylvius.

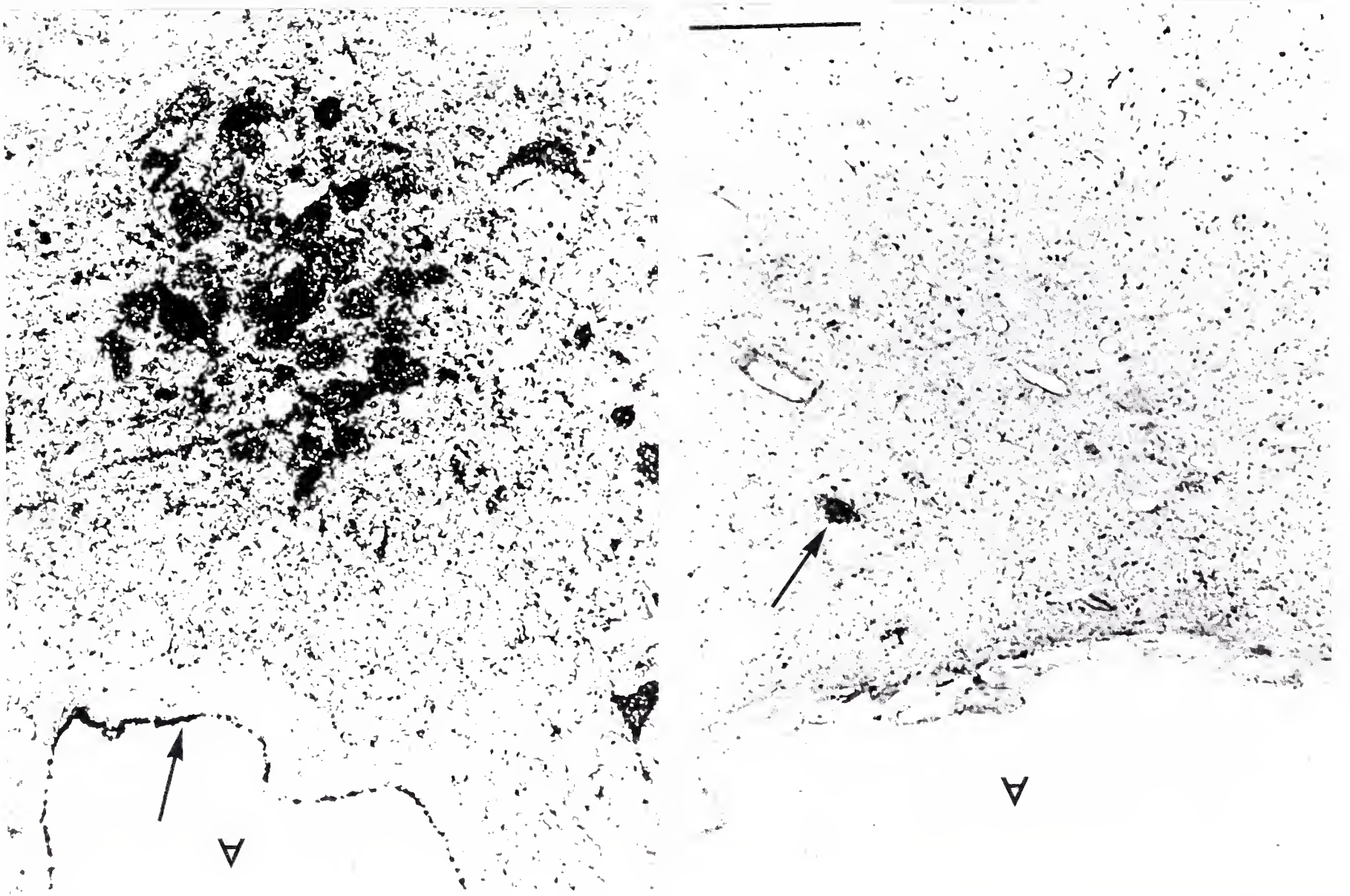
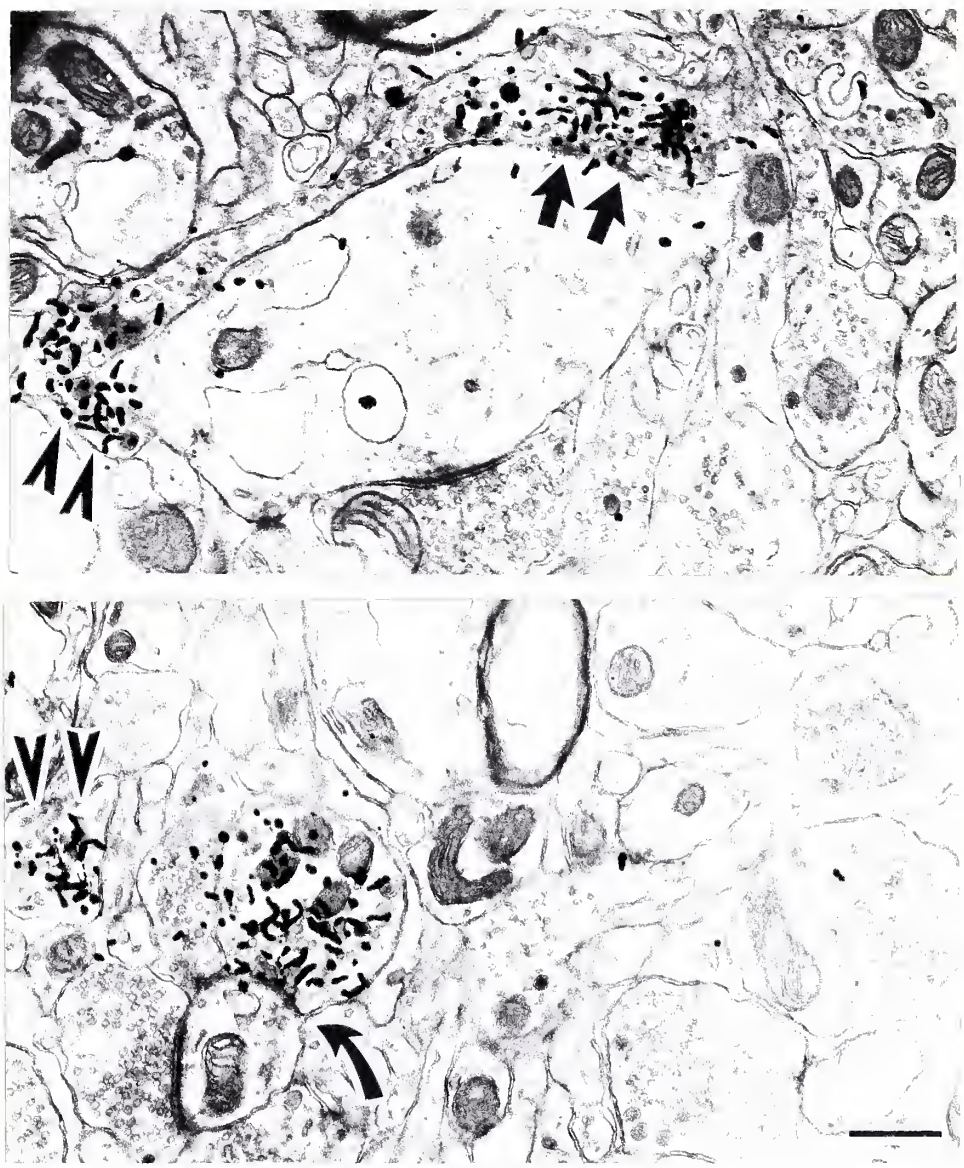


Figure 13:

H^3-NE EM autoradiography of dorsal raphe. Two examples of NE-labelled terminals are shown. In the top panel, a dendrite is innervated by a heavily labelled terminal (two arrows). Of note, a varicosity of the same axonal process is also labelled at left (two arrowheads). In the lower panel, a small dendrite is innervated by a labelled terminal (arrow) and another unlabelled terminal. In addition, a labelled unmyelinated process is also included at left (two arrowheads). Scale: 0.5 microns.



treatment markedly decreased the density of NE varicosities observed by histofluoresence in the dorsal raphe (Figure 14). When sections of the dorsal raphe were examined by electron microscopy, damaged nerve terminals were observed as previously described (Bloom, 1971; Bloom et al., 1972) and the density of NE-labelled terminals was reduced by two-thirds (Table 4).

d. H^3-NE Autoradiography: Effect of 5,7-DHT Pretreatment

In order to determine whether NE-labelled terminals innervate 5-HT cells, the effect of 5,7-DHT pretreatment (200 µg, free base, 48 hr survival) on the postsynaptic elements was examined. As previously described, this treatment produced marked, selective degeneration of 5-HT cell bodies and dendrites in the dorsal raphe, while small, presumably non-serotonergic, neurons as well as a large portion of the neuropil were not affected (Aghajanian et al., 1978). Two-thirds of the NE-labelled terminals containing synaptic specializations, innervated dendrites displaying darkened or severely disrupted cytoplasmic features, characteristic of degenerating neurons (Figure 15, bottom panel). One sixth synapsed on undamaged dendrites, and for the remaining sixth the postsynaptic profile was too small to classify reliably. Of note, the density of NE-labelled terminals observed in the dorsal raphe after 5,7-DHT pretreatment did not differ significantly from that found in unlesioned tissue (Table 4).

3. Discussion

These anatomical studies provide evidence that the NE input to the dorsal raphe directly innervates 5-HT neurons located there.

Figure 14:

NE innervation of dorsal raphe: effect of 6-OHDA treatment. Prominent NE innervation of the dorsal raphe is demonstrated in a fluorescence micrograph (left panel). 6-OHDA pretreatment (100 µg, free base; 7 days survival) markedly depleted NE innervation as shown in the right panel. Many of the remaining varicosities are swollen (arrow), characteristic of damaged monoamine processes. Scale: 100 microns. The letter, A, denotes the aquaduct of Sylvius.

A
A

Figure 15:

H^3-NE EM autoradiography: effects of 5,7-DHT treatment. An example of a NE-labelled terminal innervating an undamaged dendrite taken from unlesioned tissue, is shown in the top panel (arrow). In addition an unmyelinated axonal profile is present in the field (two arrowheads). After 5,7-DHT pretreatment (200 µg, free base; 48 hour survival; bottom panel), a damaged dendrite is shown innervated by a NE-labelled terminal (arrow). Scale: 0.5 microns.

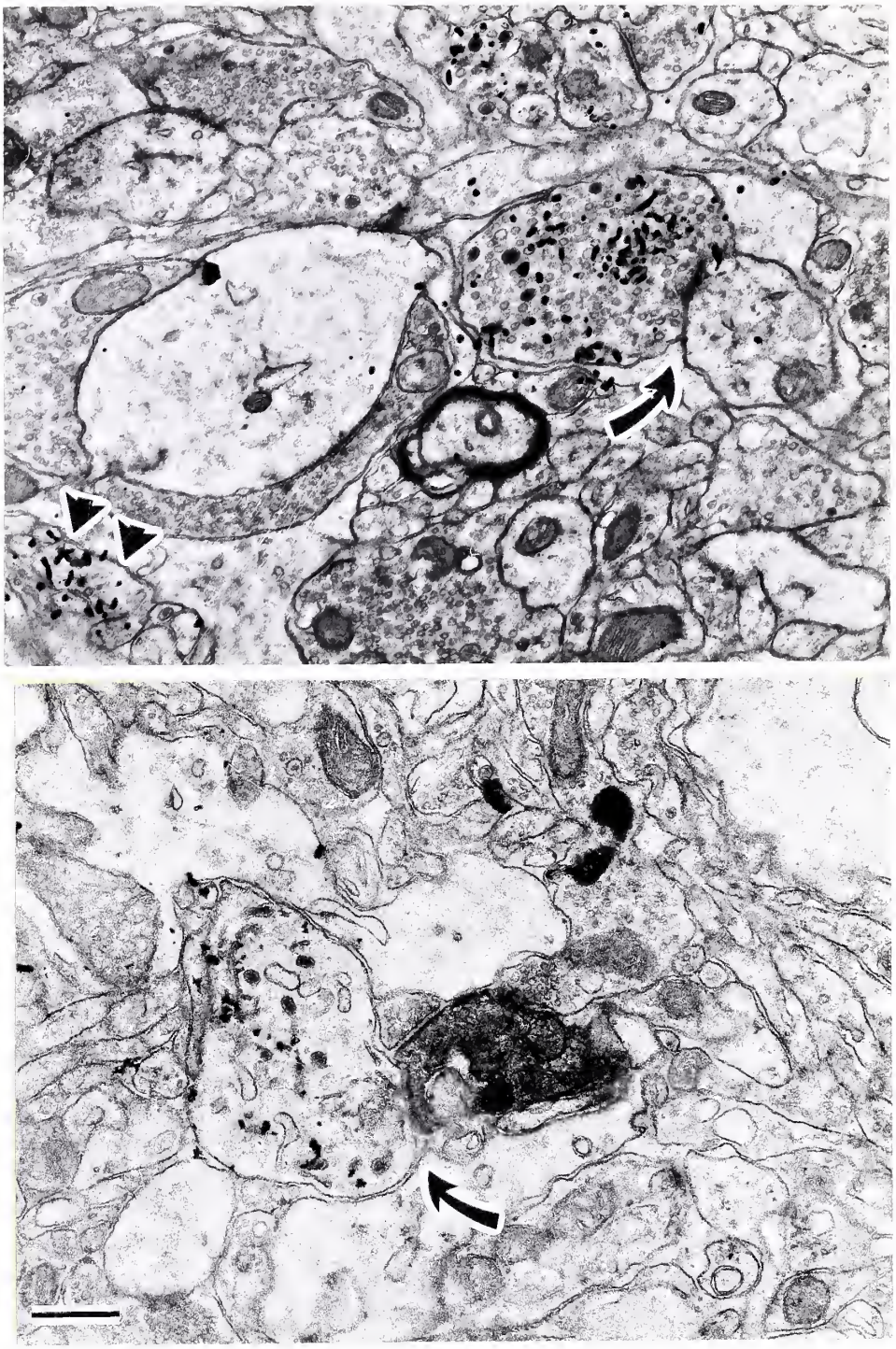


Table 4: Effects of 5,7-DHT and 6-OHDA on Density of NE-labelled Terminals in the Dorsal Raphe

	Mean Number of NE-labelled terminals per grid square ± SEM[1]	% of Terminals forming a synaptic junction
Control	1.89 ± 0.17	21
5,7-DHT (200 µg) 48 hr. survival	1.78 ± 0.14	19+
6-OHDA (100 µg) 72 hr. survival	0.58 ± 0.08*	-

* Differs significnatly from control value, $p < 0.001$.

\+ Two-thirds of the postsynaptic elements showed signs of degenerative changes

[1] Area = 2025 sq. microns. Eighty grid squares were counted in each of the groups.

NE terminals were labelled by H^3-NE EM autoradiography. Furthermore, 5-HT cells were identified following treatment with the selective 5-HT neurotoxin, 5,7-DHT. Examination of tissue in which both labelling techniques were combined, revealed that two-thirds of NE-labelled terminals, which formed synaptic junctions, innervated degenerating dendrites. This finding implies that the NE terminals directly innervate 5-HT cells of the dorsal raphe.

Several lines of evidence suggest that the EM autoradiographic method achieved selective labelling of NE terminals. 1) Lesioning of 5-HT neurons by treatment with 5,7-DHT did not reduce the density of NE-labelled terminals. 2) Treatment of animals with 6-OHDA produced a marked reduction in the density of NE-labelled terminals. The degree of NE terminal depletion following 6-OHDA treatment correlates well with the estimate provided by biochemical measures of H^3-NE retention (Breese and Taylor, 1970; Uretsky et al., 1971). 3) Furthermore, it is unlikely that NE-labelled terminals with specialized junctions are serotonergic since in parallel autoradiographic studies with 5-HT, no labelled terminals with specialized junctions were observed, even though prominent labelling of cell bodies, axons and dendrites are present.

The serotonin neurotoxin 5,7-DHT was employed to damage 5-HT neurons. Biochemical studies have provided evidence for the selectivity of this drug when used in combination with DMI (Bjorklund et al., 1975; Gerson and Baldessarini, 1975). The presence of undamaged neurons in the dorsal raphe, which are presumably non-serotonergic, provides anatomical evidence for a selective action.

As well, the density of NE-labelled terminals in the dorsal raphe was not altered by 5,7-DHT pretreatment providing further evidence for a selective action of 5,7-DHT.

Autoradiographic studies of ^{3}H-5-HT localization in the dorsal raphe allowed two observations. First, these studies provide confirmatory evidence for the presence of non-serotonergic neurons within the dorsal raphe suggested by previous studies (Aghajanian et al., 1978; Descarries et al., 1979; Gamrani et al., 1980; McGeer et al., 1979). Second, 5-HT labelled varicosities were not observed to participate in synaptic junctions. As seen with the peripheral adrenergic terminals (Grillo, 1966), transmitter release may occur in the absence of synaptic junctions. Therefore, the postulated role of 5-HT axon collaterals in the 'autoinhibition' of 5-HT cells (Wang and Aghajanian, 1977b,1978) is not ruled out by this observation.

SUMMARY AND CONCLUSIONS

Previous biochemical and physiological studies had indicated that the 5-HT cells of the dorsal raphe are regulated by the brain's NE system. The studies presented above provide evidence that the NE innervation of the dorsal raphe exerts a potent activating influence on 5-HT cell firing activity. In the experiments conducted in the first section, reserpine's suppression of 5-HT cell firing was found to stem from a decrease in adrenergic transmission. Therefore, this drug served as a useful tool in examining the effect of restoring NE tone in the vicinity of 5-HT cells. Small amounts of NE, applied iontophoretically, restored 5-HT cell firing during its suppression by reserpine, demonstrating a consistent and dramatic physiological response of 5-HT cells to NE. Blockade of this response by adrenoceptor antagonists provides evidence that NE's action is receptor-mediated. In the second section, it was found that a wide variety of alpha-adrenoceptor antagonists when applied iontophoretically totally suppressed spontaneous 5-HT cell firing. The demonstration that alpha-adrenergic agonists could reverse this suppression confirmed the suggestion that the effect of these drugs results from their blockade of adrenoceptors. This finding indicates that not only are 5-HT cells responsive to exogenously applied NE but also that NE released from terminals in the dorsal raphe is essential for maintaining the spontaneous firing activity of 5-HT neurons.

Extensive pharmacological characterization of the response of 5-HT cells to adrenergic agonists and antagonists allowed the

classification of the adrenoceptors mediating NE's action in the alpha-1 category. Since, 5-HT cells are responsive to both systemically and iontophoretically applied alpha-1 adrenergic drugs, monitoring the firing activity of 5-HT cells provides a potentially useful method for evaluating the activity of drugs at central alpha-1 adrenoceptors.

There are several possible neuronal circuits through which alpha-adrenoceptor antagonists might act to suppress 5-HT cell firing. A tonically active excitatory adrenergic input could terminate directly on 5-HT cells. The ability of NE to produce excitation at alpha-adrenoceptors has been reported in the cortex by Bevan et al. (1977). In this case, the antagonists would act by interrupting a tonically active, excitatory adrenergic pathway. Alternatively, alpha-adrenoceptor antagonists could suppress 5-HT cell firing by blocking the tonic inhibition by NE of local GABA interneurons. A similar action of NE on interneurons has been proposed to mediate the indirect effects of NE on principal neurons in both the ventral horn of the spinal cord (Jordan et al., 1977) and the lateral geniculate nucleus (Nakai and Takaori, 1974). In the dorsal raphe, the presence of very high levels of glutamate decarboxylase (Tappaz et al., 1976), within intrinsic GABA neurons (Belin et al., 1979; McGeer et al., 1979) is consistent with this model. Indeed, non-serotonergic interneurons which have a firing pattern reciprocal to that of neighboring 5-HT cells have been found which could receive the adrenergic input (Aghajanian et al., 1978). Therefore, further physiological and anatomical investigations

were aimed at defining the synaptic microcircuitry of the dorsal raphe in order to elucidate the mechanisms by which alpha-adrenoceptor antagonists suppress the firing of 5-HT neurons.

Physiological experiments presented in Section III indicated that the NE system controls 5-HT cell firing independently of GABA-ergic transmission. Application of GABA receptor antagonists did not interfere with the suppression of 5-HT cell firing produced by alpha-adrenoceptor antagonists. This finding favors the proposal that a direct innervation of 5-HT cells by NE terminals regulates 5-HT cell firing. Several observations support this alternative. A similar direct mechanism for NE's action at alpha-adrenoceptors has been proposed in the facial motor nucleus which is lacking intrinsic interneurons (McCall and Aghajanian, 1979). Also, a direct NE innervation of 5-HT cells would be consistent with the ability of low amounts of NE to invariably activate 5-HT cells when applied from drug barrels situated near the 5-HT cell soma (Section II of Experiments). If NE's principal site of action were on interneurons then, assuming a variable spatial relationship between interneurons and the drug barrel, NE might have been expected to produce a more variable response. Finally, anatomical experiments using electron-microscopic autoradiography have demonstrated that NE terminals in the dorsal raphe innervate 5-HT cells directly.

These findings provide evidence that GABA-ergic interneurons present in the dorsal raphe do not mediate NE's regulation of 5-HT cells. Instead, the activity of 5-HT cells is apparently controlled by a balance of opposing GABA-ergic (Wang et al., 1976; Wang and

Aghajanian, 1977b)and noradrenergic influences. The concept of independent and opposite NE and GABA influences on 5-HT cell firing suggests two possible mechanisms which could underlie the suppression of 5-HT cells during REM (paradoxical) sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979): either an increase in GABA-ergic tone or a decrease in noradrenergic tone could silence 5-HT cell firing. Even though the origins of the NE innervation to the dorsal raphe are not completely defined, it is of interest that NE cells in the locus coeruleus are also quiescent during REM sleep (Jones et al., 1979). If the afferent NE systems display a similar suppression during REM sleep, then this could account for the reduction in 5-HT cell firing. Further investigation will be necessary to determine whether the suppression of 5-HT cell activity during REM sleep stems from either of these suggested mechanisms.

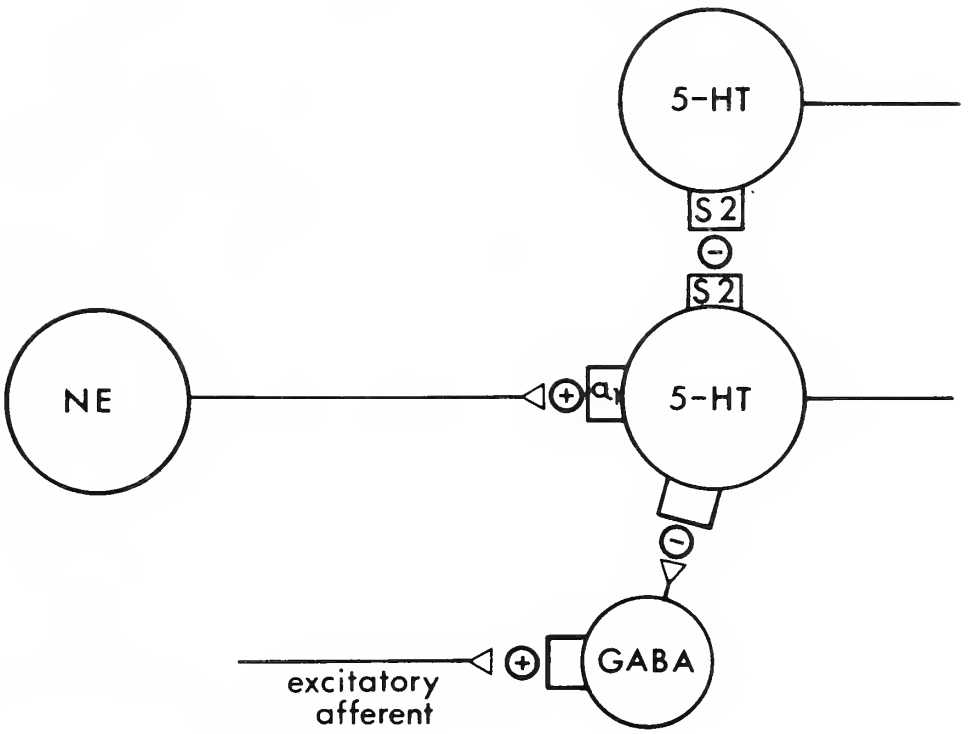
The mechanism by which a direct NE innervation of 5-HT cells could regulate their firing deserves consideration. 5-HT cells displays a characteristic rhythmic firing which could be driven by an endogenous pacemaker mechanism (Mosko and Jacobs, 1976). Alpha-adrenoceptors have been shown to mediate NE's facilitation of the excitability of principal neurons in the facial motor nucleus (McCall and Aghajanian, 1979), in the lateral geniculate nucleus (Rogawski and Aghajanian, in preparation), and in the cerebral cortex (Waterhouse et al., 1979). Therefore, NE's regulation of 5-HT cell firing could result from its facilitation of their endogenous pacemaker activity. Further investigation will be necessary in order to determine the ionic mechanisms underlying a direct noradrenergic regulation of

5-HT cell activity.

In summary, these studies form the basis for the conclusion that NE, released from terminals in the dorsal raphe activates 5-HT cells by stimulation of alpha-1 adrenoceptors, as diagrammed in Figure 16. Hopefully, this insight into the mechanism underlying the regulation of 5-HT cells by the NE system will eventually contribute to a better understanding of the role of these monoaminergic systems in the physiology and pathophysiology of the central nervous system.

Figure 16:

Circuit diagram of dorsal raphe: the 5-HT cells of the dorsal raphe are represented by two circles labelled 5-HT. These are subject to 'autoinhibition' by 5-HT acting at serotonin receptors which have been classified in the S2 category (Aghajanian, in press). In addition, a local GABA interneuron has been included which exerts an inhibitory action, denoted by, (-), on 5-HT cells. The NE innervation directly activates (+), 5-HT neurons by stimulating alpha-1-adrenoceptors (α_1).



DORSAL RAPHE
NUCLEUS

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