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Midbrain Benzodiazepine-GABA-Serotonin Interactions: Effects on Locomotor Activity in the Rat

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MIDBRAIN BENZODIAZEPINE-GABA-SEROTONIN INTERACTIONS: EFFECTS ON LOCOMOTOR ACTIVITY IN THE RAT

by

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DEDICATION

To Mother and Father, for their constant love and support,

and,

to Deanna, for making my life complete.

VITA

The author, Stephen Mitchell Sainati, is the son of Bruno Sainati and Margaret (Cook) Sainati. He was born November 28, 1952, in Chicago, Illinois.

His elementary education was obtained at Devonshire School in Skokie, Illinois, and Ogden Avenue School in La Grange, Illinois. His secondary education was obtained at Lyons Township High School in La Grange, Illinois, from which he was graduated in 1970.

In August, 1970, he entered Vanderbilt University in Nashville, Tennessee, and in May, 1974, was graduated *cum laude* with the degree of Bachelor of Arts with a major in biology.

In April, 1978, he was admitted to and granted an assistantship in the Department of Pharmacology at Loyola University of Chicago. In 1980 he became a student member of the Society for Neuroscience, and, in June, 1981, he was awarded a dissertation fellowship from the Arthur J. Schmitt Foundation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Acute microinjections of the GABA-agonist, muscimol, into the dorsal or median raphe nucleus of ether-anesthetized rats induced hyperkinesis as measured in photocell activity chambers. The median raphe nucleus is more sensitive to the effect of muscimol than the dorsal raphe nucleus. A dose-response analysis performed by injecting muscimol through cannulae chronically implanted in the dorsal or the median raphe nucleus of rats confirmed the greater sensitivity of the median raphe site. Intraperitoneal administration of the benzodiazepine, chlordiazepoxide, in a subataxic dose did not affect activity level, but enhanced the locomotor responses to low doses of muscimol injected into the median raphe nucleus. Conversely, intraperitoneal administration of a sub-convulsant dose of the GABA-antagonist, bicuculline, completely blocked the response to muscimol.

Subsequently, dose-response analyses for the water soluble benzodiazepines, chlordiazepoxide, flurazepam, and midazolam were performed by injecting the drugs through cannulae chronically implanted in the median raphe nucleus of rats. Both midazolam and flurazepam produced hyperactivity which was most prominent within the first 30 minutes post-injection. Flurazepam, moreover, proved twice as potent as midazolam. In contrast, chlordiazepoxide was without effect at any of the doses tested.

Next, animals received either saline or a sub-effective dose of flurazepam or midazolam into the median raphe nucleus 5 minutes prior to either a sub-effective dose of muscimol or saline. Only the combinations of a benzodiazepine plus muscimol produced hyperactivity. These combinations, moreover, produced effects as robust as those of a four-fold higher dose of muscimol alone.

In another experiment, animals received either saline or bicuculline methiodide injections into the median raphe nucleus 5 minutes before they received a hyperactivity-inducing dose of muscimol. Bicuculline completely blocked the hyperactivity effects of muscimol. Overall, these data suggest that the hyperkinetic effects of intra-raphe muscimol injections are due to activation of GABA receptors within the midbrain raphe, rather than at distant sites.

In a subsequent experiment, bilateral electrolytic destruction of the ventral tegmental nuclei of Gudden, which lie just dorsolateral to the median raphe nucleus, produced an increase in baseline activity level, but failed to alter the median raphe muscimol dose-response relationship.

Finally, forebrain serotonin was depleted by administering 5,7-dihydroxytryptamine intracerebrally. These lesions markedly attenuated the hyperactivity response produced by muscimol injections into the median raphe nucleus. These data suggest that midbrain GABA neurons modulate activity level through a direct action on serotonergic neurons.

CHAPTER I

INTRODUCTION

The benzodiazepines were introduced into clinical medicine in the early 1960's. They quickly became the most commonly prescribed class of pharmaceuticals. Today, some 20 years after their introduction, the benzodiazepines are used widely as sedative-hypnotic, anti-convulsant, anti-anxiety, muscle-relaxant and pre-anesthetic drugs. Since 1976, more than 100 million prescriptions for the benzodiazepines have been written annually in the United States alone (Harvey, 1980).

There is a consensus that the benzodiazepines exert their behavioral and physiological effects by facilitating the post-synaptic action of the inhibitory amino acid neurotransmitter, gamma-aminobutyric acid (GABA; Haefely et al., 1975; Costa and Guidotti, 1979; Krogsgaard-Larsen and Arnt, 1980; Olsen, 1982). For the most part, GABA releasing cells constitute local circuit neurons, or interneurons, within a given brain structure. These GABA-ergic cells, which are distributed throughout the neuraxis, modify the operation of other neurons which often utilize a neurotransmitter other than GABA. GABA interneurons thus modulate the inputs and outputs of a given brain region and, thereby, its function. As facilitators of GABA neurotransmission, it is not surprising that the benzodiazepines can produce profound behavioral effects.

It is the objective of the present work to elucidate the role of midbrain GABA and benzodiazepine receptors in the modulation of locomotor activity, and to determine whether this regulatory function is mediated by a serotonergic system. In order to provide a background for this research effort, the following topics will be reviewed: 1) the pharmacology of the benzodiazepines; 2) the behavioral effects of the benzodiazepines; 3) GABA and serotonin as neurotransmitters; 4) GABA-benzodiazepine interactions; 5) serotonin-benzodiazepine interactions; and, 6) the modulation of midbrain raphe neurons by GABA and benzodiazepine receptors.

Benzodiazepines

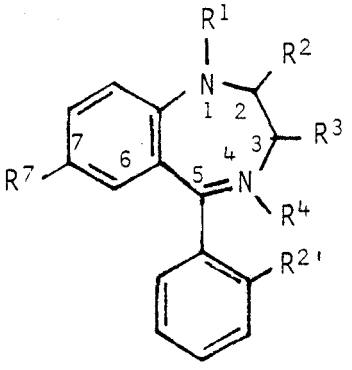
Chemistry

The nucleus of the benzodiazepine molecule is depicted in Figure 1 At the 7-position, electronegative groups enhance, and electropositive groups reduce potency. Substituents elsewhere in this ring decrease activity. A wide variety of moieties may occupy positions 1 to 3. A low electron density at the 4-nitrogen, provided by a double bond between positions 4 and 5, or by an electron-withdrawing group at position 4, is common to all clinically useful benzodiazepines. An electronegative 2'-substituent (ortho) on the 5-phenyl moiety will enhance potency (Harvey, 1980).

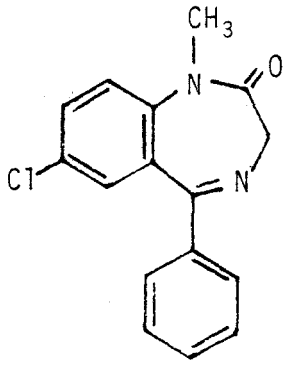
Substitutions have led to four basic classes of the benzodiazepines (Figure 1): the 1) 2-keto- ; 2) triazolo- ; 3) 3-hydroxy- ; and, 4) the 7-nitro- benzodiazepines. These different classes of benzodiaze-

pines all possess anti-convulsant, anti-anxiety, sedative-hypnotic, and muscle-relaxant properties. It is the differences in their pharmacokinetics which largely determine their clinical application (Johnson and Rising, 1979; Breimer et al., 1980). A major problem associated with the 2-keto- benzodiazepines, which include the often prescribed sedative-hypnotic, flurazepam, and the multipurpose drug, diazepam, is the production of long-acting (half-life 50 - 100 hours) active metabolites (Benet and Scheiner, 1980; Breimer et al., 1980). Certain benzodiazepines, such as prazepam, chlorazepate, and possibly chlordiazepoxide, appear to serve as pro-drugs for the formation in vivo of active 2-keto- metabolites (Curry and Whelpton, 1979). These compounds, thus, find clinical utility in the treatment of anxiety-neuroses where long-term steady-state plasma levels of drug (or active metabolite) are desirable (Baldessarini, 1980). A 7-nitro- group (as contained, for example, in clonazepam; Figure 1) increases anti-convulsant activity (Rall and Schleifer, 1980). Furthermore, the 7-nitro-benzodiazepines are reduced to inactive 7-amino- derivatives, the parent compounds having half-lives ranging between 15 and 37 hours. The 3-hydroxy-benzodiazepines have short half-lives (8.5 - 15 hours), plus the advantage of being conjugated at the 3-position to form inactive glucuronide derivatives which are excreted directly (Benet and Scheiner, 1980; Breimer et al., 1980). Finally, the triazolo-benzodiazepines have very short half-lives (2.5 - 11 hours). They are oxidized to form compounds which do not cross the blood-brain barrier and are rapidly

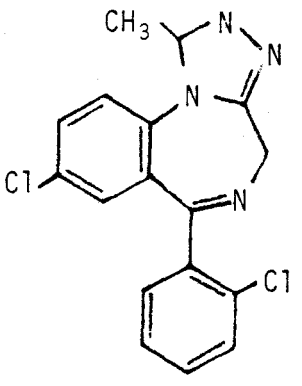
FIGURE 1: Structures of benzodiazepine nucleus and subclasses.



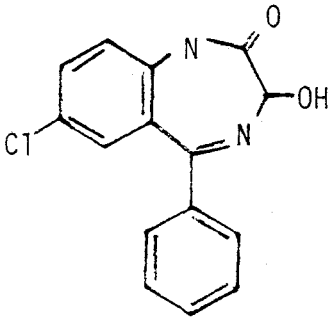
BENZODIAZEPINE NUCLEUS



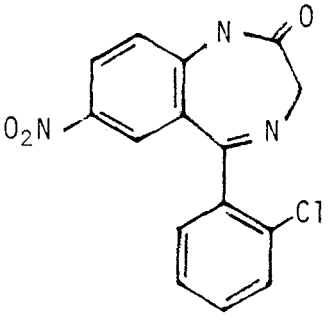
DIAZEPAM
(2-keto-)



TRIAZOLAM
(triazolo-)



OXAZEPAM
(3-hydroxy-)



CLONAZEPAM
(7-nitro-)

excreted following oxidation (Johnson and Rising, 1979; Pieri et al., 1981). For more complete reviews of benzodiazepine structure-activity relationships, the reader is referred to Sternbach (1973), Greenblatt and Shader (1974), Johnson and Rising (1979), and Breimer et al. (1980).

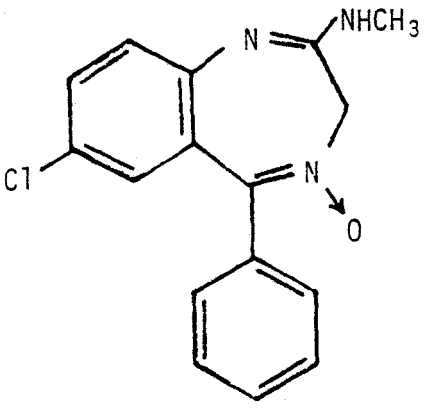
Pharmacological Properties

In the present study, three different benzodiazepines have been employed. These drugs were selected primarily because they are all water soluble, but show distinct pharmacokinetic properties. We thus will concentrate on these agents, the structures of which are shown in Figure 2: 1) chlordiazepoxide; 2) flurazepam; and, 3) midazolam.

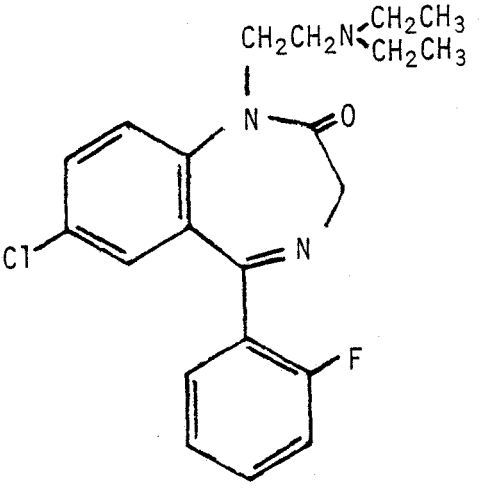
Chlordiazepoxide

Chlordiazepoxide was the first benzodiazepine marketed as a "minor tranquilzer" or anxiolytic drug. It was synthesized in 1955 by Sternbach, and its sedative-hypnotic and anti-anxiety effects were discovered not long after (Greenblatt and Shader, 1974). Unstable in solution, preparations for parenteral administration must be freshly mixed and used immediately. When injected intravenously into mice, there is immediate uptake of radiolabeled chlordiazepoxide into brain, heart, kidney, liver, and skeletal muscle. Brain radioactivity at first is more concentrated in neocortex and thalamus, but within 10 minutes after injection the label is distributed evenly throughout the brain (Placidi

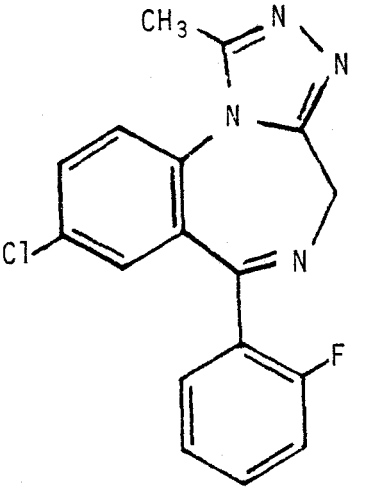
FIGURE 2: Structures of benzodiazepines used in present study.



CHLORDIAZEPOXIDE



FLURAZEPAM



MIDAZOLAM

and Cassaro, 1968). Within several hours, the label becomes concentrated in excretory organs.

Chlordiazepoxide is extensively (87-88%) bound to plasma proteins, especially albumin (Greenblatt and Shader, 1974). The half-life of chlordiazepoxide, itself, is approximately 10 hours. This figure, however, is somewhat misleading. The parent drug is converted to centrally-active metabolites, such as desmethyl-chlordiazepoxide and demoxepam, the latter compound having a reported half-life of up to 95 hours (Greenblatt and Shader, 1974). In fact, the anxiolytic effect of chlordiazepoxide in humans is better correlated with the steady-state concentrations of these metabolites than with the plasma level of chlordiazepoxide itself (Lin and Friedel, 1979).

Flurazepam

Flurazepam, a 2-keto-benzodiazepine, is very rapidly metabolized, having a plasma half-life of less than 2 hours (Greenblatt and Shader, 1974). As is the case with chlordiazepoxide, flurazepam is converted to active metabolites. Although flurazepam, itself, is centrally active, the desamino- and desalkyl- metabolites are an order of magnitude more potent, and reach higher plasma levels than the parent compound (Hasegawa and Matsubara, 1975; Harvey, 1980). The elimination half-life of the desamino- compound approximates 20 hours, while that of the desalkyl- metabolite approaches 100 hours (Breimer, 1977).

Midazolam

Midazolam is a triazolo-benzodiazepine, which is characterized by an imidazole ring fused to positions 1 and 2 of the diazepine ring. The compound first was synthesized by Walser and collaborators (1978). The imidazole ring confers a high stability in aqueous solution and a short duration of action (Pieri et al., 1981). After intravenous administration the plasma half-life of midazolam ranges from 1.3 - 2.5 hours (Lauven et al., 1981; Amrein et al., 1981). The major route of metabolism is demethylation to form alpha-hydroxymidazolam, which subsequently is conjugated with glucuronic acid. In addition, oxidation can occur at position 4 yielding 4-hydroxymidazolam and alpha,4-dihydroxy- midazolam (Walser et al., 1978.) The non-conjugated metabolites are markedly less potent than the parent compound in vivo, primarily due to their poor penetration of the blood-brain barrier (Pieri et al., 1981). Midazolam has a rapid onset of action (Heizman and Ziegler, 1981; Crevoisier et al., 1981.) This property, combined with its short duration of action, solubility in aqueous vehicle, and lack of active metabolites, make midazolam ideal for animal studies in which repeated daily injections are needed.

Benzodiazepines and Behavior

In view of the clinical effects of the benzodiazepines, it is not surprising that these compounds produce striking behavioral changes when administered to laboratory animals. In order to facilitate this review, studies concerning the behavioral effects of the benzodiazepines will be

discussed under one of the following rubrics: 1) appetitive behavior, 2) avoidance behavior, 3) conflict behavior, 4) aggressive behavior, and 5) locomotor activity.

Appetitive Behavior

The effects of the benzodiazepines on appetitive behavior are critically dependent upon the nature of the reward and the specific experimental paradigm employed. In general, two different primary positive reinforcers (reward) have been used: a) consummatory reward, and b) electrical brain stimulation reward (BSR; Olds and Milner, 1954). To date, no consistent effects of benzodiazepines on responding for consummatory reward (for example, food or water) have been demonstrated (for excellent reviews, see Gray, 1977; and, Morley, 1980). With regard to responding for BSR, the benzodiazepines have been reported both to facilitate and to depress operant responding. In general, low doses of the more potent anxiolytic benzodiazepines, such as diazepam and chlordiazepoxide, enhance responding for BSR (Olds, 1966; Panksepp et al., 1970; Wauquier, 1976), while sedative-hypnotic benzodiazepines, such as nitrazepam, produce a depression of response (Wauquier, 1976). In our own laboratory, we have noted a response enhancement following low doses (1.0 - 2.0 mg/kg, intraperitoneally) of midazolam (Sainati and Lorens, unpublished). The duration of the chlordiazepoxide-induced response enhancement greatly exceeds the presence of detectable amounts of chlordiazepoxide in serum (Wauquier, 1974; Lorens and Sainati, 1978). This probably is due to the conversion of chlordiazepoxide to active metabol-

ites having very long durations of action (see above). One possible explanation for the benzodiazepine-induced response enhancement is that these and other drugs with abuse liability may release an endorphin whose effect on opiate receptors mediates the drug-induced alterations in BSR (Lorens, 1976; Stein, 1978; Lorens and Sainati, 1978).

A drug with abuse liability in humans might be expected to be self-administered by laboratory animals. Although more difficult to establish than with opioids and stimulants, both oral and intravenous self-administration of benzodiazepines has been obtained (Harris et al., 1968; Findley et al., 1972; Davis et al., 1976; Walton and Deutsch, 1979). These findings suggest that in addition to their effects on operant responding for BSR or consummatory reward, the benzodiazepines themselves have a positively reinforcing property.

In contrast to the primary reinforcement hypothesis both Stein et al. (1977) and Wauquier (1976; 1979) have postulated that the facilitatory effects of the benzodiazepines on BSR may be due to a release from a punishing component of the stimulation. Benzodiazepine-induced behavioral facilitation is seen most clearly in situations where rewarded behavior is suppressed by punishment (Wauquier, 1979). Although the anatomical substrates which mediate BSR have yet to be deliniated, the medial forebrain bundle appears to play an important role (Olds et al., 1960; Lorens, 1976). According to Stein and coworkers (1977), BSR is mediated by catecholamine systems in the brain. Indeed, noradrenergic fibers from the locus coeruleus [(A-6, in the classification of Dahlst-

röm and Fuxe, 1964)], and dopaminergic fibers from the substantia nigra (A-9) and ventral tegmental area of Tsai (A-10), do ascend in the medial forebrain bundle (Lindvall and Bjorklund, 1978; Moore and Bloom, 1978; Bloom and Moore, 1979). Although some evidence suggests that dopamine neurons are important in the mediation of BSR, it appears that noradrenergic systems do not play a critical role (Fibiger, 1978). Ascending 5-HT fibers, originating in the mesencephalic dorsal and median raphe nuclei, also have been found in the medial forebrain bundle (Azmitia and Segal, 1978). The ascending 5-HT systems have been implicated as forming a component of a "punishment system" (Wise et al., 1972; Stein et al., 1973). The inhibition of this system theoretically would produce a release from punishment and a resultant enhancement in responding.

Avoidance Behavior

Passive Avoidance

Passive avoidance is a term used to describe the learned suppression of approach behavior toward a particular place or stimulus. For example, following receipt of electrical foot shock for entering a designated area of a testing apparatus, the animal learns to refrain from returning to that section of the apparatus. Such a paradigm involves spatial discrimination. The benzodiazepines tend to impair passive avoidance acquisition and retention (Gray, 1977). Such effects have been seen with relatively high doses of chlordiazepoxide (7.5 - 60 mg/kg) and diazepam (6-24 mg/kg) in both rats and mice (Fuller, 1970;

Morrison and Stephenson, 1970; Kumar, 1971; Oishi et al., 1972). In mice a similar disruption of passive avoidance retention has been reported following the administration of diazepam (10 mg/kg), lorazepam (20 mg/kg) and flurazepam (1.0 mg/kg) (Jensen et al., 1979).

Active Avoidance

Active avoidance is a general term used to describe experimental paradigms in which the animal must emit an operant response in order to avoid an aversive stimulus, such as an electric shock to the feet. Active avoidance paradigms may involve spatial (for example, the animal must move from one part of a test apparatus to another in order to avoid an electric shock) or non-spatial [for example, lever-pressing as in the paradigm of Sidman (1953)] operants (Gray, 1977).

One-Way Active Avoidance

In one-way active avoidance procedures, the subject is required always to move in one same direction to avoid an aversive stimulus. Thus, the spatial cues in the animal's surroundings become conditioned stimuli (CS). In most one-way avoidance test situations, benzodiazepines have little effect on acquisition of the task, but do tend to reduce the response latency (Gray, 1977).

Two-Way Active Avoidance

In two-way (shuttlebox) active avoidance, the animal is placed in one side of a two-compartment chamber. A warning signal (CS) is presented. At a given time (for example, 5 seconds) after the onset of the CS, the animal receives an unconditioned stimulus, electrical footshock, until it escapes to the other side of the apparatus. On the next trial, the process is repeated, only now the animal must return to the side where it originally was shocked. This represents a conflict between active and passive avoidance (Gray, 1977) because the subject naturally will be reluctant to return to the side from which it just fled. Accordingly, treatments which disrupt an animal's ability to utilize spatial cues may facilitate two-way avoidance acquisition.

The facilitatory effect of chlordiazepoxide on shuttlebox performance in rats and mice is well known (Fuller, 1970; Iwahara, 1971; Gray, 1977; Sansone and Vetulani, 1980). In general, this facilitation is seen only after high doses, that is, those sufficient to suppress spontaneous locomotor activity (for example, 8.0 - 20 mg/kg). In our laboratory, chlordiazepoxide in doses of 4.0 - 8.0 mg/kg tends to facilitate two-way avoidance acquisition during 50 massed trials, but this effect rarely reaches statistical significance (Sainati and Lorens, unpublished). This finding is in agreement with observations made by Takaori's group (1969), who reported that there is a tremendous variability in the performance of control animals even among littermates from the same strain. Midazolam (2.0 - 4.0 mg/kg), while failing to improve

acquisition of the two-way avoidance task, does increase the number of spontaneous inter-trial barrier crossings (Sainati and Lorens, unpublished).

Sidman Avoidance

Sidman avoidance paradigms represent a non-spatial avoidance task (Gray, 1977). Briefly, the subject is placed in a chamber outfitted with a manipulandum (Skinner box) and an electrifiable grid floor through which shocks are programmed to occur at regular intervals unless the subject performs the appropriate response (for example, lever-pressing). With a proper response, the animal can postpone the next shock for a certain period of time (Sidman, 1953; Heise and Boff, 1962). In general, low doses of benzodiazepines facilitate avoidance responding, especially in animals whose performance is poor in the non-drugged state. High doses, in contrast, suppress avoidance behavior especially in animals with good performance in the non-drugged state (Gray, 1977). Similar results have been obtained for chlordiazepoxide, diazepam (Bignami et al., 1971), nitrazepam (Takaori et al., 1969), tempazepam (Longoni et al., 1973), flurazepam (Randall and Kappell, 1973), and midazolam (Pieri et al., 1981). Gray (1977) and Bignami et al. (1971) suggest that the benzodiazepines improve performance in the Sidman avoidance paradigm by reducing the suppressant effects of the apparatus cues associated with the shock. This hypothesis renders the Sidman avoidance paradigm a type of "conflict" test, as will be discussed below.

Conflict Behavior

Of the psychopharmacological techniques available for studying the effects of anti-anxiety agents (for review see Bignami, 1976), among the most routinely employed are those involving operant conditioning techniques in a Skinner box (Gray, 1977; Sepinwall and Cook, 1978). Such methods have achieved popularity because experimental animals can be used as their own controls, owing to the stability of baseline data in trained animals (Cook and Sepinwall, 1975a). The conflict paradigm of Geller and Seifter (1960; 1962) has been used widely to study the effects of anxiolytics in laboratory animals. Briefly, this procedure involves a schedule in which periods of intermittent food reinforcement are alternated with periods during which continuous food reinforcement is available, but in combination with footshock punishment. This creates in the animal an approach- avoidance "conflict." Another "conflict" procedure is the lick-suppression test of Vogel and coworkers (1971), which consists of suppression of drinking by shocks administered through the drinking tube. The benzodiazepines, along with other clinically effective anxiolytics (for example, meprobamate), enhance responding for food reward during the punished (conflict) segment, but not during the non-punished segment. Diazepam, chlordiazepoxide, oxazepam, flurazepam, nitrazepam, flunitrazepam and midazolam, as well as other benzodiazepines, share this property (Geller et al., 1962; Margules and Stein, 1968; Stein et al., 1973; Gray, 1977; Sepinwall and Cook, 1978; Lippa et al., 1979; Malick and Enna, 1979; Pieri et al., 1981). These

drugs possess little analgesic potency (Bignami, 1976), and classical analgesics, such as morphine, possess little anti-conflict activity (Sepinwall and Cook, 1978).

The notion that the anti-conflict effect of the benzodiazepines represents an animal model for predicting the antianxiety property of a compound in humans is strongly supported by the nearly perfect correlation between the minimum effective doses of various anxiolytics in rat anti-conflict tests and the average daily clinically effective dose for the treatment of human anxiety neuroses (Cook and Sepinwall, 1975b; Lippa et al., 1978). The correlation between receptor binding potency and rat anti-conflict potency for various benzodiazepines also is excellent (Mohler and Okada, 1977a; Sepinwall and Cook, 1980). Moreover, the anti-conflict property of the benzodiazepines has been demonstrated in every species tested to date (Sepinwall and Cook, 1978).

Aggression

In various animal models of aggression, the benzodiazepines, as well as non-benzodiazepine anxiolytics, selectively decrease aggressive behavior in a number of species (Gray, 1977; Harvey, 1980). The experimental paradigms employed have included experimenter provocation, electric shock, and isolation-induced aggression (Delgado, 1973; Christmas and Maxwell, 1970; Valzelli, 1973). It has been reported, however, that rat muricidal behavior is enhanced following benzodiazepine administration (Harvey, 1980). One possible explanation for this apparent paradox is the hypothesis of Hoffmeister and Wuttke (1969) that the benzodiaze-

pines diminish defensive aggression, but enhance offensive aggression. Fighting induced by footshock or isolation would represent defensive aggression, whereas muricide would represent offensive aggression (predation).

Locomotor Activity

The effects of the benzodiazepines and other minor tranquilizers on spontaneous locomotor activity are dose-dependent (Greenblatt and Shader, 1974). At low doses, activity remains at baseline level, while at high doses, toxicity ensues and activity is reduced (Marriott and Spencer, 1965; Christmas and Maxwell, 1970; Hughes, 1972). Intermediate doses tend to increase activity level. The effects of a given dose, however, are highly variable even within a given species. In rats, activity-enhancing doses of chlordiazepoxide range between 5.0 and 30 mg/kg; the effective doses for diazepam and nitrazepam are between 0.5 and 5.0 mg/kg (Greenblatt and Shader, 1974). Flurazepam tends to enhance locomotor activity in doses from 1.0 to 10 mg/kg (File and Hyde, 1979; Crawley, 1981). Midazolam has been reported to enhance activity in lighted Animex chambers, but only after the dose of 5.0 mg/kg (Pieri et al., 1981).

When evaluating the effect of any drug on locomotor activity, it is essential to pay particular attention to the experimental conditions employed. Rats and mice will approach a novel environment cautiously and tend to choose a familiar environment when given a choice (Greenblatt and Shader, 1974; File and Hyde, 1979). It is important, thus, to

know whether the animal is familiar or unfamiliar with the test situation (Marriott and Spencer, 1965; Christmas and Maxwell, 1970; Hughes, 1972). There are many reports of increases in exploratory behavior following benzodiazepine administration (Greenblatt and Shader, 1974), or of increased preference for a novel setting over a familiar one (Marriott and Spencer, 1965; Hughes, 1972; File and Hyde, 1979; File, 1980; Crawley, 1981).

Inasmuch as rats are nocturnal animals, the lighting conditions of the experimental surrounding also have an important influence on the locomotor response to a drug. Although the benzodiazepines, in subataxic doses, tend to increase locomotor activity in a lighted arena (Marriott and Spencer, 1965; Christmas and Maxwell, 1970; Hughes, 1972), they have little effect on activity level when measured in dark photocell cages (Krsiak et al., 1970; Pieri et al., 1981). This difference could be explained by the hypothesis that novel, lighted arenas are more fear or anxiety-producing, so that the anxiolytic benzodiazepines can release a fear-induced suppression of locomotor activity.

Summary

The benzodiazepines are an important class of drugs with several clinical applications. Recently it has become clear that the benzodiazepines also are "abused", or "recreationally" used, substances, often in combination with ethanol and/or the barbiturates. The benzodiazepines appear to have positively reinforcing effects in that they are self-administered by animals and man (Woods, 1978), and can enhance

responding for brain-stimulation reward. The benzodiazepines also disinhibit punished behaviors. This is most clearly demonstrated in experimental paradigms which employ "conflict," such as the Geller-Seifter procedure and the lick-suppression test. The effects of the benzodiazepines on other behaviors controlled by painful stimuli appear to be task-dependent. Thus, for example, the benzodiazepines can facilitate two-way active avoidance acquisition, but fail to influence the acquisition of a one-way conditioned avoidance response. In addition, the benzodiazepines can affect locomotor activity, depending on the experimental parameters employed. The benzodiazepines appear to modify exploratory and locomotor activity predominantly in test situations which contain a high degree of novelty or fear-inducing stimuli. These behavioral effects of the benzodiazepines long have been viewed as reflective of their sedative and anxiolytic properties.

Neurotransmitter Role of GABA

One of the major inhibitory neurotransmitters in the central nervous system (CNS) is gamma-aminobutyric acid (GABA) (Baxter, 1970; De Feudis, 1975; Johnston, 1978). Several indices have been considered and explored in attempts to demonstrate GABA-ergic neurons (Cooper et al., 1978):

The presence and concentration of GABA, itself.

The presence and activity of the enzyme, glutamic acid decarboxylase (GAD), which catalyzes the conversion of glutamate to GABA.

The presence and activity of the metabolizing enzyme, GABA-transaminase (GABA-T).

The high-affinity uptake of GABA.

GABA receptor localization.

Biochemical (Gabellec et al., 1980), immunohistochemical (Barber and Saito, 1975; Saito, 1976) and autoradiographic (Privat, 1976) studies of these indices have shown that GABA serves as a neurotransmitter throughout the neuraxis (in the spinal cord, the cerebellum, the neostriatum, the neocortex, and many other brain regions).

Physiology

GABA functions as an inhibitory synaptic transmitter throughout the mammalian CNS. Although the net effect of GABA always is inhibitory, this effect can be produced either by pre-synaptic depolarization, such as in the spinal cord substantia gelatinosa (Nishi et al., 1974;

Nicoll and Alger, 1979; McGeer and McGeer, 1981), or by post-synaptic hyperpolarization, such as commonly seen at higher levels in the CNS (MacLeod et al., 1980; Marciani et al., 1980; Matthews et al., 1981; McGeer and McGeer, 1981). Both the pre-synaptic depolarization and the post-synaptic hyperpolarization responses produced by GABA are brought about by an increase in the permeability of membranes to chloride ions. The response of post-synaptic neurons to GABA is determined by the interaction between two distinct membrane units, the GABA receptor and the chloride ionophore. When GABA binds to its receptor, the chloride ion channel opens and allows a redistribution of chloride across the neuronal membranes along the electrochemical concentration gradient (Roberts, 1974; Costa and Guidotti, 1979; Olsen, 1982). An increase in permeability to chloride usually produces hyperpolarization due to an influx of chloride ions. The presynaptic depolarization, however, probably is due to an efflux of chloride from the nerve terminals, since these terminals contain relatively high concentrations of chloride intracellularly (Roberts and Hammerschlag, 1976).

Pharmacology

GABA is formed by the one-step decarboxylation of the alpha-amino acid, glutamate. This reaction is catalyzed by l-glutamic acid decarboxylase (GAD), an enzyme with an absolute requirement for the cofactor pyridoxal phosphate (vitamin B-6). Synaptically released GABA is taken up by specific, sodium-dependent high-affinity reuptake systems into both presynaptic nerve terminals and surrounding glial cells. The major

route of metabolism is by transamination with alpha-ketoglutarate via the enzyme GABA-transaminase (GABA-T). The resultant metabolite, succinic semialdehyde, is rapidly oxidized to succinic acid by succinic semialdehyde dehydrogenase (SSA-DH). For a review of GABA metabolism, see Roberts and Hammerschlag (1976).

Various pharmacological manipulations may be employed to alter GABA neurotransmission.

Enhancement of GABA-ergic activity

Drugs can produce GABA-mimetic effects either by acting as ligands directly at the GABA receptor, or indirectly by increasing the availability or affinity of GABA for the receptor post-synaptically.

Directly acting receptor agonists

A number of conformationally restricted GABA analogues have been reported to be GABA receptor agonists (Johnston, 1978; Enna and Maggi, 1979; Andrews and Johnston, 1979). Among the purported GABA agonists are muscimol (Johnston, 1978; Baraldi et al., 1979; Beaumont et al., 1979; Worms et al., 1979), 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridine-3-ol (THIP; Maurer, 1979; Arnt and Krogsgaard-Larsen, 1979), isoguvacine (Andrews and Johnston, 1979), kojic amine (Yarbrough et al., 1979) and SL76-002 (Bartholini et al., 1979). With the exception of SL76-002, none of these drugs crosses the blood-brain barrier in appreciable concentration (Johnston, 1978; Baraldi et al., 1979). The direct-agonist nature of SL76-002, moreover, recently has been questioned (Enna and

Maggi, 1979). For intracranial injections, muscimol has become the most widely used GABA receptor agonist due to its high affinity for the GABA receptor *in vitro*. For this reason, muscimol has been used extensively in the present series of experiments.

Indirectly acting agents

Indirectly acting GABA-mimetics include GABA reuptake inhibitors, releasing agents, inhibitors of degradation, and allosteric receptor ligands, such as the benzodiazepines.

Inhibitors of GABA uptake: GABA is taken up both into nerve terminals (Varon *et al.*, 1965) and glial elements (Henn and Hamberger, 1971; Sellstrom and Sjoberg, 1975; Varon and Somjen, 1979) by specific high-affinity membrane transport systems. These two specific systems can be distinguished pharmacologically. There are drugs which are relatively specific for nerve-terminal reuptake and others which are relatively specific for glial uptake of GABA. Among the relatively selective substrates for neuronal reuptake are 1-2,4-diaminobutyric acid, nipecotic acid, guvacine and *cis*-3-aminocyclohexane carboxylic acid (Iversen, 1978; Johnston, 1978; Wood *et al.*, 1979). Glial reuptake is inhibited by such drugs as *beta*-alanine and *beta*-proline (Johnston, 1978; Schousboe *et al.*, 1979). These specificities, however, are only relative (De Feudis *et al.*, 1979).

The high-affinity uptake of labeled GABA can be used to identify GABA-ergic structures autoradiographically (Hokfelt and Ljungdahl, 1971; Iversen, 1978). These results must be interpreted cautiously, however,

because the correlation between the rate of GABA uptake, GAD activity and GABA levels tends to vary (Storm-Mathisen, 1975; Fonnum and Storm-Mathisen, 1978).

GABA releasing agents: At present, baclofen is the only known GABA releasing drug. A sterically-hindered structural GABA analogue, baclofen is thought to exert its muscle-relaxant activity through a release of intracellular GABA stores (Andrews and Johnston, 1979).

Inhibitors of GABA degradation: Selective inhibition of the enzymes GABA-T or SSA-DH usually leads to increased levels of CNS GABA. This phenomenon is correlated with an anticonvulsant effect (Johnston, 1978). Amino-oxyacetic acid is a potent inhibitor of GABA-T, and is used very commonly to increase brain GABA levels. A non-specific carbonyl-trapping agent, amino-oxyacetic acid also inhibits GAD and many other transaminase enzymes (Johnston, 1978; Metcalf, 1979; Loscher, 1980).

Ethanolamine-O-sulfate is an excellent specific irreversible inhibitor of GABA-T. This drug, however, does not penetrate the blood-brain barrier. Gamma-acetylenic GABA, gamma-vinyl-GABA and gabaculine act quite similarly to ethanolamine-O-sulfate, and, moreover, do cross the blood-brain barrier (Johnston, 1978; Loscher, 1980; Palfreyman et al., 1981).

Sodium di-n-propylacetate (sodium valproate) is a clinically effective anti-convulsant which acts more by inhibiting SSA-DH than by any action on GABA-T (Johnston, 1978; Palfreyman et al., 1981). All of

the aforementioned agents are effective in elevating central nervous system GABA levels.

Allosteric ligands: The evidence that benzodiazepines act to facilitate GABA-ergic transmission by a modulatory effect on an allosteric post-synaptic site will be reviewed below.

Inhibition of GABA-ergic activity

Receptor antagonists

Bicuculline: This convulsant drug which has been employed in the present work, is a specific competitive GABA-receptor antagonist (Mohler and Okada, 1977b; Johnston, 1978). Being insoluble in water and unstable at physiological pH (Olsen et al., 1975), (+)-bicuculline must be dissolved in an acidic vehicle for parenteral administration. Salt derivatives which are water soluble, such as bicuculline hydrochloride and bicuculline methiodide, are more suitable for direct intracranial injection. When administered parenterally, however, these compounds, in contrast to (+)-bicuculline, do not cross the blood-brain barrier.

Picrotoxin: This convulsant does not influence the binding of GABA to synaptic membranes. Instead, it interferes with the opening of the GABA-specific chloride ionophore, which is necessary for the post-synaptic neuronal response (Johnston, 1978; Olsen et al., 1979). Thus, it seems that picrotoxin does not affect the interaction of GABA with GABA receptors, but rather the membrane molecules responsible for controlling chloride ion flux (Johnston, 1978).

Inhibitors of GABA synthesis

3-Mercaptopropionic acid is a convulsant which reversibly inhibits GAD, while at the same time activating GABA-T (Johnston, 1978; Loscher, 1979). This drug produces a rapid fall in brain GABA level. In addition, carbonyl-trapping agents, which act as inhibitors of the coenzyme, pyridoxal phosphate, will decrease the activity of GAD (Tapia, 1975).

Anatomy

Unlike the monoaminergic neuronal systems, which are organized into discrete long fiber pathways, GABA systems comprise predominantly short-fiber interneurons (Fonnum, 1978; Fonnum and Storm-Mathisen, 1978). There are three principal exceptions to the "short axon rule" for GABA neurons: 1) the striatonigral, 2) the nigrothalamic, and 3) the pallidothalamic GABA projections (Fonnum and Storm-Mathisen, 1978). Since the primary focus of the present work is on midbrain structures, only the anatomy of GABA systems in the mesencephalon will be reviewed in detail.

Substantia nigra

The substantia nigra contains the highest concentration [4.25 µmole/gram wet tissue mass (Kanazawa *et al.*, 1973; Balcom *et al.*, 1975; Fonnum and Storm-Mathisen, 1978)] of GABA in the CNS. Within the substantia nigra there is a topographical variation in both GABA concentration and GAD activity, with the highest value for each occurring in the medial and central parts of the pars reticulata (Tappaz *et al.*, 1977).

In the pars compacta, the levels are only one-half as great. In vitro autoradiographic studies have revealed a relatively high density of binding sites for the radiolabelled GABA-receptor agonist, ^{3}H-muscimol, over the substantia nigra zona reticulata, with only low densities seen over the zona compacta (Palacios et al, 1981a; 1981b). The origin of nigral GABA and GAD has been investigated intensively over the past twenty years. It is accepted almost universally that the substantia nigra receives a number of GABA-ergic fibers from the corpus striatum (Hattori et al., 1973; Ribak et al., 1976; Fonnum and Storm-Mathisen 1978). Destruction of the striatonigral pathway produces a greater than 75% fall in nigral GABA content and GAD activity (Hattori et al., 1973; Minchin and Fonnum, 1979; Gale and Iadarola, 1980). In the cat, the striatonigral GABA projection has been found to have a distinct topographic organization (Fonnum et al., 1974). Briefly, fibers from the putamen and posterior caudate terminate mostly in the posterolateral pars reticulata, with fibers from the head of the caudate projecting mainly to the anteromedial region. The fibers from the lateral part of the caudate terminate intermediately. Recently, a similar pattern of distribution has been described in the rat (Di Chiara et al., 1980).

In addition to its extrinsic GABA-ergic input, evidence has been advanced for the existence of intrinsic GABA-ergic neurons in the pars reticulata which project to the pars compacta (Cheramy et al., 1978; Cattabeni et al., 1979). Stimulation of the striatonigral tract enhances the firing rate of pars compacta neurons, presumably by inhibiting inhi-

bitory (possibly GABA-ergic) cells in the pars reticulata. Thus, neurons in the pars compacta are disinhibited (Grace et al., 1980). When iontophoretically applied directly into the pars compacta, GABA decreases neuronal firing. This evidence suggests that there is an inhibitory GABA-ergic interneuron in the pars reticulata, which terminates on pars compacta neurons (Di Chiara et al., 1979a).

The substantia nigra also may be a source of GABA-ergic efferents to other brain regions (Anderson and Yoshida, 1977; Kilpatrick et al., 1980). Using a combination of retrograde and orthograde tracing techniques, Clavier et al. (1976) have found evidence for a nigrothalamic projection. Intranigral administration of the excitatory glutamate analogue, kainic acid, causes a marked diminution of GAD activity in the ventromedial thalamus (Di Chiara et al., 1978; Di Chiara et al., 1979b; Straugham, 1979) and in the superior colliculus (Di Chiara et al., 1979b).

Ventral tegmental area

Certain analogies have been drawn between the nigro- striatal dopamine system and the mesolimbic dopamine system (Fonnum and Storm-Mathisen, 1978). Just as the corpus striatum receives a heavy dopaminergic input from the substantia nigra (A-9), the nucleus accumbens, together with other limbic structures, receives a major dopaminergic input from the mesencephalic ventral tegmental area of Tsai (A-10; Moore and Bloom, 1978). One might, therefore, expect a "limbomesencephalic" GABA projection which is analagous to the striatonigral pathway.

Indeed, a high level of GAD activity has been found in the ventral tegmental area (Fonnum et al., 1977), and iontophoretically applied GABA inhibits the activity of neurons in this area (Wolf et al., 1978). It appears, however, that GABA-ergic inhibition in the ventral tegmental area is mediated by local interneurons rather than a long fiber pathway, since interruption of descending fibers from the limbic forebrain does not alter tegmental GAD activity (Fonnum et al., 1977).

Raphe nuclei

It is now fairly well established that GABA serves as a neurotransmitter for inhibitory interneurons within the mesencephalic B-7 and B-8 5-HT cell groups (Dahlstrom and Fuxe, 1964), which constitute the principal origin of ascending 5-HT projections to the forebrain (Azmitia and Segal, 1978). These 5-HT systems are thought to regulate several physiological and behavioral processes (see below). The median and dorsal raphe nuclei are rich sources of GAD activity (Massari et al., 1976). Following intracerebroventricular administration of ^{3}H-GABA into the rat, autoradiography reveals labeled cell bodies and fibers in the dorsal raphe nucleus (Gamrani et al., 1979). Immunocytochemical localization of GAD supports the presence of GABA-containing cell bodies, fibers and nerve terminals in the dorsal raphe nucleus (Nanopoulos et al., 1980). Using a combination of horseradish peroxidase retrograde transport, a GAD activity measure, and ^{3}H-GABA autoradiographic techniques, Belin and associates (1979) have provided evidence for a GABA system in the midbrain periaqueductal and pontine ventricular gray, including the

dorsal raphe nucleus (Pujol *et al.*, 1981). *In vitro* autoradiographic studies have shown that ^{3}H-muscimol binding sites are present with a moderate density in the dorsal raphe nucleus (Palacios *et al*, 1981a; 1981b). Gallager and Aghajanian (1976), furthermore, have shown that microiontophoretically applied GABA produces a picrotoxin-reversible inhibition of the spontaneous firing of dorsal raphe 5-HT cells, which is potentiated by both systemically and locally applied benzodiazepines (Gallager, 1978).

Tegmental nuclei of Gudden

Among the prominent brainstem nuclei, the dorsal tegmental nucleus of Gudden has been found to contain the highest level of GAD activity, with the ventral tegmental nucleus of Gudden having a relatively low level (Massari *et al.*, 1976). In addition, a moderately high density of ^{3}H-muscimol binding sites over the dorsal tegmental nuclei of Gudden has been reported (Palacios *et al*, 1981a; 1981b). Nauta (1958) included these tegmental nuclei within his limbic midbrain area because of their interconnections with the limbic forebrain. These areas, such as the hippocampus and dentate gyrus, also have been shown to possess high levels of GAD activity (Tappaz *et al.*, 1976).

Neurotransmitter Role of Serotonin

Serotonin (5-hydroxytryptamine, 5-HT), formed by the enzymatic 5-hydroxylation of l-tryptophan and the subsequent decarboxylation of the resulting 5-hydroxy-l-tryptophan, appears to function as a neurotransmitter in mammalian brain (Aghajanian and Wang, 1978; Fuller, 1980). Largely confined to midbrain and hindbrain regions, distinct groups of 5-HT containing perikarya first were described by Dahlström and Fuxe (1964), and labeled caudorostrally B-1 through B-9.

Anatomy

Histofluorescence (Dahlström and Fuxe, 1964), immunocytochemical (Steinbusch, 1981; Kulmala and Lorens, 1982), retrograde dye tracing (van der Kooy and Hattori, 1980b; van der Kooy and Steinbusch, 1980), and autoradiographic (Azmitia and Segal, 1978) techniques have been used to map out the projections of 5-HT neurons. The 5-HT cell bodies largely coincide with the brainstem raphe nuclei (Taber et al., 1960). The more caudal B-1 through B-3 groups send axons principally to the spinal cord and lower medulla (Lorens, 1978; Loewy and McKellar, 1981). The B-4 through B-6 groups may provide both ascending and descending projections (Cooper et al., 1978; Lorens, 1978). The more rostral 5-HT cell groups (B-7 through B-9) are the source of extensive ascending projections to the forebrain. The B-7 cell group, which overlaps the dorsal raphe nucleus, projects primarily to the neostriatum, olfactory tubercle, amygdala, neocortex, hypothalamus and thalamus. The B-8 cell group, located predominantly in the caudal linear and median raphe nuc-

lei, is the origin of 5-HT projections to such limbic structures as the septum and hippocampus, as well as to the cortex, hypothalamus and substantia nigra (Azmitia and Segal, 1978; Lorens, 1978; van de Kar and Lorens, 1979; Dray *et al.*, 1976; 1978; van der Kooy and Hattori, 1980a).

Physiology

Serotonin appears to function primarily as an inhibitory neurotransmitter (Cooper *et al.*, 1978; Segal, 1981). Stimulation of the 5-HT containing median raphe nucleus inhibits the spontaneous activity of hippocampal neurons, especially in the heavily innervated CA-1 region (Segal, 1975). A similar effect is seen when 5-HT is applied iontophoretically. Electrical stimulation of the median raphe nucleus also inhibits the firing rates of medial septal neurons (Segal, 1976), whereas stimulation of the dorsal raphe nucleus inhibits neurons in the amygdala (Wang and Aghajanian, 1977) and neostriatum (Miller *et al.*, 1975). Furthermore, perfusion *in vitro* with 5-HT produces a potassium-dependent, non-chloride-dependent hyperpolarization of hippocampal CA-1 cells (Segal, 1981).

Microiontophoretically applied 5-HT has a powerful and direct inhibitory action on 5-HT neurons in the dorsal raphe nucleus (Aghajanian and Wang, 1978). This is thought to be due to an action of 5-HT on "autoreceptors" in the dorsal raphe nucleus. "Autoreceptors" may be defined as receptors which mediate the response of a neuron to its own transmitter. The autoreceptors are thought to reflect the presence of

5-HT collaterals. Raphe cells demonstrate a very characteristic slow, regular firing pattern (Aghajanian and Wang, 1978). All attempts to "drive" these neurons antidromically have resulted in an initial rapid burst of activity, followed by a period of post-stimulus depression. This is taken as physiological evidence for recurrent collateral inhibition. Such autoregulatory mechanisms would allow raphe 5-HT cells to maintain a tonic level of activity within a narrow range (for review, see Aghajanian and Wang, 1978).

Pharmacology

Serotoninergic neurotransmission may be altered pharmacologically in several different ways. It is clearly possible to decrease the activity of serotonin neurons and to reduce the availability of 5-HT post-synaptically. It is, however, debatable as to whether it is possible pharmacologically to enhance 5-HT neurotransmission other than by administering directly acting receptor agonists. Drugs which enhance the concentration of 5-HT in the synaptic cleft will lead to decreased firing of 5-HT neurons because 5-HT will act in an inhibitory manner at 5-HT autoreceptors (Aghajanian and Wang, 1978). Thus, drugs, whose presumed mechanism of action (such as reuptake inhibition) depends on the release of 5-HT from axon terminals by neuronal discharge, will not lead to an effective enhancement of 5-HT neurotransmission, because the subsequent increase in synaptic 5-HT concentration will result in negative feedback inhibition. By increasing the neurotransmitter concentration at other than autoreceptor sites, serotonin releasers and reuptake inhi-

bitors would, nevertheless, produce an acute serotoninomimetic post-synaptic response.

Serotonin receptor agonists

Receptor agonists include quipazine, bufotenin, lysergic acid diethylamide, N,N-dimethyl-5-methoxytryptamine and pipamperone. These drugs bind to the 5-HT receptor and produce a serotonin-mimetic effect. Quipazine has been reported to produce behavioral effects characteristic of a 5-HT receptor agonist (Green et al., 1976; Jacoby et al., 1976). It would appear, however, that the apparent agonist effects of quipazine may, in fact, be due to an antagonism of 5-HT autoreceptors (Martin and Sanders-Bush, 1982).

Among the indirectly acting compounds are the 5-HT reuptake inhibitors, releasers, precursors and inhibitors of degradation.

Inhibitors of 5-HT reuptake

The tertiary-amine tricyclic antidepressants (for example, imipramine) inhibit 5-HT reuptake at the presynaptic nerve terminal. These compounds, however, also inhibit norepinephrine reuptake to a certain extent (Baldessarini, 1980). Fluoxetine, zimelidine and paroxetine are some of the more selective 5-HT reuptake inhibitors. Zimelidine, however, is metabolized to norzimelidine, which inhibits noradrenaline reuptake (Fuller, 1980).

5-HT releasing agents

The drugs p-chloroamphetamine and fenfluramine, in addition to inhibiting serotonin nerve-terminal reuptake, release serotonin (as well as norepinephrine) from vescicular stores (Trulson and Jacobs, 1976; Fuller, 1978). These two compounds also have been reported to produce long-term reductions in CNS serotonin concentrations, presumably by inhibiting tryptophan hydroxylase (Sanders-Bush and Steranka, 1978; Fuller, 1980).

5-HT precursors

The administration of the 5-HT precursors, l-tryptophan and 5-hydroxy-l-tryptophan, leads to elevations in brain 5-HT level. Unfortunately, both of these compounds are somewhat non-specific. The essential amino acid, l-tryptophan, is taken up by virtually all cells and incorporated into their protein metabolism. 5-Hydroxy- l-tryptophan is taken up by catecholaminergic neurons, in which it can be decarboxylated to 5-HT and released ectopically (Yunger and Harvey, 1976).

Inhibitors of 5-HT degradation

The only known inhibitors of 5-HT catabolism are the monoamine oxidase inhibitors. None of these drugs, however, is selective for 5-HT degradation since monoamine oxidase also is a major enzyme in the metabolism of the catecholamines (Baldessarini, 1980).

5-HT antagonists

The drugs which decrease 5-HT neurotransmission are the 5-HT receptor antagonists, inhibitors of serotonin synthesis, and depletors of serotonin stores. Some of the recently identified 5-HT receptor antagonists are cyproheptadine, methysergide, metergoline, methiothepin, cinanserin and mianserin (Aghajanian and Wang, 1978; Fuller, 1980). These drugs, however, possess varying degrees of dopamine agonist activity. In addition, cyproheptadine is a well known histamine antagonist. Furthermore, it is uncertain whether these agents are effective antagonists at all 5-HT receptors (see Aghajanian and Wang, 1978; Martin and Sanders-Bush, 1982).

Inhibitors of 5-HT synthesis

The precursor enzyme in the synthesis of serotonin, tryptophan 5-hydroxylase is inhibited by p-chlorophenylalanine. The resultant enzymatic inhibition is irreversible and thus leads to a prolonged (up to 2 weeks) decrease in tryptophan hydroxylase activity. p-Chlorophenylalanine, however, also inhibits both tyrosine and phenylalanine hydroxylase. As mentioned above, p-chloroamphetamine and fenfluramine also produce long-lasting (several weeks) reductions in CNS 5-HT concentrations.

5-HT neurotoxins

The neurotoxins, 5,6- and 5,7-dihydroxytryptamine, when injected intracerebrally, either locally or into the ventricles, destroy 5-HT

containing perikarya and processes with a high degree of specificity (Bjorklund et al., 1973; Lorens, 1978). A secondary-amine tricyclic antidepressant, such as desmethylimipramine, however, must be used as a pretreatment in order to prevent the uptake of the dihydroxytryptamines into noradrenergic neurons. 5,7-Dihydroxy- tryptamine has several advantages over 5,6-dihydroxytryptamine (see Lorens, 1978), and thus was used in the present study.

Serotonin and Behavior

CNS 5-HT systems have been postulated as playing important roles in several psychological and physiological processes, including temperature regulation (Myers, 1975; 1981; Myers and Sharpe, 1968; Myers and Waller, 1975); modulation of luteinizing hormone, prolactin and renin release (van de Kar et al., 1980; 1981; Koenig et al., 1980); the response to painful stimuli and morphine analgesia (Messing and Lytle, 1977) and, the control of mating behavior (Karczmar, 1980). It is outside the scope of this dissertation to review all of these proposed functions. Thus we shall focus our comments on those behaviors which are modified by the benzodiazepines, possibly via an effect on the activity of raphe 5-HT neurons.

Punished behavior

The existence of a 5-HT "punishment" system has been postulated (Stein et al., 1973; 1975; Haefely, 1978). If such a system exists, one would expect pharmacological manipulation of CNS 5-HT systems to have

appreciable effects of punished behaviors. The putative 5-HT receptor antagonists, methysergide, cinanserin and cyproheptadine, have been reported to produce anti-conflict effects (Cook and Sepinwall, 1975b; Sepinwall and Cook, 1978; 1980). Serotonin synthesis inhibitors, such as p-chlorophenylalanine, moreover, have similar effects (Koe, 1979; Sepinwall and Cook, 1978; 1980). Destruction of 5-HT neurons with 5,6- and 5,7-dihydroxytryptamine also can release punished behavior as measured in conflict tests, regardless of whether these agents are administered intracerebroventricularly (Sepinwall and Cook, 1978) or injected directly into the ascending 5-HT pathways in the ventromedial mesencephalic tegmentum (Tye et al., 1977; 1979).

Locomotor activity

The effects of 5-HT depletion on locomotor activity are dependent upon the type of surgical and pharmacological intervention, as well as upon the behavioral paradigm employed (Lorens, 1978). Electrolytic lesions of the median but not of the dorsal raphe nucleus produce hyperactivity in both a familiar (home cage; Kostowski et al, 1968) and novel (open field; Srebro and Lorens, 1975; Hole et al., 1976) environment. In the open field, 5,7-dihydroxytrypt- amine lesions, in contrast to electrolytic lesions, do not alter activity level. p-Chlorophenylalanine also fails to affect open field activity (Kohler and Lorens, 1978), while p-chloroamphetamine produces a decrease in activity (for review, see Lorens, 1978). On the other hand, increases in home cage activity have been obtained after systemic injection of the tryptophan

hydroxylase inhibitors, p-chlorophenylalanine and p-chloroamphetamine, as well as following 5,7-dihydroxytryptamine lesions (Mackenzie et al, 1978). Recently, Williams and Azmitia (1981) microinjected different doses (1.0 - 10 μg) of 5,7-dihydroxytryptamine into the fornix-fimbria which contains 5-HT fibers afferent to the hippocamus. These injections produced a dose-related increase in nocturnal locomotor activity (as measured in photocell chambers) during the seven consecutive nights of testing. A reduction of ^{3}H-5-HT uptake was found in the dorsal hippocampus and was related to the dose of 5,7-dihydroxytrypta- mine. The degree of dorsal hippocampal ^{3}H-5-HT uptake was negatively correlated with the mean nocturnal activity for the seven nights of testing. These authors interpreted their results to imply that the increase in nocturnal locomotor activity produced by a generalized depletion of 5-HT in the brain may be due to disruption of hippocampal 5-HT terminals supplied by the fornix-fimbria.

It is of interest to note that bilateral electrolytic lesions in the ventral and dorsal tegmental nuclei of Gudden which lie in close proximity to the median and dorsal raphe nuclei, respectively, can produce increases in open field locomotor activity similar to those seen after electrolytic raphe lesions (Lorens et al., 1975; Lorens, 1978). This, as well as the data alluded to above, suggests the possibility that the effects of electrolytic raphe lesions on locomotor activity may not be due solely to the destruction of 5-HT containing cells. The behavioral consequences of raphe and Gudden lesions are not, however,

identical. While bilateral destruction of the tegmental nuclei of Gudden enhances the acquisition of a two-way (shuttlebox) avoidance task, only combined, not selective, electrolytic dorsal and median raphe nucleus lesions produce a similar effect (Lorens et al., 1975; Lorens, 1978).

GABA-Benzodiazepine Interactions

In light of the potent central nervous system effects of the benzodiazepines, efforts have been made to localize CNS benzodiazepine receptors. Recently, high-affinity, saturable, stereospecific binding sites for benzodiazepines have been found both *in vivo* (Williamson *et al.*, 1978; Chang and Snyder, 1978), and *in vitro* (Squires and Braestrup, 1977; Mohler and Okada, 1977a; Tallman *et al.*; 1980). The existence of such receptors for benzodiazepines has prompted a search for endogenous ligands. Several substances, including the purines, inosine and hypoxanthine; the cofactor, nicotinamide; a *beta*-carboline; and a small peptide, GABA-modulin, have been proposed as possible endogenous ligands. To date the evidence is not compelling for any one of these substances (for a review, see Paul *et al.*, 1980).

Studies of the biochemical regulation of the benzodiazepine receptor have revealed that GABA and GABA-mimetics, such as muscimol, increase, while GABA-receptor antagonists, such as bicuculline, decrease the affinity of benzodiazepines for their receptors (Tallman *et al.*, 1978; 1980). Moreover, permeable halide ions such as chloride and iodide, but not large anions such as citrate or sulfate, increase the affinity of radiolabeled benzodiazepines for their receptor (Costa *et al.*, 1979). These findings suggest that the benzodiazepine receptor is coupled to both a GABA receptor and a chloride ionophore. These biochemical data are supported by electrophysiological observations suggesting an interaction of benzodiazepines with both a GABA receptor and

a chloride ionophore (MacDonald and Barker, 1979; Costa et al., 1979; 1981). Although the morphological correlation between the distributions of putative GABA and putative benzodiazepine binding sites in vitro is not perfect, there is a significant degree of overlap (see Young and Kuhar, 1979, 1980; Palacios et al, 1981a; 1981b). These observations support the hypothesis of a receptor complex in the mammalian central nervous system which consists of a functionally coupled benzodiazepine receptor, GABA receptor, and chloride ionophore (Costa and Guidotti, 1979; Tallman et al., 1980; Paul et al., 1981a; Olsen, 1982).

The benzodiazepine binding site has a truly neuromodulatory role. The interaction of this site with its ligand produces an increase in the affinity and/or number of GABA receptors. The benzodiazepines depend on the synaptic release of GABA in order to produce their physiological effects. In the absence of GABA, the binding of a benzodiazepine to its receptor does not produce any changes in the post-synaptic membrane potential. It now appears that the benzodiazepines may exert many, if not all, of their pharmacological effects (including their anxiolytic, soporific, anti-convulsant, and muscle relaxant properties) by affecting this system (Haefely et al., 1975; Costa and Guidotti, 1979; Krogsgaard-Larsen and Arnt, 1980; Paul et al., 1981; Paul and Skolnick, 1981; Olsen, 1982).

Serotonin-Benzodiazepine Interactions

Benzodiazepines exert several effects on 5-HT metabolism. Firstly, 5-HT synthesis and turnover rates are decreased. Secondly, removal of the 5-HT metabolite 5-hydroxy-3-indoleacetic acid (5-HIAA) from the brain is inhibited. Steady-state levels of 5-HT may or may not be increased (Chase et al., 1970; Koe, 1979).

A slower rate of turnover most likely represents a decrease in 5-HT release due to a reduction in the activity of serotonergic neurons. Benzodiazepines reduce accumulation of ^{3}H-5-HT and ^{3}H-5-HIAA after intravenous ^{3}H-tryptophan (Dominic et al., 1975). They also retard the disappearance of radiolabeled 5-HT following intracisternal injection of ^{14}C-5HT. Interference with transport of 5-HIAA from the brain has been inferred from the slower disappearance of labeled 5-HIAA from the brain following intracisternal injection of either ^{14}C-5-HT or ^{14}C-5-HIAA (Chase et al., 1970). Furthermore, tolerance does not develop to the reduction in 5-HT turnover produced by repeated daily injections of oxazepam (Wise et al., 1972; Stein et al., 1975). The ability of the benzodiazepines to decrease 5-HT turnover and to decrease serotonergic activity in the brain may underlie several of their behavioral effects.

Midbrain Benzodiazepine-GABA-Serotonin Interactions

As mentioned above, there is a good deal of evidence to support the role of GABA as a neurotransmitter within the mesencephalic dorsal and median raphe nuclei (Gamrani et al., 1979; Nanopoulos et al., 1980; Belin et al., 1979;). Using an immunocytochemical double-labeling technique, Pujol and coworkers (1981) claim to have found neurons in the dorsal raphe nucleus which contain both GAD and 5-HT. Close examination of their data, however, reveals that the two stains are not uniformly distributed within the double-labeled cells. Their appearance and pattern of distribution, moreover, suggests the presence of GAD-positive terminals on 5-HT perikarya, rather than the coexistence of GAD and 5-HT within the same cell bodies.

Young and Kuhar (1980) recently have demonstrated moderate to dense benzodiazepine binding in the periaqueductal gray, the dorsal raphe nucleus, the dorsal and ventral tegmental nuclei of Gudden, and the more caudally located raphe pontis and raphe magnus nuclei. We have found, in addition, moderate benzodiazepine receptor labelling in the region of the median raphe nucleus (Sainati et al., 1982).

When microiontophoretically applied to spontaneously firing dorsal raphe 5-HT cells in the rat, GABA produces a picrotoxin-reversible inhibition of spontaneous firing, which is potentiated by both systemically and locally applied benzodiazepines (Gallager and Aghajanian, 1976; Gallager, 1978). In addition, systemic diazepam and chlordiazepoxide have been found to produce a dose-dependent suppression of raphe unit activ-

ity in freely-moving cats (Preussler et al., 1981). Moreover, Przewlocka and coworkers (1979) have reported that injection of muscimol into the dorsal raphe nucleus significantly reduces 5-HT and 5-HIAA levels in the hypothalamus, but not in the neostriatum. Forchetti and Meek (1981), furthermore, recently have examined the effects of GABA agonists and antagonists, locally applied to the median raphe nucleus, on hippocampal 5-HT and 5-HIAA concentrations in probenecid pretreated rats. They found that muscimol (100 ng) decreased 5-HT turnover 90 minutes post-injection, whereas picrotoxin (2.0 µg) and bicuculline methiodide (2.0 µg) produced opposite results. The hippocampal content of 5-HT was not affected. Parenteral diazepam (5.0 mg/kg, intraperitoneally) blocked the effects of the GABA antagonists and potentiated the action of muscimol. These results provide evidence for a tonic inhibition by GABA neurons of the firing rate of 5-HT perikarya in the median raphe nucleus.

If serotonin and GABA constitute a bipole system, and as inactivation or destruction of CNS 5-HT systems produces certain specific behavioral changes (see above), then GABA-ergic inhibition of these systems should produce similar changes. Intracisternal administration of the GABA-transaminase inhibitor, ethanolamine-O-sulfate, has been reported to produce an increase in exploratory locomotor activity (File, 1977). In addition, injection of the GABA analogue, muscimol, directly into the dorsal raphe nuclei of ether-anesthetized rats, has been observed to greatly increase their locomotor activity (Przewlocka et al., 1979).

These effects were antagonized by both local and systemic injections of bicuculline and picrotoxin.

Experimental Plan

The literature reviewed above suggest that some of the clinical and behavioral effects of the benzodiazepines may be due to their enhancement of GABA-ergic neurotransmission in the midbrain raphe nuclei. The data suggest, furthermore, that benzodiazepine-enhanced GABA-ergic inhibition of serotonergic neurons may produce striking changes in appetitive behavior, avoidance behavior, punished behavior and locomotor activity. We, therefore, undertook a series of experiments designed to investigate the extent to which GABA-ergic modulation of midbrain raphe serotonergic neurons influences locomotor activity in the rat.

First we replicated the findings of Przewłocka and associates (1979) that the acute microinjection of muscimol (100 ng) into the dorsal raphe nucleus produces hyperactivity. Secondly, we found that the acute microinjection of muscimol (100 ng) into the median raphe nucleus produced increases in activity which were 4 times greater than those seen after similar injections into the dorsal raphe nucleus. A dose-response analysis using animals with chronically-indwelling cannulae, and a complex Latin square design, showed that the median raphe nucleus indeed was more sensitive to the effects of muscimol. Thus, subsequent experiments utilized only cannulae implanted chronically in the median raphe nucleus.

To determine whether the hyperkinesis produced by the intra-raphe injection of muscimol was due to activation of GABA receptors, we attempted to potentiate the effects of muscimol by administering intraperitoneally the benzodiazepine, chlordiazepoxide, and to block the effect with the GABA antagonist, bicuculline.

The success of these experiments prompted us to determine whether the effects of the peripherally administered bicuculline and chlordiazepoxide were due to their binding to the same neuronal membranes as the intra-raphe muscimol. We thus conducted the following series of experiments. First, intra-raphe dose-response analyses for the water soluble benzodiazepines, chlordiazepoxide, flurazepam and midazolam, were performed. A similar study was carried out using bicuculline methiodide. Subsequently, animals received sub-effective doses of flurazepam or midazolam, followed by a sub-effective dose of muscimol, into the median raphe nucleus. Finally, animals received a combination of bicuculline methiodide and muscimol into the median raphe nucleus to determine whether the former drug would block the effects of the latter. The results from these experiments supported the view that the intra-raphe injection of muscimol produces hyperkinesis via an activation of local GABA receptors.

Since destruction of the ventral tegmental nuclei of Gudden produces hyperactivity, and since these nuclei have been reported to contain a high density of benzodiazepine receptors, we investigated the effect such lesions on the intra-raphe muscimol dose-response relation-

ship. To ascertain the role of ascending 5-HT systems in the mediation of the hyperkinetic effect of intra-raphe muscimol, we examined the muscimol dose-response relationship following 5,7-dihydroxytryptamine induced degeneration of these projections.

The results obtained from these experiments, overall, strongly suggest that the activation of midbrain GABA receptors produces a hyperkinetic response in rats which depends on the integrity of an ascending serotonin system for its expression.

CHAPTER II

MATERIALS AND METHODS

Animals

The experimental subjects were male Sprague-Dawley rats (King Animal Farms, Orange, WI), 90 - 120 days old and weighing 310 - 375 grams at the time of surgery. The animals were housed individually in a temperature (22 +/- 1°C), humidity (40 - 52%), and illumination (12 hour light-dark cycle) controlled room. Food and water were available *ad libitum* in the home cage.

Surgery

A Kopf stereotaxic instrument was used. The incisor bar was set 3.2 mm above the interaural plane. At the time of surgery, each animal received 50 mg/kg ampicillin (Omnipen-N, Wyeth) and 0.4 mg/kg atropine sulfate (Lilly), intramuscularly, and chloramphenicol (Chloromycetin, 1% ophthalmic ointment; Parke-Davis) topically around the wound. For the acute intra-raphe microinjections, ether (Malinckrodt) was employed as an anesthetic, and the wound margins were infiltrated with 0.1 ml of lidocaine hydrochloride (Xylocaine; Astra, Worcester, MA) in order to minimize post-operative pain. For other surgical procedures intraperitoneal pentobarbital sodium (50 mg/kg; Butler, Columbus, OH) was used as an anesthetic. All wounds were closed with autoclips (Clay Adams).

Acute Intra-Raphe Injections

Muscimol (100 ng in 0.5 µl of vehicle) or saline (0.5 µl) was administered via a 5.0 ul Hamilton syringe with a 30-gauge needle (0.35 mm o.d.) oriented mid-sagittally at an angle of 47° caudal to the vertical plane. The solution was delivered slowly (in about 60 seconds). The needle was left in situ for 2 min following completion of the injection. Coordinates for the dorsal raphe placement were 6.2 mm caudal and 9.6 mm ventral to the skull surface 1.0 mm rostral to lambda (L + 1). Coordinates for the median raphe placement were 8.1 mm caudal and 12.1 mm ventral to L + 1.

Chronic Intra-Raphe Cannula Placement

A guide cannula (0.46 mm o.d. and 0.25 mm i.d.; Plastic Products Co., Roanoke, VA) was implanted in either the dorsal or the median raphe nucleus. A stylet (0.23 mm dia) then was inserted such that its tip was flush with that of the guide cannula. This assembly then was cemented onto stainless steel anchoring screws embedded in the skull. The coordinates were the same as mentioned above.

Electrolytic Lesions

A 0.25 mm diameter stainless steel wire insulated with Expoylite except at the cross section of its tip was used as the lesion electrode. This electrode was connected to the positive pole of a Grass model DCLM-5A direct current lesion maker (Grass Medical Instruments Co., Quincy, MA). An alligator clip attached to the wound margin served as

the ground. Lesions were produced bilaterally in the ventral tegmental nuclei of Gudden (VTG) by passing 2.0 milliamperes direct current for 2 seconds via the anode. The anode was inserted stereotactically at an angle 47° caudal to the vertical plane. The coordinates used were 0.5 mm lateral to the midsagittal suture, and 7.5 mm caudal and 11.7 mm ventral to the skull surface at L + 1.

Neurotoxic Lesions

The specific serotonin neurotoxin, 5,7-dihydroxy- tryptamine creatinine sulfate (5,7-DHT; Sigma Chemical Co., St. Louis, MO), was dissolved in 0.2% ascorbate in 0.9% saline, and was injected bilaterally either into the lateral cerebral ventricles (75 µg free base in 5.0 µl vehicle), or into the ventromedial mesencephalic tegmentum (4.0 µg base in 2.0 µl) at a rate of 0.5 µl/minute. The coordinates used were 0.5 mm rostral to bregma, 1.2 mm lateral to the midline, and 5.5 mm ventral to the skull surface for the lateral ventricle injections; and, an angle of 47° caudal to the vertical plane, 5.5 mm caudal to lambda, 0.6 mm lateral to the midline, and 11.0 mm ventral to the reading at lambda for the mesencephalic tegmentum injections. These subjects all received desmethylimipramine hydrochloride (Merrell, Cincinnati, OH), 20 mg/kg intraperitoneally, 30 - 45 minutes prior to surgery. Because desmethylimipramine potentiates the anesthetic effects of pentobarbital, the dose of the anesthetic for these animals was 35 mg/kg.

Drugs

Intraperitoneal Administration

Chlordiazepoxide hydrochloride (Roche, Nutley, NJ) was dissolved in 0.9% saline to final concentrations (as the base) of 3.1, 6.3, 12.5, 25.0 and 50.0 umole/ml (0.9, 1.9, 3.8, 7.5 and 15.0 mg/ml). (+)-Bicuculline (Sigma) was dissolved in acidified vehicle (pH = 5.5 - 6.0) to final concentrations of 0.19, 0.38, 0.75, 1.50 and 3.00 umole/ml (0.04, 0.07, 0.14, 0.27, 0.55 and 1.10 mg/ml).

Intracranial Administration

All drugs injected intracranially were dissolved in 0.9% saline. Muscimol (Sigma) was prepared in concentrations of 0.22, 0.44, 0.88, 1.75 and 3.50 nmole/ 0.5 µl (25, 50, 100, 200 and 400 ng/ 0.5 µl). Bicuculline methiodide (Pierce Chemical Co., Rockford, IL) was prepared in concentrations (as the base) of 0.22, 0.44 and 0.88 nmole/ 0.5 µl (81, 161 and 323 ng/ 0.5 µl). Chlordiazepoxide hydrochloride (Roche), flurazepam dihydrochloride (Roche), and midazolam maleate (Roche) were prepared in concentrations of 0.11, 0.22, 0.44, 0.88 and 1.75 nmole/ 0.5 ul. These concentrations, expressed in terms of mass (of the base), are: chlordiazepoxide - 44, 88, 175, 350 and 700 ng; flurazepam - 43, 85, 170, 340 and 680 ng; and midazolam - 36, 71, 143, 286 and 571 ng.

Biochemical Analysis

The animals were killed by decapitation and their brains quickly removed and dissected on a glass plate over dry ice as described by Lor-

ens and Guldberg (1974). The hippocampi and striata were wrapped in aluminum foil, flash frozen in liquid nitrogen, and stored in a -70°C freezer for no more than 3 weeks prior to assay. The brainstems were placed in phosphate-buffered formalin and fixed for at least 2 weeks prior to sectioning.

Serotonin (5-HT) and 5-hydroxy-3-indoleacetic acid (5-HIAA) levels were determined by high-performance liquid chromatography (HPLC; Mefford, 1981). Each tissue sample was sonicated for 20 sec in 500 ul of 0.1 N perchloric acid which contained 1.0 mM sodium EDTA, 0.3 mM thioglyoxylic acid, and 50 µg of the internal standard, N-methyl-5-hydroxytryptamine (NM5HT). The homogenate was centrifuged at 15,000 x g for 10 min, then microfiltered by centrifugation using millipore tubes. The filtrate was drawn into a six port rotary valve (Rheodyne Model No. 7125) and injected into an HPLC system (BioAnalytical Systems, Inc., West Lafayette, IN) utilizing a reverse phase column (10µm µ-Bondapak C-18, Waters Associates, Milford, MA) and a Waters Model No. M-45 pump. The mobile phase consisted of 0.15 M monochloroacetic acid, 10 mM octanylsulfonic acid and 1.0 mM EDTA. The flow rate was 1.5 ml/min. Electrochemical detection was accomplished with an LC-4 amperometer (BioAnalytical Systems). Retention times were: 5-HIAA - 24.8 min, 5-HT - 29.8 min, and NM5HT - 32.3 min.

Histology

Those animals not requiring biochemical determinations of 5-HT and 5-HIAA levels were perfused transcardially with 100 ml of saline followed by 100 ml of phosphate-buffered formalin. Their brains then were removed and post-fixed for at least one week prior to sectioning. After the tissue was well fixed, it was transferred to a solution containing 5% sucrose in 0.1 M phosphate buffer for 24 - 48 hours. The tissue then was frozen with dry ice and cut on a sliding microtome. Every fourth section (50 μm) was retained, mounted on a gelatin coated slide and stained using the cresylecht violet procedure (Powers and Clark, 1955).

Apparatus

Activity level was measured in enclosed cylindrical photocell chambers (46 cm dia x 42 cm high; Model No. PAC-001, Lehigh Valley Electronics, Inc., Beltsville, MD) with wire mesh floors. The interiors of the walls and covers of these chambers were painted flat black. Interruption by the animal of any one of six photocell beams located at the base of the chamber activated an electromechanical counter.

Statistical Analysis

The data from Experiment II were analyzed using a complex Latin square design (Bruning and Kintz, 1977, pp. 92-106). Data from Experiments III and IV were analyzed using an analysis of variance (ANOVA) with a repeated measures - two factors design (ibid, pp. 48-54). The

data from the biochemical and the remaining behavioral experiments were analyzed by an ANOVA, two-factor mixed design with repeated measures on one factor (ibid, pp. 55-61); and, a three-factor mixed design with repeated measures on two factors (Kirk, 1968; Bruning and Kintz, 1977, pp. 73-84). Individual between-group comparisons were performed, when merited, by Newman-Keuls' multiple range test (Newman, 1939; Keuls, 1952; Bruning and Kintz, 1977, pp. 119-122), or by an F-test for simple effects (ibid, pp. 140-142). For within group comparisons, a Newman-Keuls test for related measures (Keuls, 1952; Bruning and Kintz, 1977, pp. 137-138) was used. Statistical analyses were performed on a Hewlett-Packard HP-85 minicomputer with a No. 90053 statistical program package, as well as with Nos. 300-0027, 300-0029, 300-0035 and 300-0043 ANOVA programs (Hewlitt-Packard Corp., Corvallis, OR).

CHAPTER III

RESULTS

Experiment I

ACUTE INTRA-MIDBRAIN MICROINJECTIONS OF MUSCIMOL

It has been reported (Przewlocka et al., 1979) that acute injections of muscimol (50 and 100 ng) into the dorsal raphe (DR) nucleus of rats produce elevations in locomotor activity. In our first experiment, we attempted to replicate these findings. In addition, the effects of muscimol injections into the DR were compared to those following injections into the median raphe nucleus (MR).

Procedure

Rats were anesthetized with ether and injected with muscimol (100 ng in 0.5 µl of saline) or saline (0.5 µl) into either the DR (n = 7) or the MR (n = 7) as described in the Materials and Methods section (Chapter II). Four additional animals served as sham-operated controls. These animals were treated in the same manner as the injected rats, except that a needle was not lowered into their brains. Fifteen minutes post-injection, the rats, fully awake, were placed in the photocell chambers, and their activity counts recorded every 15 minutes for 90 minutes.

Results

Histological analysis

Of the 7 animals which received muscimol injections into the MR, one was eliminated from the study prior to analysis of the behavioral data because the needle tract terminated 1.5 mm laterally in the mesencephalic reticular formation. Of the remaining animals, the needle tips in 4 terminated in the rostral extent of the B-8 5-HT cell group (Dahlström and Fuxe, 1964; Steinbusch, 1981; Kulmala and Lorens, 1982) just dorsal to the rostral one-third of the interpeduncular nucleus. The needle tips in the other 2 rats were localized in the MR at the level of the ventral tegmental nucleus of Gudden (Figure 3). The needle tips of the 3 animals which received vehicle injections into the MR were similarly placed at the level of the ventral tegmental nucleus of Gudden.

Of the 7 animals which received muscimol injections into the DR, one was rejected from further analysis because the needle tip was localized within the lumen of the cerebral aqueduct. In the remaining animals, the needle tips terminated in the rostral DR at the level of the third and fourth cranial nerve nuclei (Figure 4). The animals which received vehicle injections into the DR had similar needle placements.

Figure 3: Photomicrograph of median raphe (MR) needle tip. Cresylecht violet stained coronal section (50 µm) through the caudal midbrain of a MR-muscimol injected rat from Experiment I. The needle tip (black arrow) appears as a small cavity rimmed by a glial scar, and, in this animal, terminates in the caudal portion of the B-8 5-HT cell group at the level of the VTG. Bar represents 1 mm. Abbreviations: Aq = cerebral aqueduct of Sylvius; IPN = interpeduncular nucleus; ML = median lemniscus; MLF = medial longitudinal fasciculus; VTG = ventral tegmental nucleus of Gudden.

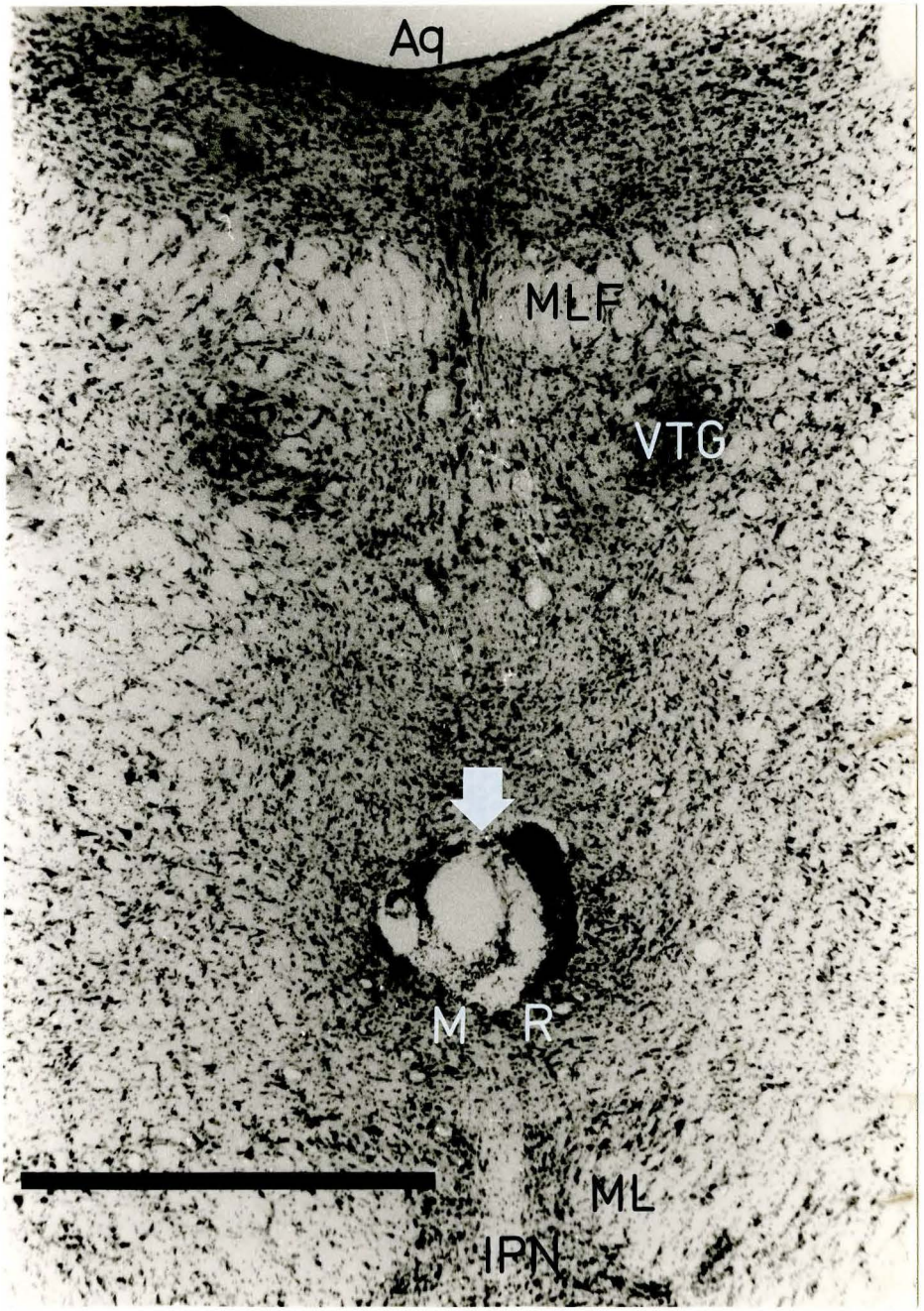
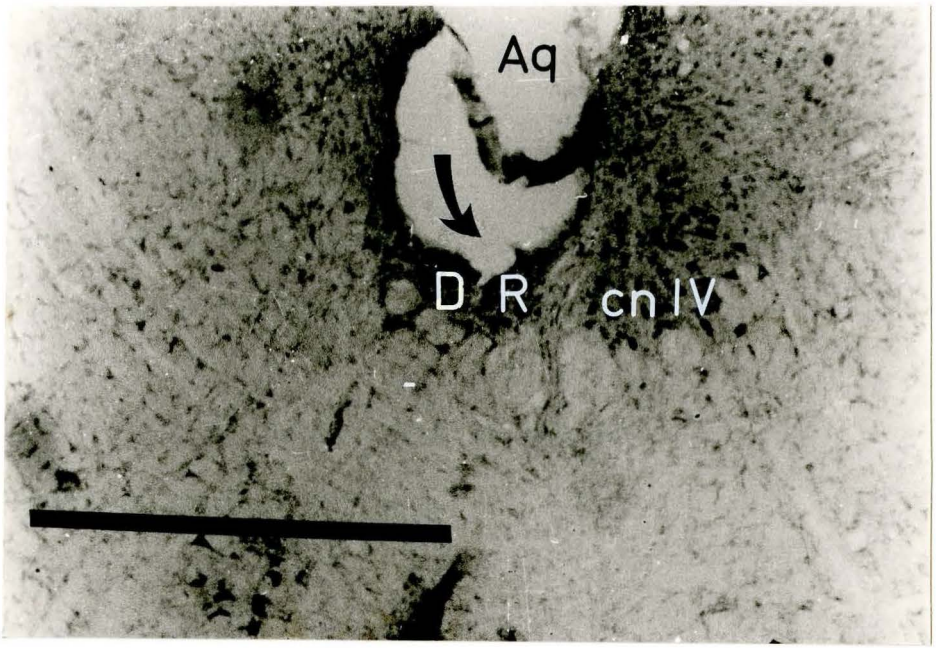

Aq
MLF
VTG
M
R
ML
IPN

Figure 4: Photomicrograph of dorsal raphe (DR) needle tip. Nissl stained coronal section (50 μm) through the midbrain of a DR-muscimol injected rat from Experiment I. The needle tip (black arrow) appears as a small cavity rimmed by a glial reaction, and, in this animal, terminates in the rostral part of the B-7 5-HT cell group at the level of cn IV. Bar represents 1 mm. Abbreviations: Aq = cerebral aqueduct of Sylvius; cn IV = trochlear nucleus.


Aq
D R
cn IV

Activity level

An ANOVA of the number of counts per 15 minutes failed to demonstrate any significant differences between the 4 sham-operated, the 3 MR-vehicle injected, and the 3 DR-vehicle injected animals. These groups, therefore, were combined into a single control group. Subsequently an ANOVA revealed significant treatment [$F(2,20)=23.3$, $p<0.001$], time [$F(14,140)=13.4$, $p<0.001$], and interaction [$F(14,140)=7.6$, $p<0.001$] effects between the control and muscimol injected groups. The cumulative activity scores per 15 minutes for each group are shown in Figure 5.

Between-group comparisons of the total 90 minute activity scores showed that both the MR- and DR-muscimol injected groups were significantly more active than the control group. The MR-muscimol injected group, furthermore, was significantly more active than the DR-muscimol injected group.

Individual group comparisons of the activity scores for each 15 minute period (Figure 5) revealed that the MR-muscimol injected group was significantly more active during the first 15 minutes in the apparatus and throughout the remainder of the 90 minute test session. The activity level of the DR-muscimol injected group did not differ from control during the first 15 minute segment, but was significantly higher than control for the remainder of the test session. The hyperactivity induced by muscimol injections into the MR, thus, was more rapid in onset and of a greater magnitude, than that produced by the DR-muscimol injections. In fact, at the end of the 90 minute session, the cumula-

tive activity scores of the MR-muscimol injected group was approximately four times greater than that of the DR-muscimol injected group.

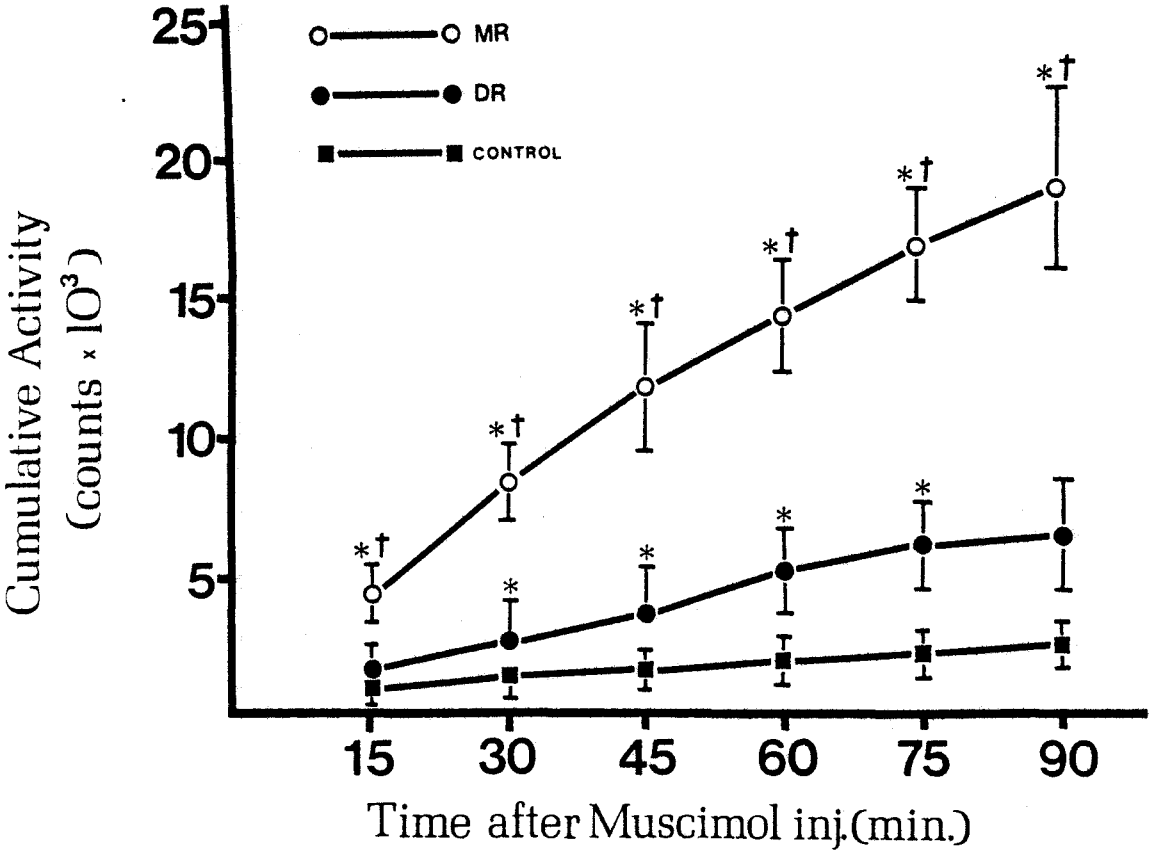
Conclusion

In this first experiment we were able to confirm the report of Przewłocka et al. (1979) that muscimol injections into the DR produce hyperactivity. In addition, we found that muscimol injections into the MR also produced a hyperkinetic effect, and, that this effect not only appears earlier after injection, but is of a much greater magnitude.

Figure 5: Activity scores after acute muscimol injection. Group (Mean +/- S.E.M.) cumulative activity scores in rats following acute microinjection of vehicle (control, n = 10) or muscimol into the dorsal (DR, n = 6) or median (MR, n = 6) raphe nucleus.

* significantly different from control group ($p<0.01$);

† significantly different from DR-muscimol injected group ($p<0.01$, Newman-Keuls' multiple range test).


MR
DR
CONTROL
25
20
15
10
5
Cumulative Activity
(counts × 10³)
15
30
45
60
75
90
Time after Muscimol inj.(min.)

Experiment II

MUSCIMOL DOSE-RESPONSE RELATIONSHIP IN ANIMALS WITH CHRONICALLY INDWELLING CANNULAE

In order to rule out the possible interactions of the ether anesthesia and the acute surgical trauma with the effects of intra-midbrain muscimol injections on locomotor activity, we performed a dose-response analysis using animals having cannulae chronically implanted in the DR or the MR. Furthermore, since each animal served as its own control and received each drug dose, a complex Latin square design was employed in order to determine whether a given dose schedule produced effects significantly different from another.

Procedure

Beginning 1 week post-operatively, the animals were adapted to the test apparatus and injection procedure (Jacquet, 1975) for 3 consecutive days (Wednesday - Friday). The subjects were placed in the photocell chambers for 30 minutes, removed and wrapped in a towel for 30 - 60 seconds, then returned to the chambers for an additional 2 hours. During the subsequent three weeks, the animals were tested Monday through Friday, muscimol or vehicle injections being performed on Tuesdays and Thursdays. The animals were placed in the apparatus for 30 minutes and their activity scores recorded for each 15 minute segment. The rats then were removed, wrapped in a towel, and injected (on drug days only) over 30 seconds with 0.5 µl of drug solution. The animals then were placed back in the chambers for an additional 2 hours. Activity counts

were recorded 15, 30, 60, 90 and 120 minutes post-injection. Muscimol (0, 25, 50, 100, 200 and 400 ng in 0.5 µl saline) was injected into the MR or the DR according to a complex Latin square design (Bruning and Kintz, 1977, pp. 92 - 106), with at least 10 animals receiving each of the 6 dose sequences.

Results

Histological analysis

Prior to performing any statistical analyses of the behavioral data, we screened the cannula placements. Of the 60 animals implanted with MR cannulae, 30 were found acceptable. The cannulae in these animals terminated within the B-8 5-HT cell group coinciding with the caudal linear and median raphe nuclei, just dorsal to the interpeduncular nucleus. The histological appearance of the cannula placements in these animals was similar to the needle tracts obtained in Experiment I (see Figure 3). The cannula tips of the rats rejected from the study terminated at least 1.5 mm lateral, dorsal, and/or caudal to the B-8 5-HT perikarya.

Of the 72 rats which were implanted with DR cannulae, only 30 had acceptable placements. The cannula tips in these animals all were localized within the boundaries of the DR (B-7 5-HT cell group). The cannulae in the animals eliminated from the study terminated at least 0.75 mm dorsal and/or lateral to the DR. The histological appearance of the DR cannula tracts was indistinguishable from that of the DR needle tracts obtained in Experiment I (see Figure 4).

Activity level

An ANOVA of the activity scores per 15 minutes for the 30 minute pre-drug session on each of the 6 drug days showed that there were no significant cannula placement, days, or interaction effects. There was a significant time effect [$F(1,58)=63.20$, $p<0.001$] due to the greater activity of the animals during the first 15 minutes of the 30 minute pre-drug period. These results show that the animals' activity levels were comparable during the pre-drug sessions on each of the 6 drug days. Subsequently, a complex Latin square analysis of the total 120 minute post-injection activity scores was performed. This analysis revealed a significant overall effect of placement site [$F(1,48)=5.04$, $p<0.05$], a significant overall effect of the muscimol dose [$F(5,240)=29.08$, $p<0.0001$], and a significant interaction between dose and injection site [$F(5,240)=22.21$, $p<0.0001$]. Importantly, the effects of order of treatment were not significant.

As shown in Figure 6, injections of muscimol (50 - 400 ng) into the MR produced significant increases in activity. The peak effect was seen after the 100 ng dose. In contrast, only the two highest doses of muscimol (200 and 400 ng) induced hyperactivity following injection into the DR. The temporal effects of intraraphe muscimol injections are shown in Figure 7. For clarity, only the data are presented which were obtained following the saline injections and following the doses of muscimol which produced optimal effects (Figure 6) after injection into either the MR (100 ng) and DR (200 ng). The muscimol-induced hyper-

Figure 6: Dose-activity effects of intra-raphe muscimol. Total activity scores (Mean +/- S.E.M.) for the 2 hour period following muscimol injections via chronically indwelling median (MR, n = 30) or dorsal (DR, n = 30) raphe cannulae. The muscimol was administered according to a Latin square design with 5 animals receiving each of the dose sequences.

* significantly higher than control condition ($p<0.01$, Newman-Keul's multiple range test);

† significantly higher than DR-injected group ($p<0.0001$),

significantly higher than MR-injected group ($p<0.0001$, F-test for simple effects).

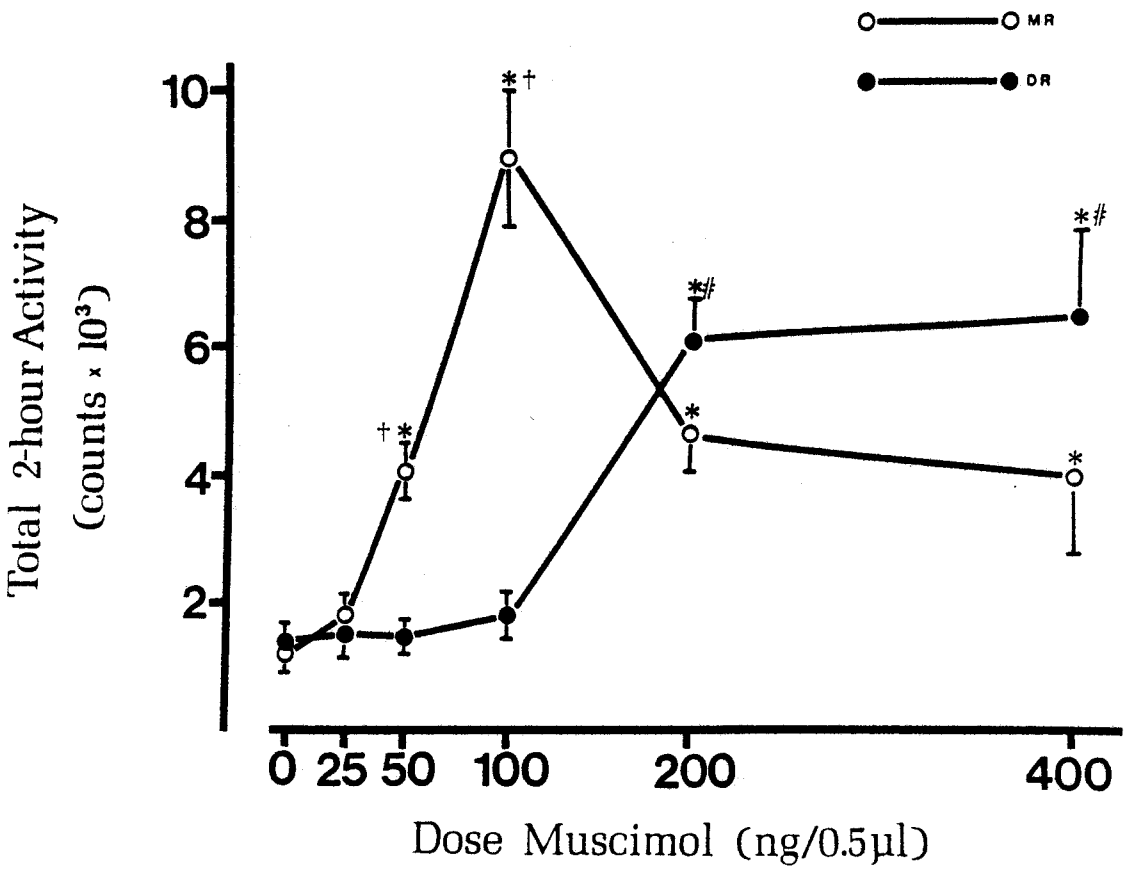
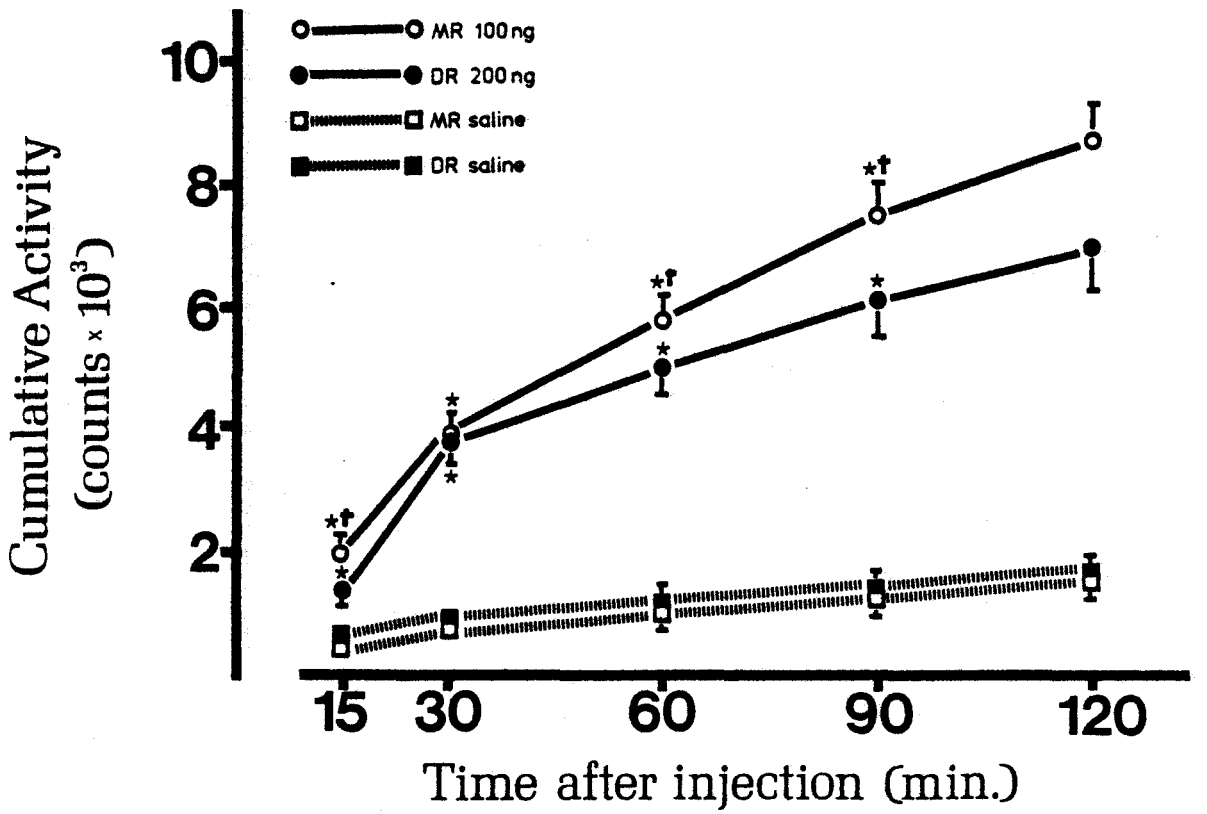

MR
DR
10
8
6
4
2
Total 2-hour Activity
(counts x 10³)
0 25 50 100 200 400
Dose Muscimol (ng/0.5µl)

Figure 7: Temporal effects of intra-raphe muscimol injections.

Cumulative activity scores (Mean +/-S.E.M.) 15, 30, 60, 90 and 120 minutes following the injection of opitmal doses of muscimol through cannulae chronically implanted in the median (MR - 100 ng, n = 30) or the dorsal (DR - 200 ng, n = 30) raphe nucleus. The MR- and DR-saline scores were those obtained following saline (0.5) µl injections.

* significantly elevated over corresponding saline-injected control condition ($p<0.01$, Newman-Keuls' test for related measures);

† significantly elevated over DR-muscimol (200 ng) injected condition ($p<0.05$, Newman-Keuls' multiple range test).


MR 100 ng
DR 200 ng
MR saline
DR saline
Cumulative Activity
(counts x 10³)
10
8
6
4
2
15
30
60
90
120
Time after injection (min.)

activity was most prominent during the first 30 minutes post-injection, and diminished in magnitude over the subsequent 90 minutes.

Conclusion

These observations indicate that injection of muscimol into the MR produces a significant elevation in activity level at a dose (50 ng) one-fourth that required to produce a similar effect when injected into the DR. The magnitude of the hyperkinetic effect following the injection of an optimal (100 ng) dose of muscimol into the MR, moreover, is at least 1.5 times greater than after such an injection of an optimal dose (200 - 400 ng) into the DR. These results suggest the possibility that the hyperkinetic effect of muscimol following injection into the DR may be due to diffusion of the drug to an effective MR site.

Inasmuch as the MR proved to be more sensitive to muscimol than the DR, we decided to concentrate our subsequent efforts on the MR site. Furthermore, since we found in Experiment II that the order of administration of the drug treatments did not significantly affect the experimental outcome, we also decided to employ the more cost-effective randomized block designs (Kirk, 1968) in our subsequent experiments.

Experiment III

INTERACTIONS OF PERIPHERAL BICUCULLINE AND CHLORDIAZEPOXIDE WITH INTRA-RAPHE INJECTIONS OF MUSCIMOL

In the previous two experiments it was shown that intra-raphe injections of muscimol produce hyperactivity as measured in photocell chambers. If the hyperkinetic effect of muscimol is due to the activation of GABA receptors, then this effect should be attenuated by the peripheral administration of the GABA receptor antagonist, bicuculline, and potentiated by the facilitator of GABA-ergic neurotransmission, chlordiazepoxide. It was the objective of the present experiment to test these hypotheses.

In a preliminary study (Sainati and Lorens, 1981) we determined that chlordiazepoxide, when administered intraperitoneally in doses greater than 3.8 mg/kg produced decreases in locomotor activity as measured in dark photocell chambers. Following these doses, the animals appeared ataxic and sedated. Doses ranging between 1.2 - 3.8 mg/kg did not affect activity as compared to control. We also found that the intraperitoneal injection of bicuculline in doses ranging between 0.1 - 1.1 mg/kg had no effect on activity level. Higher doses regularly produced seizures, the convulsant dose for 50 percent of the cases (ED-50) being 2.2 mg/kg. In the following experiment, therefore, the 3.8 mg/kg dose of chlordiazepoxide, and the 1.1 mg/kg dose of bicuculline were used.

Procedure

Cannulae were implanted in the MR of 18 animals as described in the Materials and Methods section (Chapter II). Groups of 9 rats each were assigned to the studies of the chlordiazepoxide-muscimol interaction, and of the bicuculline-muscimol interaction. The testing procedure was identical to that described in Experiment II, except that the activity counts were recorded every 15 minutes for only one hour post-injection.

Fifteen minutes prior to receiving muscimol (0, 25, or 50 ng) injections into the MR, one group of rats received either saline (1.0 ml/kg) or chlordiazepoxide (3.8 mg/kg) intraperitoneally. Fifteen minutes prior to receiving intra-raphe muscimol (0, 50, or 100 ng), the other group of rats received either the acidified vehicle (1.0 ml/kg) or (+)-bicuculline (1.1 mg/kg) intraperitoneally.

A repeated measures - two factors ANOVA design was used in the statistical analysis of the behavioral data. This is an analysis which can be used when all treatments in a two-factor experiment are administered to each subject (Bruning and Kintz, 1977, pp. 48-54). The effects of each treatment can be discerned individually, and the interaction between the two can be ascertained. Because no statistical test for simple effects exists specifically for the repeated measures - two factors ANOVA design, individual comparisons between means were made with Studnet's t-test for related measures (Student, 1927; Bruning and Kintz, 1977, pp. 13-16).

Results

Histological analysis

Two rats were eliminated from the chlordiazepoxide-muscimol study and one rat was eliminated from the bicuculline-muscimol study prior to statistical analysis of the behavioral data, since the cannula placements in these animals were localized 1.5 mm lateral to the MR. The cannula tips in the accepted animals terminated in the B-8 5-HT cell group dorsal to the interpeduncular nucleus.

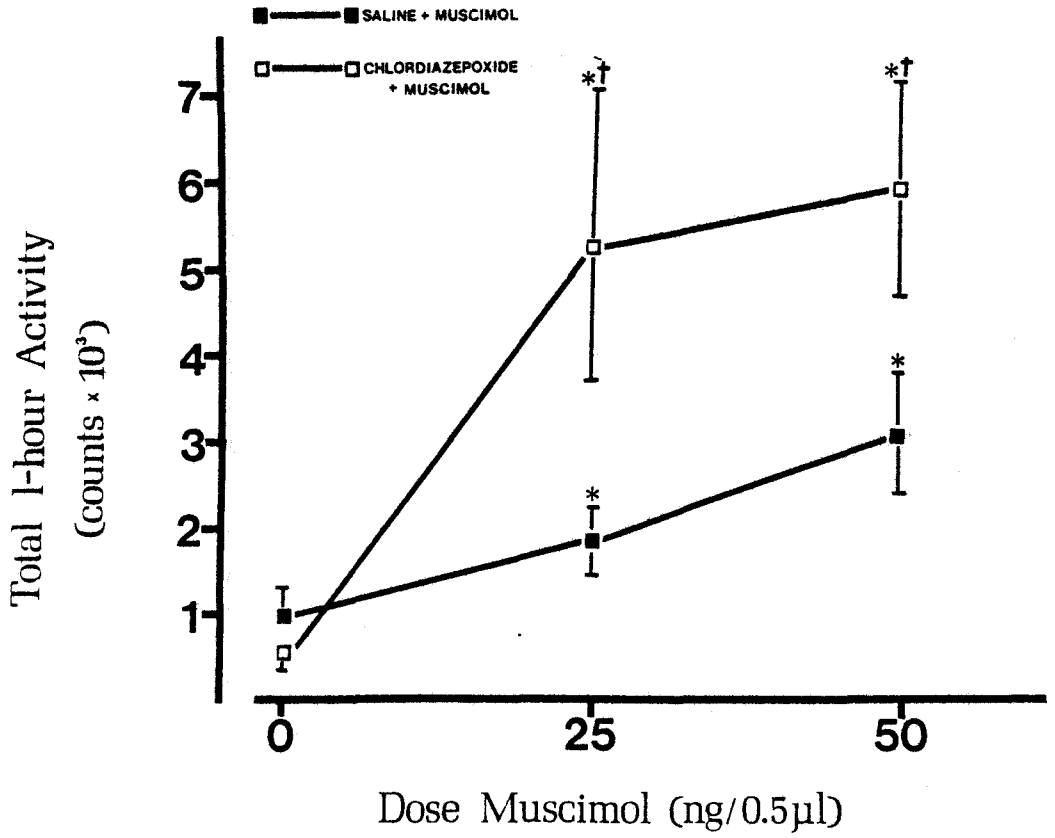
Locomotor activity

Chlordiazepoxide-Muscimol Interaction. An ANOVA of the total 60 minute post-injection activity scores failed to demonstrate a significant effect of chlordiazepoxide alone. The effect of muscimol, however, was significant [$F(2,16)=7.37$, $p<0.01$], as was the muscimol - chlordiazepoxide interaction [$F(2,16)=6.30$, $p<0.01$]. Thus, as shown in Figure 8, the parenteral injection of chlordiazepoxide potentiated the effect of intra-raphe muscimol (25 and 50 ng) on locomotor activity.

Figure 8: Chlordiazepoxide potentiates the muscimol effect. Total activity scores (Mean +/- S.E.M.) for the one hour period following the injection of muscimol (0, 25 or 50 ng) into the median raphe nucleus (n = 7). Fifteen minutes prior to receiving muscimol, the animals received either chlordiazepoxide (3.8 mg/kg) or saline (1.0 ml/kg) intraperitoneally.

* significantly greater than the corresponding control condition ($p<0.01$);

† significantly higher than the corresponding saline plus muscimol effect ($p<0.05$, Students t-test, 2-tailed).


SALINE + MUSCIMOL
CHLORDIAZEPOXIDE + MUSCIMOL
Total 1-hour Activity (counts × 10³)
7
6
5
4
3
2
1
0
25
50
Dose Muscimol (ng/0.5µl)

Bicuculline-Muscimol Interaction. An ANOVA of the total 60-minute post-injection activity scores failed to show a significant effect of bicuculline by itself. However, the effect of muscimol was significant [$F(2,16)=8.64$, $p<0.005$], as was the bicuculline - muscimol interaction [$F(2,16)=6.69$, $p<0.05$]. Figure 9 depicts the blockade of the hyperkinetic effect of intra-raphe muscimol (50 and 100 ng) by the peripheral administration of bicuculline.

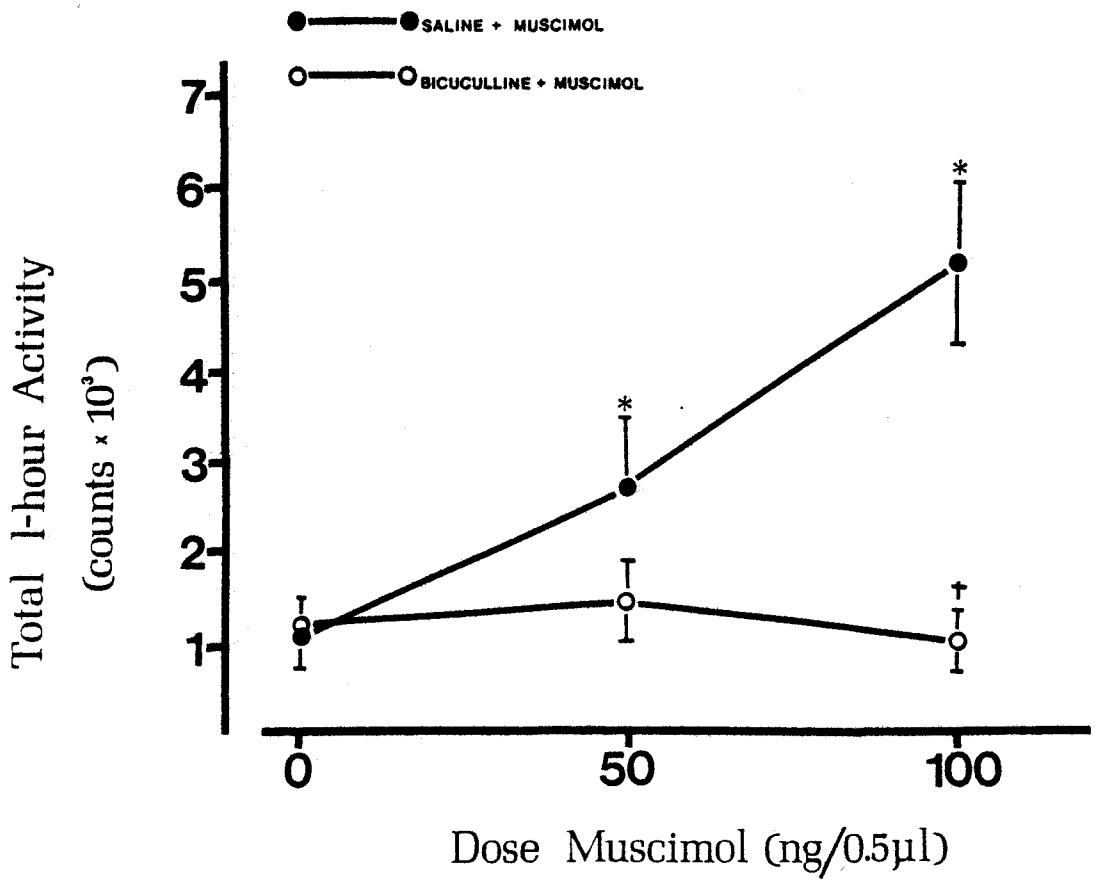
Conclusion

These results support the view that the hyperkinetic effect of intra-raphe muscimol injection is due to an activation of GABA receptors.

Figure 9: Bicuculline blocks the muscimol effect. Total activity scores (Mean +/- S.E.M.) for the one hour period following injection of muscimol (0, 50, or 100 ng) into the median raphe nucleus (n = 8). Fifteen minutes prior to receiving muscimol the animals received either bicuculline (1.1 mg/kg) or acidified vehicle (1.0 ml/kg) intraperitoneally.

* significantly higher than corresponding vehicle-injected control condition ($p<0.01$);

† significantly lower than corresponding vehicle plus muscimol effect ($p<0.002$, Student's t-test, 2-tailed).


SALINE + MUSCIMOL
BICUCULLINE + MUSCIMOL
Total 1-hour Activity
(counts x 10³)
7
6
5
4
3
2
1
0
50
100
Dose Muscimol (ng/0.5µl)
*
*
†

Experiment IV

In Experiment III it was shown that the peripheral injection of chlordiazepoxide potentiated, whereas the systemic administration of bicuculline blocked, the hyperactive effect of intra-raphe muscimol. These data support the view that the hyperkinetic effect of intra-raphe muscimol is due to the activation of GABA receptors. However, since GABA and benzodiazepine receptors are localized throughout the neuraxis, we cannot be certain that the effects of bicuculline and chlordiazepoxide are due to their binding allosterically to the same GABA receptors as those occupied following the intra-raphe injection of muscimol. In order to determine whether the effects of peripherally-administered bicuculline and chlordiazepoxide are due to their interaction with muscimol at the same receptor sites, we performed the following series of experiments: a) an intra-raphe benzodiazepine dose-response analysis; b) an intra-raphe bicuculline dose-response analysis; c) a test of the interactions between intra-raphe benzodiazepines and muscimol; and, d) a test of the interactions between intra-raphe bicuculline methiodide and muscimol.

IVa:

INTRA-RAPHE BENZODIAZEPINE DOSE-RESPONSE ANALYSIS

There is a substantial amount of evidence which indicates that GABA serves as a neurotransmitter in both the DR (Gallager, 1978; Nanopoulos *et al.*, 1980) and the MR (Forchetti and Meek, 1981). The benzodiazepines, moreover, are thought to act by facilitating GABA-ergic neurotransmission (Gallager, 1978; Costa and Guidotti, 1979). We hypothesized, therefore, that intra-raphe injections of benzodiazepines also should produce hyperactivity by facilitating the effects of endogenously released GABA.

Procedure

Three water soluble benzodiazepines (chlordiazepoxide, midazolam and flurazepam) were chosen for intra-raphe dose-response analysis. Cannulae were implanted in the MR of 18 rats as described in the Materials and Methods section (Chapter II). Six of the rats were assigned to the chlordiazepoxide group, six to the midazolam group, and six to the flurazepam group. The subjects were tested using the same procedure as in Experiment II. Activity counts were recorded 15 and 30 minutes before and 15, 30, 60, 90 and 120 minutes after injection. On drug days, animals received saline or a dose of the appropriate benzodiazepine (0, 0.22, 0.44, 0.88 or 1.75 nmole). A three-factor split-plot ANOVA with repeated measures on two factors (Kirk, 1968; Bruning and Kintz, 1977, pp. 73-88) was used to analyze the results of this experiment.

Results

Histological analysis

The behavioral data from 2 rats (one from the chlordiazepoxide group and one from the midazolam group) were eliminated prior to statistical analysis, since the cannula tips in these animals were localized 1.0 - 2.0 mm lateral to the MR. The cannula tips in the accepted animals terminated in the rostral portion of the B-8 5-HT cell group, dorsal to the interpeduncular nucleus.

Locomotor activity

An ANOVA of the behavioral data demonstrated differences in the response to the three drugs [$F(2,15)= 20.25$, $p<0.0002$]. The effects of the different drug doses also were significant [$F(5,75)=13.79$, $p<0.0001$]. The subjects' performances changed as a function of time after injection. [$F(3,45)=668.26$, $p<0.00001$]. In addition, the drug and dose [$F(10,75)=4.08$, $p<0.0005$], the drug and time [$F(6,45)=31.19$, $p<0.0001$], the dose and time [$F(15,225)=7.64$, $p<0.0001$], and the drug, dose and time [$F(30,225)=4.01$, $p<0.0001$] interactions all were significant. Individual comparisons demonstrated that injections of flurazepam and midazolam into the MR produced a dose-dependent elevation in locomotor activity (Figure 10). Flurazepam was twice as potent as midazolam, the former having a peak effect at 0.44 nmole, the latter at 0.88 nmole. Chlordiazepoxide was without effect. As shown in Figure 11, the benzodiazepine-induced hyperactivity was most prominent during the first 30 minutes post-injection.

Figure 10: Intra-raphe benzodiazepine dose-response relationship.

Total activity scores (Mean +/- S.E.M.) for the 2 hour period following injection of 0, 0.22, 0.44, 0.88 or 1.75 nmole of flurazepam (FLU; n = 6), midazolam (MID; n = 5) or chlordiazepoxide (CDP; n = 5) through cannulae chronically implanted in the median raphe (MR) nucleus.

Asterisks indicate significant elevation over the corresponding saline-injected control (dose = 0) condition (*$p<0.05$, **$p<0.01$, Newman-Keuls' multiple range test).

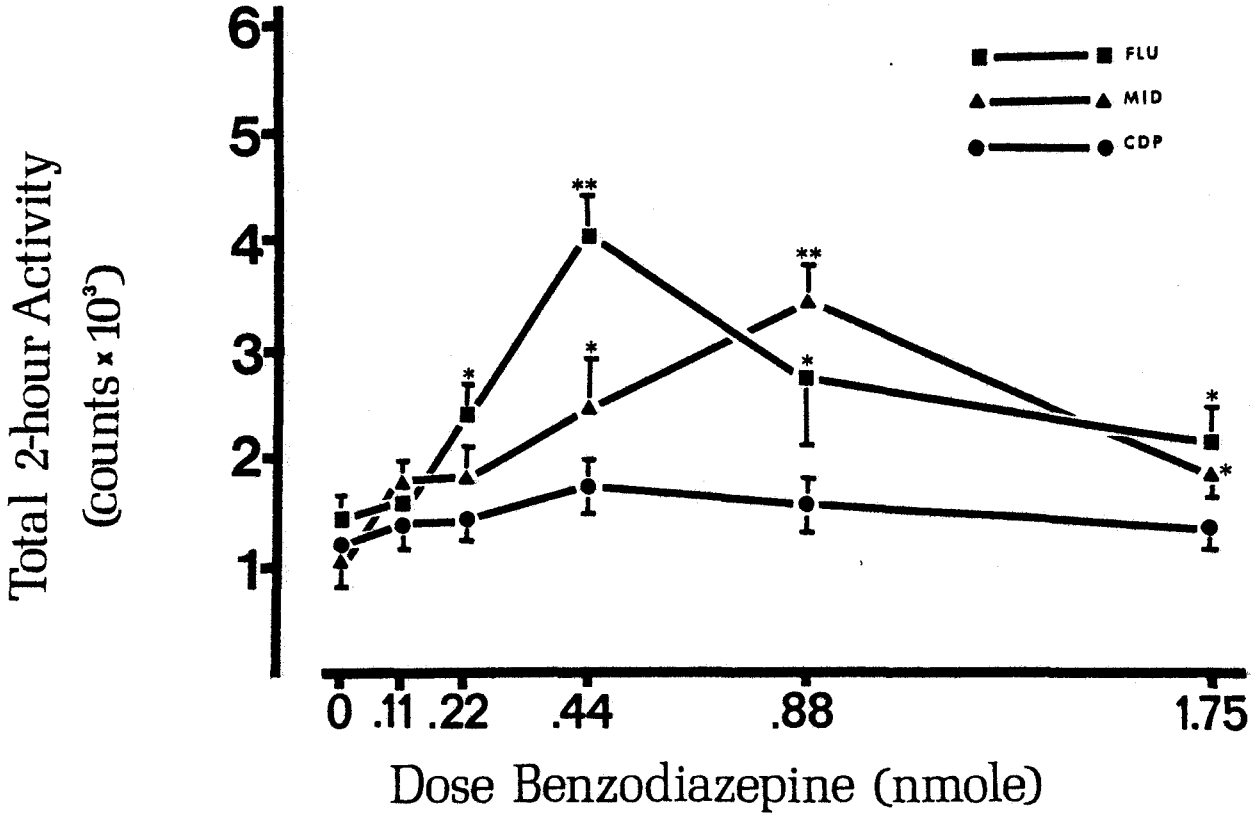
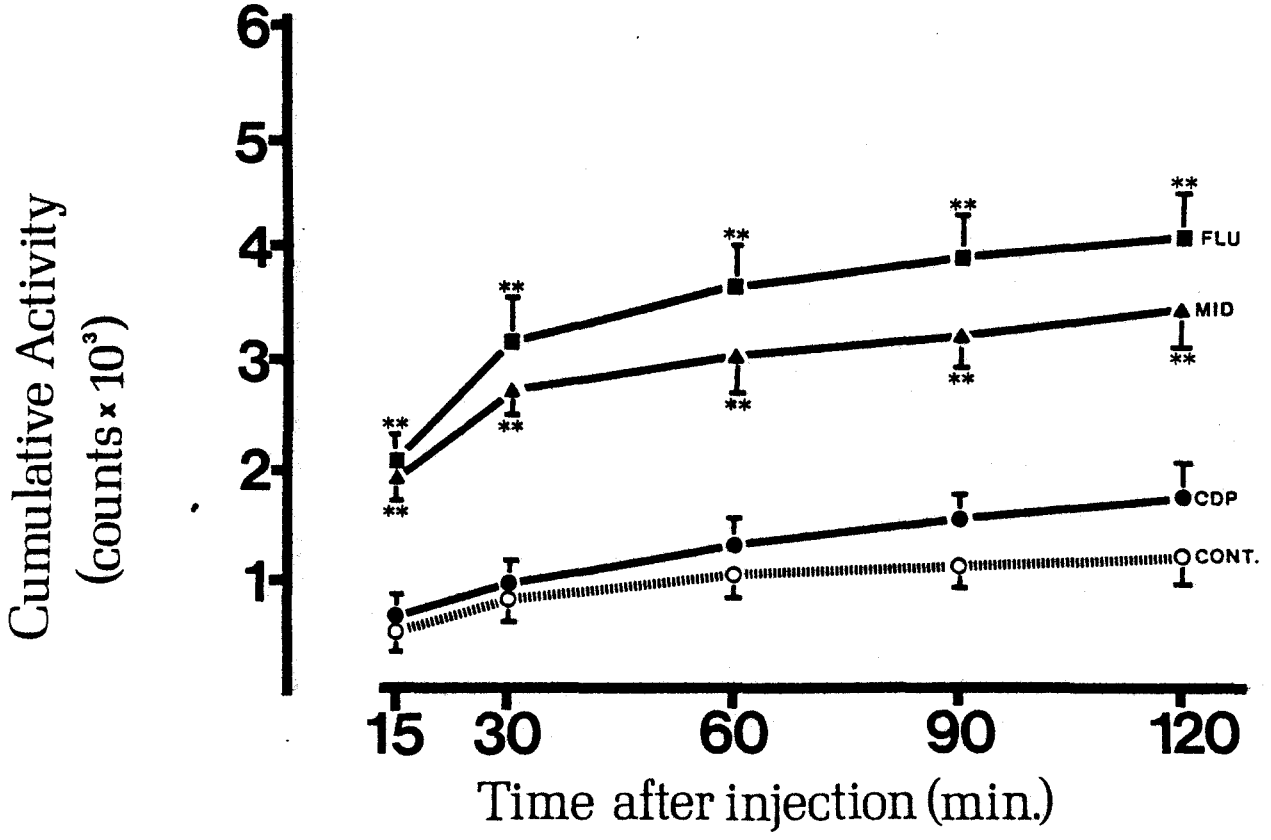

Total 2-hour Activity
(counts x 10³)
6
5
4
3
2
1
FLU
MID
CDP
0
.11
.22
.44
.88
1.75
Dose Benzodiazepine (nmole)

Figure 11: Temporal effects of intra-raphe benzodiazepines. Cumulative activity scores (Mean +/- S.E.M.) 15, 30, 60, 90 and 120 minutes following the injection of optimal doses of flurazepam (FLU, 0.44 nmole); midazolam (MID, 0.88 nmole); or chlordiazepoxide (CDP, 0.44 nmole) through cannulae chronically implanted in the median raphe nucleus. For the sake of clarity, the post-saline control (CONT.) scores for the 3 groups have been combined.

** significantly elevated over the corresponding saline-injected control condition ($p<0.01$, Newman-Keuls' test for related measures).


Cumulative Activity
(counts x 10³)
6
5
4
3
2
1
FLU
MID
CDP
CONT.
**
15
30
60
90
120
Time after injection (min.)

Conclusion

Facilitation of midbrain GABA neurotransmission by intra-MR injections of the benzodiazepines, flurazepam and midazolam, led to an increase in locomotor activity. Although qualitatively similar to the effects of intra-MR muscimol, the magnitude and duration of the benzodiazepine effects were somewhat less. This is not surprising, however, since the effects of the benzodiazepines depend on the release of endogenous GABA, whereas muscimol, as a receptor agonist, does not. The effective pharmacological doses of muscimol no doubt produce a greater concentration of receptor ligand than does the amount of GABA normally available for release.

Interestingly, in contrast to midazolam and flurazepam, chlordiazepoxide failed to alter activity level following its injection into the MR. This observation supports the view that chlordiazepoxide is a prodrug, similar to prazepam and chlorazepate, which must be oxidized *in vivo* to produce a centrally active metabolite (Johnson and Rising, 1980; Breimer *et al.*, 1980). Recent autoradiographic studies in our laboratory (Sainati *et al.*, 1982) support this conclusion. Briefly, we found that both midazolam and flurazepam displaced the binding of ^{3}H-flunitrazepam from brain stem tissue sections *in vitro*. Chlordiazepoxide, however, failed to do so. It would appear, therefore, that chlordiazepoxide itself is not active at CNS benzodiazepine receptor sites.

IVb:

INTRA-RAPHE BICUCULLINE DOSE-RESPONSE ANALYSIS

Procedure

Because the free base form of bicuculline must be dissolved in a vehicle too acidic for direct intracranial injections, this experiment was performed with the water-soluble salt, bicuculline methiodide. Seven rats were outfitted with chronically indwelling cannulae in the MR. Animals were tested as described in the procedure for Experiment II. On drug days, subjects received intracranial injections of either saline or bicuculline methiodide (0.22, 0.44 or 0.88 nmole; 81, 161 or 323 ng as the base) according to a randomized block design.

Results

Histological analysis

Of the 7 rats tested, only 5 were found to have acceptable cannula placements. The cannula tract and tip locations in these rats were indistinguishable from those in the previous experiments, as they terminated within the B-8 5-HT cell group dorsal to the interpeduncular nucleus.

Locomotor activity

A two-factor split-plot ANOVA with repeated measures on one factor (Kirk, 1968; Bruning and Kintz, 1977, pp. 55-61) failed to reveal a significant effect of bicuculline methiodide on locomotor activity. Only

the time after injection effect was significant [$F(4,96)=22.24$, $p<0.0001$]. Seizures were not observed in any of the animals following the administration of the bicuculline doses used.

Conclusion

If direct activation of GABA receptors (as produced by muscimol) or facilitation of GABA-ergic transmission (such as produced benzodiazepines) in the MR results in hyperactivity, then blockade of GABA receptors should have the opposite effect. The data obtained in the present experiment, however, do not support this view. Intra-MR muscimol may inhibit the firing rates of local 5-HT neurons. According to the present hypothesis, the resultant decrease in neurotransmitter release from MR 5-HT efferent nerve terminals would result in the observed hyperkinesis. Blockade of the inhibitory effects of endogenously released GABA at the same receptor sites (that is, on serotoninergic neurons), nevertheless, may not result in the opposite condition: although it is possible that MR 5-HT perikarya may increase their rate of discharge immediately following their release from a tonic GABA-ergic inhibition, this enhancement most likely would be followed by an almost immediate return (within a fraction of a second) to the baseline due to collateral feedback inhibition (Aghajanian and Wang, 1978). Any transient increase in 5-HT release from MR efferent nerve terminals would, thus, not be of long enough duration to bring about a hypokinetic response.

IVc:

INTERACTIONS OF INTRA-RAPHE BENZODIAZEPINES WITH MUSCIMOL

Procedure

Cannulae were implanted in the MR nuclei of 14 rats as described in the Materials and Methods section (Chapter II) Seven rats were assigned to the flurazepam - muscimol group and 7 to the midazolam - muscimol group. In this experiment, animals received either saline vehicle, or a sub-effective dose (0.22 nmole) of flurazepam or midazolam into the MR 5 minutes prior to the administration either of saline (0.5 µl) or of a sub-effective dose of muscimol (0.22 nmole). Otherwise, the procedure was the same as that in Experiment II.

A repeated measures - two factors ANOVA design was used for the statistical analysis of the behavioral data (Bruning and Kintz, 1977, pp. 48-54).

Results

Histological analysis

The behavioral data from 3 rats were eliminated prior to statistical analysis, since the cannula placements in these animals were localized 1.5 - 2.5 mm lateral to the MR. The cannula tips terminated in the B-8 5-HT cell group dorsal to the interpeduncular nucleus in the 5 rats comprising the flurazepam - muscimol group, and in the 6 rats forming the midazolam - muscimol group.

Locomotor activity

Flurazepam-muscimol interaction. An ANOVA of the total 2 hour post-injection activity scores demonstrated significant effects both of muscimol [$F(1,26)=187.73$, $p<0.0001$] and of flurazepam [$F(1,26)= 173.38$, $p<0.0001$]. The effects of flurazepam and muscimol, moreover, interacted [$F(1,26)=161.00$, $p<0.0001$]. Individual comparisons, however, showed that only the combined injection of flurazepam and muscimol produced significant elevations over the vehicle-injected control condition (Table 1). This observation accounts for the significant effects of muscimol and flurazepam mentioned above.

Midazolam-muscimol interaction. An ANOVA of the total 2 hour post-injection activity scores demonstrated significant effects both of muscimol [$F(1,27)= 168.06$, $p<0.0001$] and of midazolam [$F(1,27)=137.64$, $p<0.0001$]. Similar to the results above, the effects of midazolam and muscimol interacted [$F(1,27)=165.76$, $p<0.0001$]. Individual comparisons showed that only the sequential injections of midazolam and muscimol together produced activity scores significantly higher than those after vehicle injections (Table 1). This observation accounts for the significant effects of muscimol and midazolam reported above.

TABLE 1

Interaction of intra-raphe benzodiazepines with muscimol.

Total activity scores (Mean +/- S.E.M.) following injection of muscimol (0.22 nmole) or saline (0.5 µl) through cannulae chronically indwelling in the median raphe nucleus. Five minutes prior to receiving muscimol, the animals were injected with flurazepam (0.22 nmole; n = 5), midazolam (0.22 nmole; n = 6) or saline (0.5 µl).

TOTAL 2-HOUR POST-INJECTION ACTIVITY SCORES

DOSE BENZODIAZEPINE	DOSE MUSCIMOL	
	VEHICLE	0.22 NMOLE
FLURAZEPAM VEHICLE	1848 ± 175	2251 ± 366
FLURAZEPAM 0.22 NMOLE	2078 ± 174	5769 ± 392*
MIDAZOLAM VEHICLE	1200 ± 182	1201 ± 165
MIDAZOLAM 0.22 NMOLE	1006 ± 95	5530 ± 126†

* Significantly different from muscimol vehicle plus flurazepam vehicle condition ($p<0.001$);

† Significantly different from muscimol vehicle plus midazolam vehicle condition ($p<0.001$, Student's *t*-test, 2-tailed).

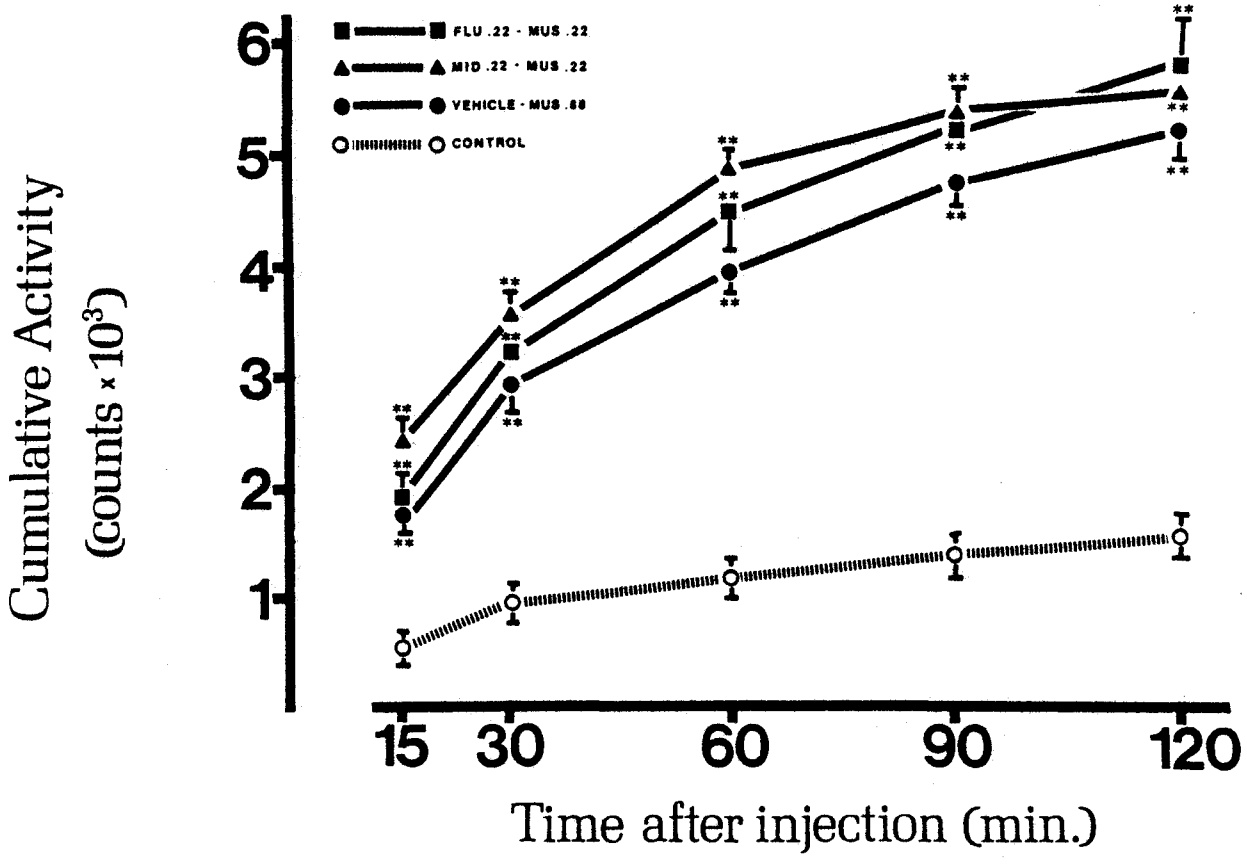
Conclusion

Our previous dose-response analysis (Experiment II) had demonstrated that muscimol in a dose of 0.22 nmole (25 ng) did not produce significant elevations in activity level. The 0.22 nmole dose of either flurazepam and midazolam also was below that required to induce hyperactivity (Experiment IVa). However, when the subeffective dose (0.22 nmole) of muscimol was injected immediately following the intra-raphe administration of either benzodiazepine, a hyperkinetic effect was produced which was of the same magnitude as that of a four-fold higher dose (0.88 nmole) of muscimol alone, as illustrated in Figure 12. These observations support the conclusion that the benzodiazepines act by facilitating the post-synpatic effects of endogenously released GABA and of GABA agonists.

Figure 12: Interaction of intra-raphe benzodiazepines with muscimol

Cumulative activity scores (Mean +/- S.E.M.) 15, 30, 60, 90 and 120 minutes following injection of muscimol (0.22 nmole) or saline (0.5 µl) through cannulae chronically indwelling in the median raphe nucleus. Five minutes prior to receiving muscimol, the animals were injected with flurazepam (0.22 nmole; n = 5), midazolam (0.22 nmole; n = 6) or saline (0.5 µl). The vehicle-muscimol 0.88 nmole data were obtained in Experiment IVd, which was conducted at the same time as the present experiment. The control means (+/- S.E.M.) were obtained in the same manner as reported in Figure 11.

** significantly elevated over the corresponding saline-injected control condition ($p<0.01$, Newman-Keuls' test for related measures).


Cumulative Activity
(counts x 10³)
FLU .22 - MUS .22
MID .22 - MUS .22
VEHICLE - MUS .88
CONTROL
1
2
3
4
5
6
15
30
60
90
120
Time after injection (min.)

IVd:

INTERACTIONS OF INTRA-RAPHE BICUCULLINE METHIODIDE AND MUSCIMOL

Procedure

Cannulae were implanted in the MR nuclei of 7 rats as described in the Materials and Methods section (Chapter II). In this experiment, animals received either saline vehicle or bicuculline methiodide (0.88 nmole; 323 ng) into the MR 5 minutes prior to either muscimol (0.88 nmole; 100 ng) or saline injection. The procedure otherwise was identical to that employed Experiment II.

Results

Histological analysis

Of the 7 rats studied, 6 were found to have acceptable cannula placements in the rostral MR.

Locomotor activity

An ANOVA of the total 2 hour post-injection activity scores demonstrated that the effect of muscimol was significant [$F(1,27)=814.13$, $p<0.0001$], whereas that of bicuculline methiodide alone was not. Furthermore, the effects of bicuculline methiodide and muscimol interacted [$F(1,27)=238.87$, $p<0.0001$]. As shown in Table 2, intra-MR bicuculline methiodide completely blocked the hyperactivity produced by a subsequent injection into the MR of an equimolar dose of muscimol.

Conclusion

The results from Experiments IV a - d suggest that the effects of peripheral administration of benzodiazepines and bicuculline on intraraphe muscimol-induced hyperactivity probably are due to their interactions at the same GABA receptors within the midbrain.

TABLE 2

Intra-raphe bicuculline interactions with muscimol.

Total activity scores (Mean +/- S.E.M.) for the 2 hour period following the injection of muscimol (0.88 nmole) or saline (0.5 µl) through cannulae chronically implanted in the median raphe nucleus (n = 6). Five minutes prior to receiving muscimol or saline, the animals received bicuculline methiodide (0.88 nmole) or saline.

TOTAL 2-HOUR POST-INJECTION ACTIVITY SCORES

DOSE BICUCULLINE METHIODIDE	DOSE MUSCIMOL	
	VEHICLE	0.88 NMOLE
VEHICLE	1638 ± 224	5210 ± 246*
0.88 NMOLE	1715 ± 235	1827 ± 103

* Significantly different from muscimol vehicle plus bicuculline vehicle condition ($p<0.001$, Student's *t*-test, 2-tailed).

Experiment V

MUSCIMOL DOSE-RESPONSE CURVE IN ANIMALS WITH LESIONS OF THE VENTRAL TEGMENTAL NUCLEI OF GUDDEN

In vitro autoradiographic studies have demonstrated that whereas the DR and MR are sites of only moderate benzodiazepine binding, the ventral tegmental nuclei of Gudden (VTG) show extremely dense benzodiazepine labelling. (Young and Kuhar, 1980; Young et al., 1981). Since the benzodiazepines are thought to bind allosterically to a membrane complex including the GABA receptor, there is a correlation between the loci of benzodiazepine and GABA binding sites. The VTG, in contrast to the DR and MR, are devoid of 5-HT perikarya (Steinbusch, 1981; Kulmala and Lorens, 1982). Bilateral electrolytic lesions of the VTG result in increased open field locomotor activity (Lorens et al., 1975). In contrast, 5,7-dihydroxytryptamine lesions of the ascending serotonin pathways do not affect activity level in the open field (Lorens et al., 1976; Lorens, 1978). Since the VTG lie immediately dorsolateral to the MR, it is possible that the locomotor effects of muscimol injections into the MR may be due to diffusion to the VTG. Activation of GABA receptors located on VTG neurons could result in hyperpolarization and suppressed rates of firing. This transient inhibition, or "functional lesion," could lead to locomotor hyperactivity. We, therefore, ablated the VTG electrolytically in order to determine whether or not this manipulation would affect the dose-response relationship for muscimol.

Procedure

Three groups of animals were employed. One sham-operated control group of 8 animals was anesthetized, the scalps incised and retracted, and craniotomies performed. The lesion electrode was not lowered into the brain, nor were intracranial cannulae implanted. In the other 2 groups, electrolytic VTG lesions (n = 15) were produced or sham-operations (n = 8) were performed first; immediately thereafter MR cannulae were implanted as described in the Materials and Methods section (Chapter II). Behavioral testing began 2 weeks later. Since bilateral electrolytic destruction of the VTG has been reported to produce hyperactivity in the open field and to facilitate acquisition of a two-way (shuttlebox) conditioned avoidance task (Lorens et al., 1975; 1976), these two tests were used to screen the animals behaviorally for effective VTG lesions prior to the initiation of the intra-raphe muscimol study. Thus, only lesion animals with both open field activity scores and total avoidance responses above control range subsequently were tested for a muscimol dose-response relationship in the photocell activity cages. In addition, the locus and effectiveness of the VTG lesions were assessed histologically and biochemically as described previously (Lorens et al., 1975).

Open field activity

Open field activity was tested in a 100 x 100 cm arena with 40 cm high walls. Illumination was provided by a 15-watt bulb centered 120 cm above the arena. The floor of the open field was painted white and

divided by black lines into 25 equal squares. Animals naive to the apparatus were placed in the center square at the start of the test. Crossings from square to square with all four limbs were recorded by the experimenter at 3 minute intervals for 9 minutes.

Two-way conditioned avoidance

Rats were trained to avoid footshock in a 100 cm long, 24 cm wide and 30 cm high shuttlebox illuminated with a 40-watt fluorescent tube mounted outside the rear wall. The front wall was composed of clear Plexiglas, while the remaining surfaces were of translucent white Plexiglas. The test chamber was divided by a 7.5 cm high metal hurdle into two equal sized compartments. Each side of the chamber contained a grid floor connected by a selector switch to a Grayson-Stadler model E-1064-GS shocker and scrambler. A 4 ohm speaker was mounted in each wall for the delivery of a 75 decibel white noise conditioned stimulus (CS). The ambient noise level approximated 50 - 55 decibels.

Acquisition of the two-way avoidance response was examined beginning 7 days after completion of open field testing. A single session of 50 massed trials was given to each VTG lesion animal which was hyperactive in the open field, and to each cannulated and non-cannulated control animal. The first training trial started 3 minutes after the rat was placed in one of the compartments. The intertrial interval was 30 - 45 seconds. The CS was terminated when the rat crossed into the opposite compartment. The unconditioned stimulus, a 0.8 milliampere continuous footshock, was administered if the animal failed to cross the bar-

rier within 5 seconds after CS onset. Intertrial barrier crossings were recorded, but not punished. Escape and avoidance latencies were measured with a stopwatch.

Muscimol dose-response analysis

Beginning 1 week after completion of two-way avoidance testing, the animals were tested for locomotor activity in the photocell chambers using a procedure identical to that described in Experiment II, with the exception that activity levels were recorded every 15 minutes for only one hour post-injection. The doses of muscimol employed were 0, 50, 100 and 200 ng.

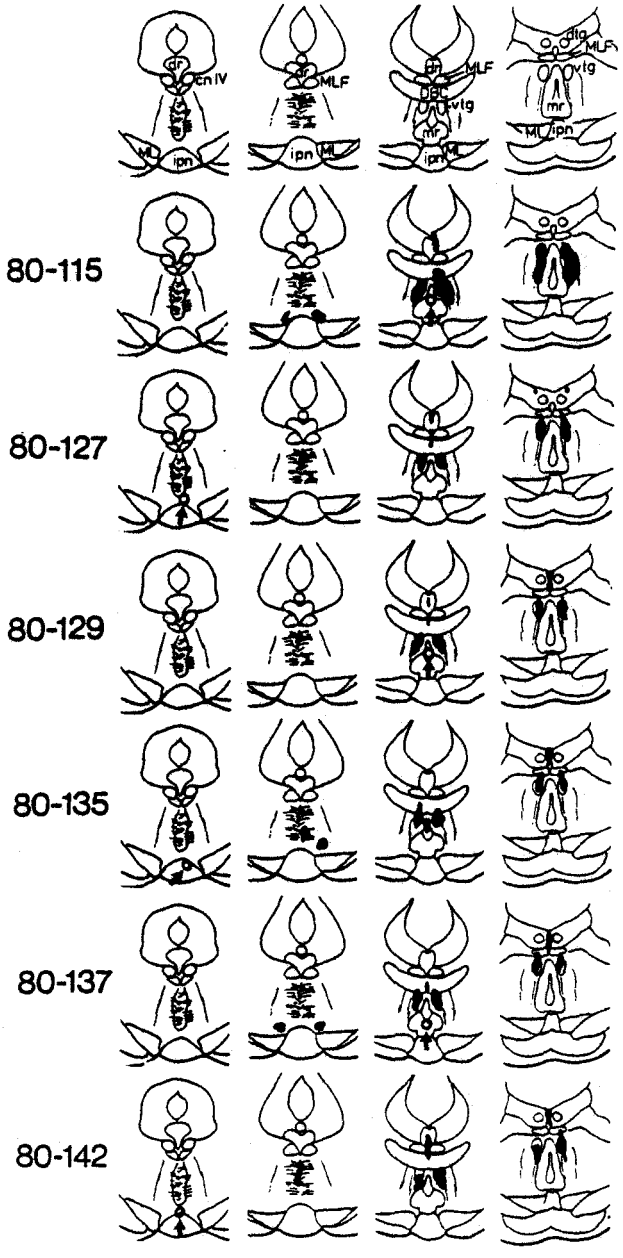
Results

Histological analysis

Of the 15 animals on which both VTG lesions and MR cannulations were performed, only 10 that met the behavioral criteria for effective VTG lesions and included in the drug study. Of these, only 6 had well placed cannulae in the B-8 5-HT cell group dorsal to the interpeduncular nucleus (Figure 13) and subsequently were analyzed behaviorally and biochemicaly. Since the cannula placements in the 8 control animals were similar to those in the accepted lesion subjects, none of these animals was rejected from the study.

Figure 13: VTG lesion and MR cannula placement sites. Reconstruction of damage (blackened area) in the VTG lesion rats. Black circles with arrows indicate loci of cannula tips. Numbers identify individual animals.

Abbreviations: cnIV = trochlear nucleus, dr = dorsal raphe nucleus, dtg = dorsal tegmental nucleus of Gudden, ipn = interpeduncular nucleus, mr = median raphe nucleus, vtg = ventral tegmental nucleus of Gudden; DBC = decussation of brachium conjunctivum, ML = medial lemniscus, MLF = medial longitudinal fasciculus.


80-115
80-127
80-129
80-135
80-137
80-142
dr
cn IV
MLF
ipn
ML
dtg
vtg
mr
DBC

Biochemical analysis

The striatal and hippocampal concentrations of 5-HT and its metabolite, 5-HIAA, were assayed by HPLC. The following groups were compared: the animals with acceptable VTG lesions and MR cannulae (n = 6), the sham-operated animals with MR cannulae (n = 8), and the sham-operated non-cannulated controls (n = 8). As shown in Table 3, hippocampal 5-HT levels in the VTG lesion animals were significantly reduced in comparison with both the sham-operated controls (57%) and the MR cannulae implanted controls (52%). The hippocampal 5-HIAA levels in the VTG lesion rats showed similar reductions. In contrast, the striatal 5-HT concentrations of the 3 groups did not differ significantly. The striatal 5-HIAA level in the VTG lesion rats, however, was significantly lower (18%) than the control level. These observations are in agreement with those reported by Lorens and coworkers (1975). Importantly, the MR cannula placements by themselves did not significantly affect the concentration of 5-HT in either the hippocampus or the striatum. Although the hippocampal 5-HIAA level of the sham-operated MR cannulated rats was slightly but significantly lower than control (24%), these data suggest that the MR cannulae did not significantly damage the 5-HT perikarya comprising, or the 5-HT fibers emanating from, the B-8 5-HT cell group.

TABLE 3

Effects of VTG lesions on regional 5-HT and 5-HIAA levels.

Hippocampal and striatal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations (Mean +/- S.E.M. ng/g wet tissue mass) in VTG lesion and control animals. Data entries represent Mean +/- S.E.M.

STRIATAL AND HIPPOCAMPAL 5-HT AND 5-HIAA LEVELS
IN VTG LESION AND NON-LESION ANIMALS

TREATMENT	n	HIPPOCAMPUS		STRIATUM	
		5-HT (ng/g)	5-HIAA (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
SHAM-OPERATED CONTROL	8	416 ± 27	743 ± 55	607 ± 22	937 ± 34
MR-CANNULA ALONE	8	370 ± 33	564 ± 30*	600 ± 22	898 ± 37
VTG LESION + MR-CANNULA	6	179 ± 31*†	343 ± 28*†	619 ± 26	767 ± 35*

* Significantly different from sham-operated controls ($p<0.01$);

† Significantly different from MR-cannula alone ($p<0.01$, Newman-Keuls' multiple range test).

Behavioral analysis

Open Field Activity and Conditioned Avoidance Acquitision. These two behavioral tests were used to screen the effectiveness of the VTG lesions. Only the ranges of scores obtained from the rats accepted for the intra-raphe muscimol dose-response analysis are presented. The ranges of total 9-minute open field activity scores were as follows: MR cannula implanted plus VTG lesion group (n = 6), 365 - 521; sham-operated MR cannula implanted group (n = 8), 128 - 298; sham-operated non-cannulated group (n = 8), 52 - 235. The ranges for the total number of conditioned avoidance responses emitted during the 50 massed trials were: animals with VTG lesions and implanted MR cannulae, 28 - 43; sham-operated rats with MR cannulae, 9 - 27; sham-operated controls, 1 - 26. The group of rats with VTG lesions plus MR cannulae also tended to show more inter-trial spontaneous barrier crossings (2 - 248) during the entire test session (about 45 minutes in length) than either the non-lesion MR cannula implanted (0 - 22) or the sham-operated non-cannulated control (0 - 14) groups.

Muscimol Dose-Response Analysis. An ANOVA (three factor mixed design - repeated measures on two factors; Bruning and Kintz, 1977, pp. 62 - 72) of the 30 minute pre-injection activity levels of the rats with VTG lesions and MR cannulae, versus the non-lesion rats with implanted MR cannulae for the 4 drug days revealed both significant group [$F(1,10)=30.66$, $p<0.0005$] and time [$F(1,10)= 112.44$, $p<0.0001$] effects. This latter effect was due to the greater level of activity of the animals during the first 15 minutes in the apparatus.

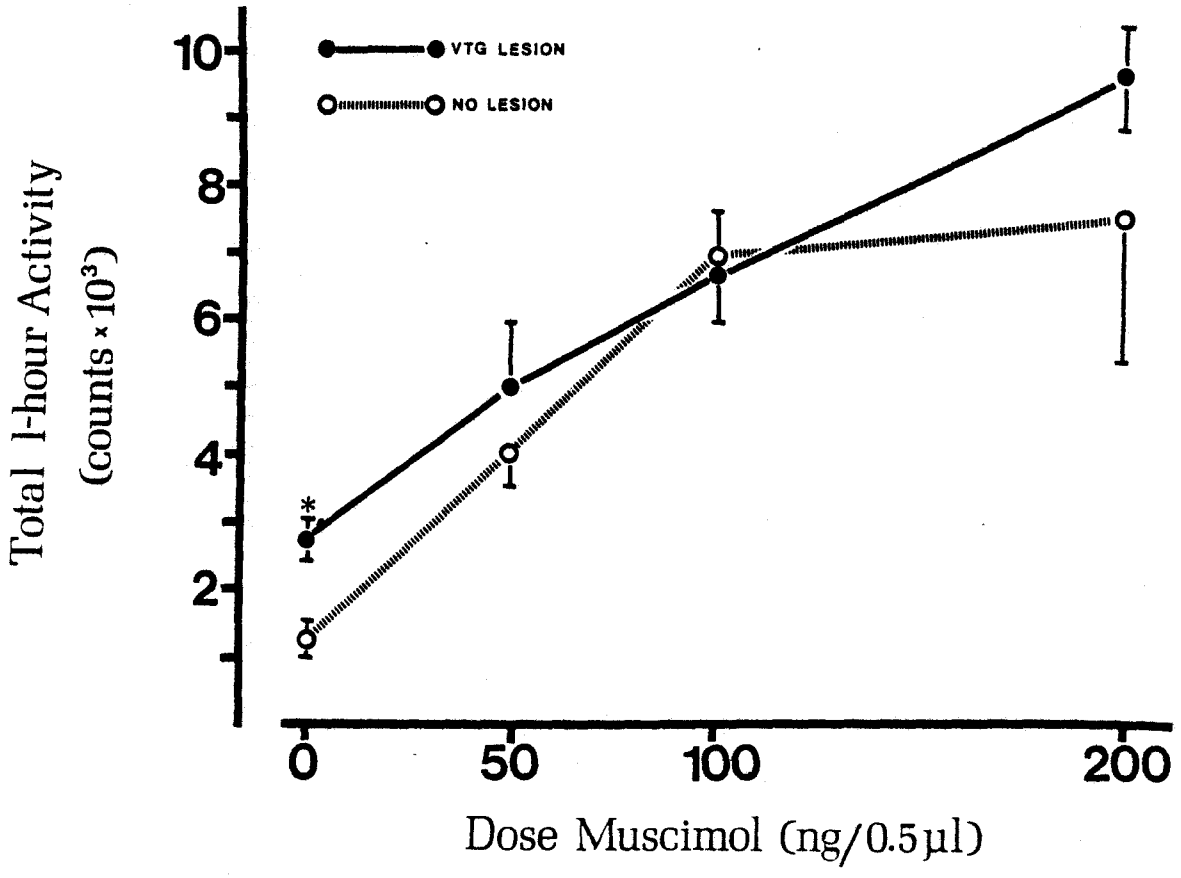
A subsequent ANOVA of the total 60 minute post-injection activity scores for the 4 drug days showed neither significant group nor interaction effects. As expected, the dose effects were significant [$F(3,30)=11.86$, $p<0.0001$]. Muscimol produced similar dose-dependent elevations in activity level in both groups (Figure 14). Because the animals with VTG lesions evidenced higher baseline levels of activity than the non-lesion subjects, we reexamined the post-muscimol effects after taking this difference into consideration. For this purpose the activity scores obtained by each rat during the last 15 minutes of the pre-drug control period were subtracted from each of its four 15-minute post-injection scores. An ANOVA of these data also failed to demonstrate any significant difference between the VTG lesion and control groups. As expected, the muscimol dose effect was significant [$F(3,30)=16.68$, $p<0.0001$], but the group-dose interaction was not.

Conclusion

These data suggest that the GABA and benzodiazepine receptors located on neuronal cell bodies or fibers within the VTG do not mediate the hyperkinetic effects of intra-raphe muscimol administration.

Figure 14: Effects of VTG lesions on muscimol-induced hyperactivity.

Dose-response relationship for muscimol injections through cannulae chronically implanted in the median raphe nucleus of rats with bilateral lesions in the ventral tegmental nuclei of Gudden (VTG, n = 6) or control operations (n = 8). Total activity scores (Mean +/- S.E.M.) for the one hour period following muscimol injection.


VTG LESION
NO LESION
10
8
6
4
2
0
50
100
200
*
Total 1-hour Activity
(counts × 10³)
Dose Muscimol (ng/0.5µl)

experiment VI

MUSCIMOL DOSE-RESPONSE CURVE IN ANIMALS WITH LESIONS OF THE ASCENDING 5-HT PROJECTIONS

We have found benzodiazepine binding sites in the MR, although not as dense as in the VTG (Sainati *et al.*, 1982). Since destruction of the VTG had no effect on the locomotor response to intra-raphe muscimol, and since the peripheral administration of benzodiazepines has been reported to diminish 5-HT turnover in the CNS, we decided to determine the effect of forebrain 5-HT depletion on the effects of intra-raphe muscimol.

Procedure

The ascending 5-HT projections were destroyed by injecting 5,7-dihydroxytryptamine (5,7-DHT) intracerebroventricularly (n = 12) or intramesencephalically (n = 12) as described in the Materials and Methods section (Chapter II). These animals, as well as vehicle-injected controls, were implanted with MR cannulae during the same operative session. Beginning 2 weeks post-operatively the animals were tested as in Experiment II, again with activity counts recorded every 15 minutes for one hour post-injection. At the end of the experiment, the animals were sacrificed by decapitation, their hippocampal and striatal 5-HT levels assayed by HPLC, and their brainstems fixed in buffered formalin for histological verification of cannula and mesencephalic 5,7-DHT injection sites.

Results

Histological analysis

Of the animals which survived the entire experiment, 7 rats which had been injected intraventricularly with 5,7-DHT, 7 animals which had received intraventricular injections of the vehicle, 7 which had been injected intramesencephalically with 5,7-DHT, and 6 which had been given vehicle injections into the mesencephalon had cannulae well localized within the B-8 5-HT cell group, dorsal to the interpeduncular nucleus. In addition, the sites of the intramesencephalic 5,7-DHT and vehicle injections were examined and all found to be appropriately localized within the course of the ascending 5-HT projections through the ventral tegmental area of Tsai, or immediately dorsal to the rostral tip of the interpeduncular nucleus (Figure 15). The histological appearance of the 5,7-DHT and vehicle injection sites were virtually indistinguishable.

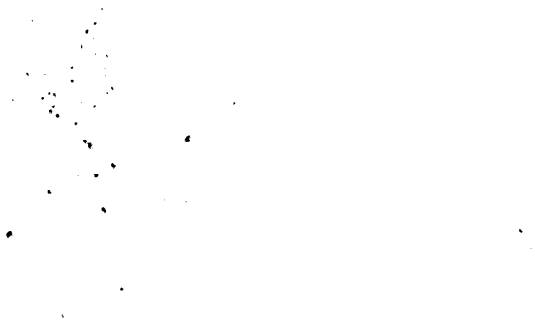
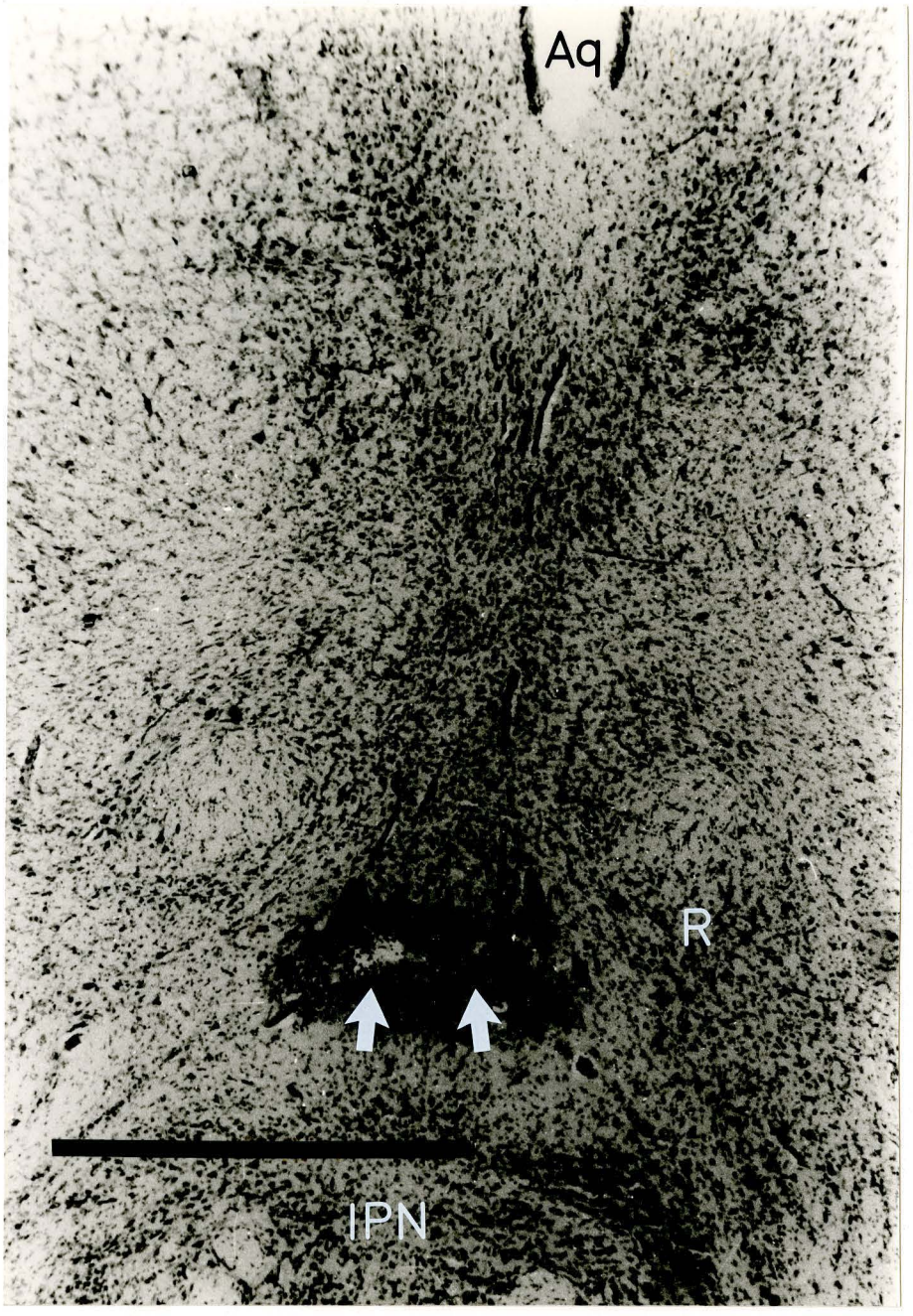


Figure 15: Photomicrograph of a midbrain 5,7-DHT injection site.

Site of a typical intra-mesencephalic 5,7-dihydroxytryptamine (5,7-DHT) injection (4.0 µg in 2.0 µl vehicle, bilaterally) is demarcated by a glial scar (arrows). Cresylecht violet stained 50 µm coronal section. Bar represents 1 mm. Abbreviations: Aq = cerebral aqueduct of Sylvius, IPN = interpeduncular nucleus, R = red nucleus.


Aq
R
IPN

Biochemical analysis

The 5-HT and 5-HIAA concentrations in the neostriata and hippocampi of the control animals which had received intracerebroventricular vehicle injections (Table 4) were virtually the same as those for the sham-operated control animals in Experiment V (Table 3). Intraventricular 5,7-DHT reduced the 5-HT concentrations in both the striatum (95%) and hippocampus (83%) as compared to intraventricular vehicle injections. The intramesencephalic 5,7-DHT injections also significantly reduced the 5-HT contents of the striatum (77%) and the hippocampus (59%) as compared to the intra-midbrain vehicle injections. Similar reductions were seen in the regional concentrations of the 5-HT metabolite, 5-HIAA. Intramesencephalic vehicle injections in comparison to the intraventricular vehicle injections, produced a small (22%), insignificant reduction in the hippocampal 5-HT concentration, but a significant (37%) fall in hippocampal 5-HIAA content (Table 2). This observation, supported by the histological data, suggests that the intra-midbrain vehicle injections interrupted some of the 5-HT fibers which innervate the hippocampus.

TABLE 4

Effect of 5,7-DHT on regional 5-HT and 5-HIAA levels.

Hippocampal and striatal 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations (Mean +/- S.E.M. ng/g wet tissue mass) in control animals and animals with intra-midbrain and intraventricular 5,7-dihydroxy-tryptamine (5,7-DHT) lesions.

STRIATAL AND HIPPOCAMPAL 5-HT AND 5-HIAA LEVELS
IN ANIMALS WITH INTRAVENTRICULAR OR
INTRA-MIDBRAIN 5,7-DHT LESIONS

TREATMENT	n	HIPPOCAMPUS		STRIATUM	
		5-HT (ng/g)	5-HIAA (ng/g)	5-HT (ng/g)	5-HIAA (ng/g)
INTRAVENTRICULAR VEHICLE	7	464 ± 49	741 ± 64	526 ± 61	824 ± 76
INTRAVENTRICULAR 5,7-DHT	7	76 ± 13*†§	60 ± 18*†§	28 ± 6*†§	102 ± 23*†§
INTRA-MIDBRAIN VEHICLE	6	362 ± 34	470 ± 62*	542 ± 99	757 ± 69
INTRA-MIDBRAIN 5,7-DHT	7	148 ± 15*§	168 ± 41*§	123 ± 35*§	327 ± 54*§

* Significantly different from intraventricular vehicle ($p<0.01$);

† Significantly different from intra-midbrain 5,7-DHT ($p<0.01$);

§ Significantly different from intra-midbrain vehicle ($p<0.01$, Newman-Keuls' multiple range test).

Behavioral analysis

An ANOVA (three factor mixed design - repeated measures on one factor; Bruning and Kintz, 1977, pp. 62 - 72) of the pre-drug baseline activity levels of the 4 groups of rats failed to reveal any significant 5,7-DHT lesion effect, regardless of the mode of its production (intraventricular versus intramesencephalic). However, an ANOVA (three factor mixed design - repeated measures on two factors; Bruning and Kintz, 1977, pp. 73 - 84) of the effects of intra-raphe muscimol injections showed the following. The effects of the dose of muscimol were significant [$F(3,72)=6.28$, $p<0.002$], as were the effects of time after injection [$F(3,72)=14.59$, $p<0.0001$]. In addition, the effects of lesion group and of muscimol dose interacted [$F(9,72)=7.26$, $p<0.001$]. Individual comparisons of the total 60 minute post-injection activity level for each group confirmed that both types of forebrain 5-HT-reducing lesion markedly attenuated the effects of intra-raphe muscimol (Figure 16).

Conclusion

The data obtained from the present experiment suggest that the hyperkinetic effect of intra-midbrain raphe injections of muscimol depend on an intact ascending 5-HT fiber system.

Figure 16: Forebrain 5-HT depletion blocks the effect of muscimol.

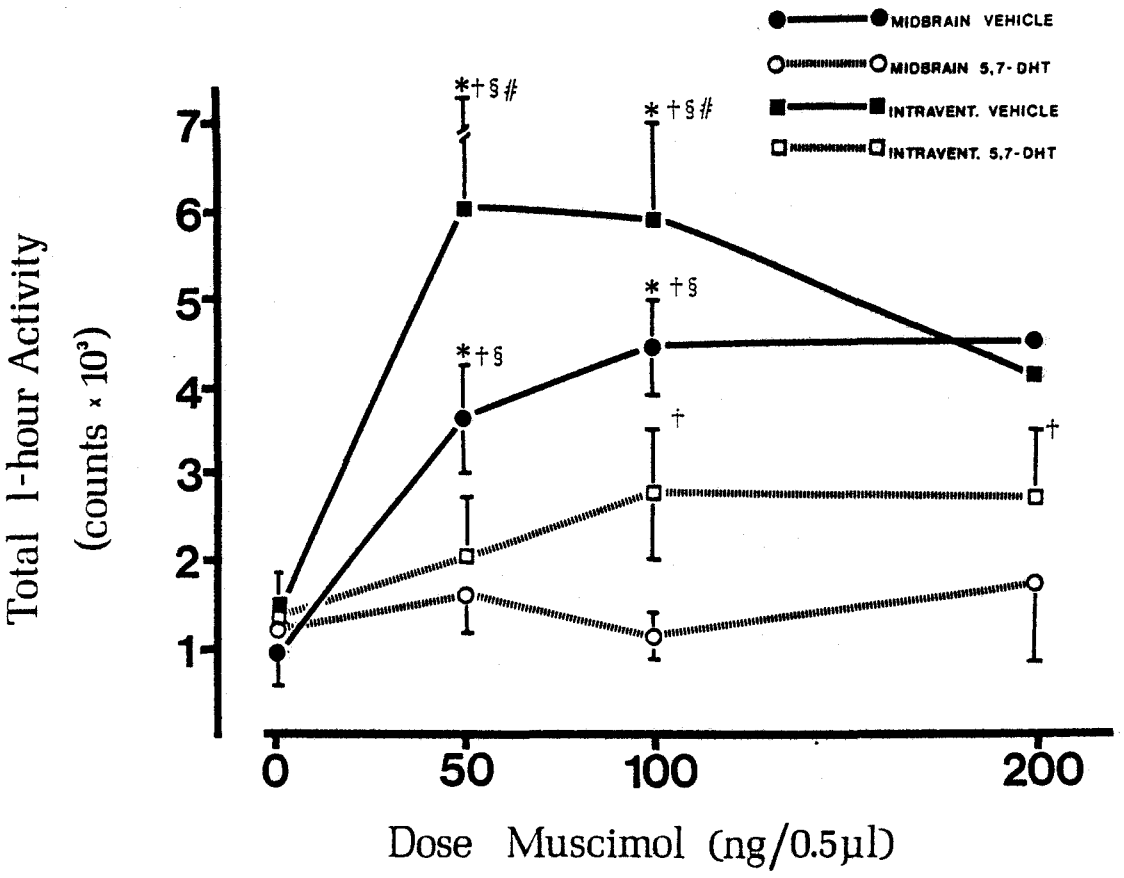
Dose response relationship for the injection of muscimol (0, 50, 100 or 200 ng) through cannulae chronically implanted in the median raphe nucleus of rats which had received 5,7-dihydroxytryptamine (5,7-DHT) injections into the cerebral ventricles (75 µg bilaterally), or into the ascending 5-HT fibers at the level of the red nucleus (4.0 µg bilaterally). Group mean (+/- S.E.M.) activity scores for the 1 hour post-injection period are presented.

* significantly elevated over saline-injected control condition ($p<0.01$, Newman-Keuls' test for related measures);

† significantly elevated over intra-midbrain 5,7-DHT,

§ significantly elevated over intra-ventricular 5,7-DHT,

significantly elevated over intra-midbrain vehicle ($p<0.01$, Newman-Keuls' multiple range test).


MIDBRAIN VEHICLE
MIDBRAIN 5,7-DHT
INTRAVENT. VEHICLE
INTRAVENT. 5,7-DHT
Total 1-hour Activity
(counts × 10³)
7
6
5
4
3
2
1
0
50
100
200
Dose Muscimol (ng/0.5µl)

CHAPTER IV

DISCUSSION

General Discussion

Overall, the present series of experiments indicates that the injection of the GABA agonist, muscimol, into the mesencephalic raphe results in a dose-dependent hyperkinetic response which is mediated by an ascending serotonergic system. The present observations also suggest that GABA receptors within the midbrain raphe regulate the discharge rate of 5-HT neurons whose forebrain efferents modulate motor systems, and that the benzodiazepines can modify the activity of these systems centrally.

The first experiment confirmed previous findings (Przewłocka et al. 1979) that acute microinjection of muscimol (100 ng) into the dorsal raphe nucleus produces hyperactivity as measured in photocell chambers. In addition, the median raphe nucleus was found to be even more sensitive to muscimol (100 ng) than the dorsal raphe nucleus, the magnitude of the hyperkinetic effect being four times greater after injection into the former site. The acute injection paradigm employed by Przewłocka and associates (1979), and in Experiment I, required that the subjects be tested immediately upon awakening from the ether anesthetic. Thus, it was not possible to discern whether the hyperactivity produced was due to muscimol alone, or to the interaction between muscimol, ether and the trauma of surgery.

In order to circumvent this problem, a muscimol dose response-analysis was conducted using animals with cannulae implanted chronically into either the dorsal or the median raphe nucleus (Figure 5). Since each animal received each drug dose, this experiment used a Latin square design in order to determine any order of treatment effects. Accordingly, injection of muscimol into the median raphe nucleus produced a significant elevation in activity level at one-fourth the dose (50 ng) required to produce a similar effect when injected into the dorsal raphe nucleus. The hyperkinetic response following the injection of an optimal dose of muscimol (100 ng) into the median raphe nucleus, moreover, was at least 1.5 times more marked than the response to an injection (200 ng) of muscimol into the dorsal raphe nucleus (Figure 6). As there was no effect of order of treatment, subsequent experiments were performed using a randomized block design.

The observed four-fold greater sensitivity of the median raphe nucleus as compared to the dorsal raphe nucleus raises the possibility that the hyperkinesis following muscimol injections into the dorsal raphe nucleus may be due, at least in part, to a spread of the drug to the more sensitive median raphe site. We have begun a series of _in vivo_ autoradiographic studies to determine the time course and the extent of the spread of radiolabel following injections of various doses of ^{3}H-muscimol through cannulae chronically implanted in the dorsal and median raphe nuclei of rats. Our preliminary results suggest that fol-

lowing injections of ^{3}H-muscimol (50 ng) into the median raphe nucleus, the greatest density of the label remains concentrated around the guide cannula tip, even 60 minutes post-injection. A light, but significant density of label, however, has spread by this time nearly 1.0 mm ventrally, caudally and rostrally. Such a spread of muscimol from the tip of a cannula in the dorsal raphe nucleus includes the median raphe nucleus. It is of interest to note in this regard that Przewłocka et al. (1979) found decreases in 5-HT and 5-HIAA concentrations in the hypothalamus, but not in the neostriatum, following the injection of muscimol (25 - 100 ng) into the dorsal raphe nucleus. As reviewed in Chapter I, substantial evidence has been advanced demonstrating that 5-HT fibers afferent to the neostriatum arise predominantly in the dorsal raphe nucleus (B-7 5-HT cell group), whereas the 5-HT projection to the hippocampus originates principally in the B-8 5-HT cell group, which overlaps the caudal linear and median raphe nuclei. In addition, Forchetti and Meek (1981) have reported that muscimol injections into the median raphe nucleus reduce 5-HT turnover in the hippocampus. Thus, the hyperkinetic response to intra-dorsal raphe muscimol injections observed by Przewlocka and collaborators might have been due to a spread of the muscimol to the median raphe nucleus (B-8 5-HT cell group). This hypothesis currently is under investigation in our laboratory.

As stated earlier, several reports implicate GABA as a neurotransmitter in both the dorsal (Belin et al., 1979; Forchetti and Meek, 1981; Gallager, 1978; Gallager and Aghajanian, 1976; Massari et al.,

1976; Paul et al., 1981) and the median raphe nucleus (Forchetti and Meek, 1981). Many of the behavioral and physiological effects of the benzodiazepines are believed to be due to their facilitation of GABA-ergic transmission (Cook and Sepinwall, 1975; Costa and Guidotti, 1979; Costa et al., 1981; Gallager, 1978; Paul et al., 1981). If the effects of intra-mesencephalic administration of muscimol were due to activation of GABA receptors in the raphe nuclei, they should be potentiated by benzodiazepines (for example, chlordiazepoxide) and blocked by the GABA-receptor antagonist, bicuculline.

In a preliminary study (Sainati and Lorens, 1981) we found that intraperitoneal administration of chlordiazepoxide in doses greater than 3.8 mg/kg produced decreases in locomotor activity as measured in dark photocell chambers to which the animals had been habituated previously. Lower doses produced no effect on activity level. These findings are consistent with other reports demonstrating that although the benzodiazepines enhance exploratory behavior in novel environments, they have little effect on activity level when the animals are tested in settings to which they have been habituated (Marriott and Spencer, 1965; Christmas and Maxwell, 1970; Kršiak et al., 1970; Pieri et al., 1981).

It was found (Experiment III) that peripheral administration of chlordiazepoxide, in the sub-ataxic dose of 3.8 mg/kg, did not itself affect activity level, but enhanced the locomotor activity response to low doses (25 and 50 ng) of muscimol injected into the median raphe nucleus. Conversely, a subconvulsant dose of bicuculline (1.1 mg/kg) completely blocked the response to 50 and 100 ng of muscimol.

If benzodiazepine compounds do act to facilitate GABA-ergic transmission, it seemes plausible to hypothesize that intra-raphe administration of representative benzodiazepines might produce increases in locomotor activity. We tested (Experiment IVa) this hypothesis by injecting three different water-soluble benzodiazepines (chlordiazepoxide, flurazepam and midazolam) directly into the median raphe nucleus through chronically indwelling cannulae. Water soluble compounds were chosen in order to avoid any potential behavioral effects that might ensue from the use of a non-aqueous vehicle. The ethanol-propylene glycol vehicle commonly used to dissolve non-water soluble benzodiazepines most likely would have deleterious effects if injected directly into the brain. We found that injections of flurazepam and midazolam directly into the median raphe nucleus both produced dose-dependent increases in locomotor activity (Figure 10). Flurazepam, moreover, produced a maximal hyperkinetic effect at a dose one-half as great as that required for midazolam. This observation is consistent with the hypothesis that these compounds act on two different allosteric sites on a benzodiazepine-GABA-receptor complex (Costa and Guidotti, 1979; Olsen, 1982). The greater potency of flurazepam over midazolam in inducing hyperactivity also is in keeping with the relative order of potencies of these drugs in displacing radiolabeled benzodiazepines from binding sites <u>in vitro</u> (Möhler and Okada, 1977; Braestrup and Squires, 1978; Brasetrup and Nielsen, 1980).

The failure of chlordiazepoxide both to induce hyperactivity when injected into the median raphe nucleus *in vivo*, and to displace ^{3}H-flunitrazepam binding *in vitro* (Sainati *et al.*, 1982), is further evidence that chlordiazepoxide is a pro-drug which must be converted to active metabolites in order to have an effect in the central nervous system (Greenblatt and Shader, 1974; Johnson and Rising, 1979; Breimer *et al.*, 1980; Harvey, 1980).

The benzodiazepines are thought to act post-synaptically by binding at a site on a large receptor-ionophore complex allosteric to the GABA binding site, and thereby increase the affinity of the latter for its ligand. If so, then the GABA agonist, muscimol, and the centrally active benzodiazepines should have additive hyperkinetic effects when injected one after the other directly into the median raphe nucleus. Indeed combining a sub-effective dose of muscimol with a sub-effective dose of either flurazepam or midazolam produced hyperkinetic effects as robust as a four-fold higher dose of muscimol alone (Figure 12).

If facilitation of GABA-ergic neurotransmission in the median raphe nucleus produced hyperactivity, then blockade of GABA receptors might be expected to have the opposite effect. However, intra-raphe bicuculline injections did not affect activity level. This observation is not surprising, since release from tonic GABA-ergic inhibition (Forchetti and Meek, 1981) would result in an initial brief increase in the firing rates of 5-HT neurons, followed by an almost immediate return to baseline due to collateral feedback inhibition (Aghajanian and Wang,

1978). On the other hand, the intra-raphe injection of bicuculline did block the hyperkinetic effect of muscimol, providing additional evidence that the muscimol effect is due to a direct activation of GABA receptors within the raphe.

The ventral tegmental nuclei of Gudden (VTG) lie just dorsolateral to the median raphe nucleus. Bilateral electrolytic lesions in the VTG produce hyperactivity similar to that seen after electrolytic lesions in the median raphe nucleus. The VTG are sites of dense benzodiazepine receptor localization (Young and Kuhar, 1980). In vitro autoradiographic studies currently in progress in our laboratory confirm this finding (Sainati et al., 1982). Furthermore, benzodiazepine receptors usually are associated with GABA receptors (Costa and Guidotti, 1979; Paul et al., 1981). Although not a particularly rich source of GABA or GAD (Massari et al., 1976), the VTG receive a significant afferent projection from the ipsi- and contra-lateral dorsal tegmental nuclei of Gudden (Briggs and Kaelber, 1971; Petrovicky, 1973), which contain high levels of GAD. It seemed entirely possible, therefore, that the hyperactivity induced by intra-raphe muscimol injections could be due to diffusion of the drug to GABA-ceptive sites located in the VTG. We, therefore, destroyed the VTG in order to determine whether this manipulation would attenuate the hyperkinetic effect of intra-raphe muscimol injections. We found, in agreement with earlier findings (Lorens et al., 1975), that the VTG lesions increased open field activity and facilitated the acquisition of a two-way conditioned avoidance response. In

the darkened activity chambers, the VTG lesion animals also manifested higher baseline levels of activity than controls, but the lesions failed to attenuate the facilitatory effects of muscimol. Thus, it appears that the effects of intra-raphe muscimol on locomotor activity and of VTG lesions on this behavior, are independent phenomena.

Subsequently, forebrain 5-HT was depleted by injecting the specific serotonin neurotoxin, 5,7-dihydroxytryptamine, into either the lateral cerebral ventricles or into the midbrain tegmentum. These lesions markedly attenuated the locomotor response produced by muscimol injections into the median raphe nucleus. These data suggest that midbrain GABA neurons modulate activity level in the rat through a direct action on mesencephalic serotonergic neurons.

As a result, we hypothesize that when muscimol is injected directly into the midbrain raphe nuclei, it suppresses the firing rate of serotonergic neurons by activating local GABA receptors. This, in turn, elicits locomotor hyperactivity by releasing hippocampal (Williams and Azmitia, 1981) or substantia nigra (see below) neurons from a tonic serotonergic inhibitory influence. Intraventricular and intramesencephalic 5,7-dihydroxytryptamine lesions, however, did not alter baseline activity level in our photocell chambers. Biochemical analysis showed that these lesions resulted in a degeneration of forebrain 5-HT efferents, but histological analysis indicated that their perikarya of origin were spared, possibly because of sustaining collaterals. It may be that in order to induce hyperactivity the 5-HT cell bodies associated

with the midbrain raphe nuclei themselves must be destroyed. Also, during the 2 week post-lesion recovery period, the motor systems of the animals may adapt to the loss of the ascending 5-HT projections. Nonetheless, the link between the midbrain GABA-ceptive site normally responsible for inducing hyperactivity, and the areas which control motor behavior still are lost. Thus, the acute inhibition of 5-HT neurons following intra-raphe muscimol produces hyperactivity, whereas a lesion which chronically destroys ascending 5-HT projections allows a new, compensated "steady state" to develop.

The neuronal mechanisms by which a GABA-ergic inhibition of raphe 5-HT cells might produce elevations in locomotor activity is largely a matter of speculation. There is a substantial amount of evidence to support the existence of inhibitory 5-HT projections from the dorsal and median raphe nuclei to the substantia nigra in the rat (Dray et al., 1976; 1978; Nicolaou et al., 1979; van der Kooy and Hattori, 1980a). Giambalvo and Snodgrass (1978) report that 5,7-dihydroxytryptamine injections into the median raphe nucleus, if placed 0.3 mm lateral to the midline, produce a "unilateral" lesion. Such lesions cause rats to rotate in a direction contralateral to the site of injection, the rotation being blocked by haloperidol. They found a significant correlation between the rate of rotation, the decrease in cortical 5-HT turnover, and the increase in striatal dopamine turnover. Moreover, they found that injections of 5,7-dihydroxytryptamine into the substantia nigra itself produced biochemical and behavioral changes similar to those following 5,7-dihydroxytryptamine injections into the median raphe nucleus.

The nigrostriatal dopamine pathway is an important component of the extrapyramidal motor system. The indirect dopamine agonist, amphetamine, and the dopamine-receptor agonist, apomorphine, both can induce turning in rats with unilateral lesions in the nigrostriatal dopamine system (Ungerstedt and Arbuthnott, 1970; Ungerstedt, 1971). These drugs, however, induce turning in opposite directions. Amphetamine is thought to produce ipsilateral turning by releasing dopamine from intact contralateral striatal nerve terminals, while apomorphine is thought to induce contralateral turning by stimulation of supersensitive dopamine receptors in the denervated ipsilateral striatum.

If unilateral stimulation of striatal dopamine receptors produces rotation in a direction contralateral to the site of stimulation, then bilateral stimulation of striatal dopamine receptors might produce a non-rotational hyperactivity. In our experiments, midline intra-raphe injections of muscimol hypothetically would suppress the inhibitory raphe-substantia nigra 5-HT pathway, producing a bilateral increase in the firing rates of nigral dopamine neurons. This, in turn, would lead to a non-rotational, stereotypic hyperkinesis.

In summary, the present results suggest that midbrain raphe 5-HT neurons exert a tonic inhibitory effect on CNS systems which influence locomotor behavior. Temporary removal of this inhibition by administering the GABA agonist, muscimol, or the benzodiazepines, flurazepam and midazolam, into the mesencephalic raphe results in hyperactivity. Thus, this forebrain 5-HT system appears to be regulated by raphe GABA inter-

neurons, whose post-synaptic effects can be facilitated following the administration of benzodiazepine drugs.

Proposals for Future Research

If, as stated above, intra-raphe injections of muscimol produce hyperactivity by suppressing an inhibitory raphe-substantia nigra 5-HT pathway, then the effect of muscimol should be prevented by the administration of a dopamine antagonist, such as haloperidol. Haloperidol would be expected to block the hyperkinetic effects of the hypothesized increased dopamine release from the nigro-striatal pathway. Furthermore, nigral lesions using 5,7-dihydroxytryptamine might produce elevations in activity level, and should prevent the hyperkinetic effect of intra-raphe muscimol injections.

Although the dorsal and median raphe nuclei (Taber, 1960) contain serotoninergic neurons, such cells are not the sole constituents of these two nuclear groups (Aghajanian et al., 1978; Descarries et al., 1979; Steinbusch et al., 1980; van der Kooy and Steinbusch, 1980; Steinbusch, 1981; Kulmala and Lorens, 1982). The following series of experiments is proposed, therefore, to determine the relative importance of the 5-HT efferents from the dorsal and median raphe nuclei in the mediation of intra-raphe muscimol induced hyperkinesis.

Muscimol dose response analysis will be performed in animals with selective 5,7-dihydroxytryptamine lesions of the dorsal or median raphe nucleus, as well as in subjects with combined lesions of both nuclei. These results will be compared to those obtained from appropriate con-

trol animals, and to data from rats with ibotenic acid lesions of the dorsal and/or median raphe nucleus. Ibotenic acid is an excitotoxic isoxazole compound which selectively destroys neuronal cell bodies at the site of application while sparing axons of passage and nerve terminals of extrinsic origin (Schwarcz, et al., 1979a; 1979b). It appears, moreover, that the somata of neurons which use a biogenic amine (such as 5-HT) as a neurotransmitter are relatively resistant to the toxic effects of ibotenic acid (R. Schwarcz, personal communication; S.A. Lorens, unpublished observations). Thus, these two neurotoxins, 5,7-dihydroxytryptamine and ibotenic acid, could be used to produce relatively specific lesions of midbrain 5-HT- and non-5-HT-containing neuronal perikarya, respectively.

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APPENDIX A

APPROVAL SHEET

The dissertation submitted by Stephen Mitchell Sainati has been read and approved by the following committee:

Dr. Stanley A. Lorens, Director
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

8/27-82
Date

Stanley A Lorens, Ph.D.
Director's Signature