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## MECHANISM OF TRIAZOLO-BENZODIAZEPINE AND BENZODIAZEPINE ACTION IN ANXIETY AND DEPRESSION: BEHAVIORAL STUDIES WITH CONCOMITANT *IN VIVO* CA<sub>1</sub> HIPPOCAMPAL NOREPINEPHRINE AND SEROTONIN RELEASE DETECTION IN THE BEHAVING ANIMAL

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### Abstract

Broderick, Patricia A., Omotola Hope and Pierrot Jeannot: Mechanism of Triazolo-Benzodiazepine and Benzodiazepine Action in Anxiety and Depression: Behavioral Studies with Concomitant *In Vivo* CA<sub>1</sub> Hippocampal Norepinephrine and Serotonin Release Detection in the Behaving Animal. Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 1998, **22**, pp. 353-386. © 1998 Elsevier Science Inc.

1. *Real time, in vivo* microvoltammetric studies were performed, using miniature carbon-based sensors, to concurrently detect norepinephrine (NE) release and serotonin (5-HT) release, in 2 separate electrochemical signals, within CA<sub>1</sub> region of hippocampus in the freely moving and behaving, male, Sprague Dawley laboratory rat.
2. Concurrently, four parameters of open-field behavior, i.e. Ambulations, Rearing, Fine Movements and Central Ambulatory behavior (a measure of anxiety reduction behavior), were assayed by infrared photobeam detection.
3. Time course studies showed that the mechanism of action of the triazolobenzodiazepine (TBZD), adinazolam, (Deracyn®) is dramatically different from that of the classical benzodiazepine (BZD), diazepam (Valium®), i.e., adinazolam increased, whereas diazepam decreased, 5-HT release within CA<sub>1</sub> region of hippocampus in the freely moving and behaving rat.
4. Adinazolam initially increased NE release and then decreased NE release in CA<sub>1</sub> region of hippocampus in the freely moving and behaving rat whereas diazepam only decreased the electrochemical signal for NE; the decrease in NE produced by adinazolam was greater than the decrease in NE release produced by diazepam.
5. The Behavioral Activity Patterns, derived from same animal controls, simultaneously with detection of *in vivo* microvoltammetric signals for NE release and 5-HT release, showed that the BZD, diazepam, exhibited more potent sedative properties than did the TBZD adinazolam.
6. Hippocampal 5-HT and NE release effects of the TBZD, adinazolam, concomitant with behavioral effects lends explanation to the dual anxiolytic/antidepressant properties of the TBZDs.

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**Keywords:** adinazolam (Deracyn®), carbon paste microelectrode, diazepam (Valium®), freely moving animal, hippocampus, infrared photocell beams, *in vivo* microvoltammetry, norepinephrine, open-field behavior, serotonin, stearate.

**Abbreviations:** benzodiazepine (BZD), dorsal raphe (DR), gamma-aminobutyric acid (GABA), gamma-butyrolactone (GBL), intraperitoneal (i.p.), locus coeruleus (LC), norepinephrine (NE), platelet activating factor (PAF), serotonin (5-HT), triazolobenzodiazepine (TBZD), tricyclic antidepressant (TCA).

### Introduction

The benzodiazepines (BZDs) comprise treatment for the generalized anxiety disorders. Alprazolam, a triazolobenzodiazepine (TBZD) and a dual anxiolytic/antidepressant pharmacotherapy (Zarcone *et al.*, 1994; Petty *et al.*, 1995) is included in this listing (Baldessarini, 1996). In addition, differential types of anxiety disorders such as panic disorder and phobia, have shown a favorable clinical response to the TBZD adinazolam (Fleishaker *et al.*, 1994), as well as to the tricyclic antidepressants (TCAs) and to the monoamine oxidase inhibitors (Sheehan *et al.*, 1980; Pohl *et al.*, 1982; Charney *et al.*, 1986). These clinical data which indicate that anxiety can be alleviated by “antidepressant” therapy, are supported by preclinical data. Using an animal model of anxiety, “novelty suppressed feeding”, Bodnoff *et al.*, (1988) showed that the chronic administration of TCAs, amitriptyline and desmethylimipramine, effectively alleviated anxiety, albeit that this anti-anxiety effect, produced by TCAs, was shown to be specific to a novel environment and was not mediated through gamma-aminobutyric acid (GABA) receptors. Furthermore, in the same study, two BZD compounds were tested, i.e. the BZD, diazepam, and the TBZD, adinazolam; after acute administration, these compounds expectedly alleviated anxiety and expectedly acted via GABA and in a manner which was not specific to novelty. Therefore, the behavioral mechanism of action for “anxiety” and for “depression”, appears to be superimposed and it is reasonable to hypothesize that an overlap between neural transmitter mechanisms may exist as well.

A pivotal focus to the present studies, is the distinction between anti-anxiety agents which are called “typical” i.e., a BZD, like diazepam (Valium®), and those which are called “atypical”, i.e., a triazolobenzodiazepine (TBZD) such as adinazolam. (Deracyn®). One distinction between the typical and atypical BZD’s is seen in chemical structure; diazepam has a methoxy group in place of the triazolo group. Furthermore, the TBZDs, such as alprazolam (Xanax®) and triazolam (Halcion®), not only differ in structure from the BZDs but also differ in metabolic pathways (Gall *et al.*, 1978). Yet, TBZDs are very potent members of the BZD class and the TBZDs share with the BZDs, the same favorable therapeutic ratio. The actual discovery of the dual anxiolytic and antidepressant activity of the TBZDs

was made during the broad pharmacological and clinical screening of the TBZD series of compounds (Hester et al., 1971; Hester et al., 1980; Pyke et al., 1983; Hoehn-Saric, 1982). Indeed, the sister compound to adinazolam, alprazolam, differing from adinazolam only by a terminal aminoethyl group, has been commonly used in the clinical management of mild depressive syndromes with anxiety and insomnia (Sheehan, 1980; Chouinard et al., 1982; Shader et al., 1982; Fawcett et al., 1987).

Clearly, the discovery of the TBZDs represents a strategic step in the therapy of patients who present with anxiety concomitantly with depressive affect. Diazepam, the typical BZD, on the other hand, has no antidepressant activity clinically (Lafaille et al., 1991). The situation remains though, that the clinical results derived from the TBZDs on depression, have virtually never been explained mechanistically from a preclinical viewpoint. For example, although the NE and 5-HT monoamines are integral to current thinking about the neurochemistry of depression (Sulser, 1983; Asnis et al., 1992), adinazolam has been reported to have only weak inhibitory effects on NE reuptake (Sethy and Harris, 1981), no specific effect on inhibiting the reuptake of 5-HT (Lahti et al., 1983) and no effect on dorsal raphe (DR) 5-HT neuronal firing (Turnel and de Montigny, 1984). The present hypothesis was that the previous *in situ*, *in vitro* and anesthetized studies used, had inherent limitations in the technologies, which masked the detection of changes in the monoamines produced by adinazolam. Therefore, *in vivo* microvoltammetry with a miniaturized three-microelectrode potentiostat system was utilized to study the effects of adinazolam on CA<sub>1</sub> hippocampal NE and 5-HT release while infrared photocell beams, surrounding the faradaic shielded chamber, performed a computerized digital and analog record of each separate component of open-field behavior. Our hypothesis was that the uniquely high temporal and spatial resolution of both behavioral and neurochemical technologies would aid in revealing the neurochemical and behavioral effects of adinazolam.

Validation for the on-line NE signal came from the following studies which showed that (a) the NE peak increased after the administration of the  $\alpha_2$  adrenergic antagonist, yohimbine, and decreased after the administration of the  $\alpha_2$  adrenoceptor agonist, clonidine (Broderick, 1991a; Broderick, 1997) and (b) that the NE peak can be detected electrochemically by an oxidation reaction of this monoamine (Mermet et al., 1990; Sauad-Chagny et al., 1990). Validation for the 5-HT peak came from the following: (a) studies which showed that the 5-HT peak increased after the administration of the 5-HT precursor, l-tryptophan (Broderick and Jacoby, 1988), and (b) studies which showed that the 5-HT peak decreased after the administration of the 5-HT<sub>1A</sub> agonists, buspirone and ipsapirone (Broderick et al. 1989; Broderick and Piercey, 1990).

Multiple neurotransmitters are detected in one voltammogram or recording in a matter of seconds with *in vivo* microvoltammetry. In the present paper, NE and 5-HT are the focus because of their integral relationship with anxiety and depression. Detection of synaptic concentrations of NE and 5-HT in the CA<sub>1</sub> area of hippocampus, are release mechanisms, primarily. This conclusion is based not only on data, mentioned above, that adinazolam has only weak effects on NE reuptake and no effects on 5-HT reuptake *in vitro*, but also on data which showed that gamma-butyrolactone (GBL), a neuronal depolarization blocker (Walters and Roth, 1972), decreased 5-HT concentrations in the chloral hydrate anesthetized animal (Broderick, 1991b; Broderick, 1992).

The hippocampal formation is organized into subcortical subdivisions; CA<sub>1</sub> region of hippocampus, selected as the neuroanatomical site for these studies, is one subdivision within the subcortical region. The subcortical hippocampal formation is divided into hippocampus proper, also known as *Cornu Ammonis* (hence, CA or CA pyramid), the dentate gyrus and the subiculum; CA<sub>1</sub>, CA<sub>2</sub>, CA<sub>3</sub>, and CA<sub>4</sub> are further subdivisions of *Cornu Ammonis*. CA<sub>1</sub> pyramids are lamellar and the *moleculare*, the *radiatum*, the *pyramidale*, the *oriens* and the *alveus* comprise a pyramid (Ramon y Cajal, 1955). Pyramidal neurons of the CA<sub>1</sub> region, the *regio superior*, are medium sized and have intrinsic and extrinsic connections which innervate the subiculum and provide the origin of the hippocampal outflow in the fornix; longer projections innervate the nucleus accumbens of the ventral striatum, brain reward circuits, through the fornix (Amaral and Insausti, 1990).

Neurochemical studies of the CA<sub>1</sub> region have demonstrated that this region of hippocampus is densely innervated with 5-HT projections from 5-HT somatodendrites, DR (Jacobs and Azmitia, 1992). Moreover, a 5-HT innervation to the locus coeruleus (LC) has been demonstrated as well (Pickel *et al.* 1977); LC is a primary source of noradrenergic innervation to the hippocampus. Hippocampal pyramidal neurons of the CA<sub>1</sub> region exhibit a pharmacological profile similar to that of the 5-HT<sub>1p</sub>, 5-HT<sub>3</sub> and 5-HT<sub>4</sub> class (Andrade and Chaput, 1991). Importantly, the miniature sensors (microelectrodes used in these studies, have a sensitive spatial resolution, amenable for detection of NE and 5-HT within the CA<sub>1</sub> region of *Cornu Ammonis*. (Broderick, 1997).

Anxiety and depression are personified within the act of behaving. Anxiety and depression can be expressed in a variety of ways which are behaviorally dependent on the environment and specific behavioral tests and responses to novelty have played a substantial role in animal models of affective

disorders. Although open-field behaviors have been reliable assays in these animal models (Gray, 1971), simply monitoring a parameter like Ambulations in an open-field paradigm becomes a complex measure of both anxiety and exploratory behavior (Gray, 1982). Therefore, a separation of open-field behaviors was undertaken in these studies, to decipher four separate parameters of open-field behaviors, i.e., Ambulations, Rearing, Fine Movements and Central Ambulatory behavior.

Both hippocampal NE and 5-HT have been implicated in the process of behaving in an open-field paradigm. Interestingly, Oades considers hippocampal NE as a neuromodulator that can inhibit CA<sub>1</sub> neurons to simultaneously censor and select specific sensory stimuli (Oades, 1985). Moreover, in the *Behavioral Inhibition Theory*, hippocampal 5-HT has been considered significant as relevant to the “shift” mechanism changing from ongoing behavior to new behavior in a novel environment (Soubrie, 1986).

Therefore, the purpose of the present paper was to compare TBZDs (adinazolam) with BZDs (diazepam) in an *in vivo* paradigm with which we were able to use same animal control to examine electrochemical signals for NE and separate electrochemical signals for 5-HT, each within seconds of release, during time course studies. The purpose was to detect changes in synaptic concentrations of CA<sub>1</sub> hippocampal NE and 5-HT during the time in which open-field behavioral changes were actually occurring, in order to decipher a possible preclinical mechanism of action for the clinically documented dual antianxiety- antidepressant activity of adinazolam.

## Methods

### Animals

The studies were done with unrestrained, freely moving, male, Sprague Dawley rats (Charles River, Kingston, NY) (weight range 312 to 417 grams at the time of the *in vivo* electrochemical and behavioral (adinazolam) studies; 300 to 440 grams at the time of the *in vivo* electrochemical and behavioral (diazepam) studies). The animals were fed Purina Rat Chow and water *ad lib.* and were group housed before surgery and individually housed after surgery. A twelve hour dark/light cycle was maintained both during the housing of the laboratory rats and throughout the experimental studies. The animals were tested free from the following viruses: Sendai Virus, Kilham Rat Virus, Reo Virus Type 3, Sialodacryoadenitis Virus, Rat Corona Virus, Toolan's H1 Virus, Micro Plasma Pulmonis Virus, Lymphocytic Choriomeningitis Virus, Hantaan Virus and Encephalitozoon Cuniculi Virus. Virus free animals allow a safer surgical procedure and a more efficacious and faster recovery from surgery.

### Surgery

The general anesthetic, pentobarbital Na (50 mg/kg intraperitoneally (i.p.)) was used to produce surgical anesthesia. A supplemental dose of pentobarbital Na (0.10 cc of the same 6% solution) was administered once after the first two hours of surgery and another supplemental dose (0.05 cc) was administered in each of the two subsequent hours of surgery in order to maintain adequate anesthesia. Animals were tested for corneal, pinna, and leg flexion responses; the absence of these reflexes denoted adequate anesthetic induction. Body temperature was continuously monitored with a rectal thermometer (Fisher Sci., Fadem, NJ) and was maintained at  $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  with an aquamatic K module heating pad (Amer. Hosp. Supply, Edison, NJ). Animals were stereotaxically implanted with Broderick Probe® microelectrodes (made in this laboratory) within CA<sub>1</sub> region of hippocampus (AP=-2.6, ML=+2.25, DV=-2.7) (Pellegrino *et al.*, 1979). (Stereotaxic equipment was purchased from Kopf Stereotaxic, Tujunga, CA). Ag/AgCl reference microelectrodes and stainless steel auxiliary microelectrodes (made in this laboratory) were placed in contact with cortex. The three microelectrode assembly was held in place with dental acrylic (Kadon Cavity Liner, Caulk, Becker-Parkin Dental Supply Co. Inc., NY). Animals recovered in a bedded Plexiglas chamber (dimensions 12" x 12" x 18"). Animals were treated with physiological saline (body weight in cc/kg i.p.) immediately after surgery.

### *In Vivo* Electrochemical (Microvoltammetric) Biotechnology:

The methods for the manufacture of the three *in vivo* electrochemical microelectrodes are published by this laboratory (Broderick, 1989). A review of the historical and technical aspects of the field of *in vivo* electrochemistry is cited (Broderick, 1990). The ability to distinguish between DA and NE, by *in vivo* microvoltammetry has been published (Broderick, 1988). *In vivo* electrochemical current was measured in picoamperes (pA) as a function of the semidifferentiation of time in seconds. Current was directly proportional to the concentration of each neurotransmitter according to the Cottrell Equation, which equates concentration of molecules oxidized with the amplitude of the oxidation peak which denotes electrochemical current. Logarithmic regression analysis indicated that the lowest detection limits for NE and 5-HT *in vitro*, approached 6 nmoles and 3 nmoles, respectively in the first preconcentration step (Broderick, 1989; Broderick, 1993; Broderick, 1997). The current produced by the oxidation of NE within CA<sub>1</sub> region of hippocampus was detected in 10-15 seconds whereas that produced by 5-HT was detected in 10-12 seconds in each *in vivo* microvoltammetric scan. NE release and 5-HT release, at each data point, represented a measurement in pA of NE and 5-HT current within CA<sub>1</sub> region of hippocampus. Current for NE and 5-HT, was detected within the same synaptic environment.

*In vivo* microvoltammetric (semidifferential) studies on freely moving and behaving Sprague Dawley rats were begun approximately 18 to 21 days after the aseptic surgical procedures were performed. On each experimental day, an animal was placed in a Faradaic, Plexiglas chamber (dimensions: 24" x 18" x 23.5"). The three microelectrode assembly, enclosed within the animal's prosthetic acrylic cap, was connected to a CV37 detector (BAS, West Lafayette, IN) by means of a mercury commutator (Br. Res. Instr., Princeton, NJ), a flexible cable, and a mating connector (BJM Electronics, Staten Island, NY). The CV37 was electrically connected to a Minigard surge-suppressor (Jefferson Electric, Magnetek, NY), which was then connected to an isolated electrical ground. *In vivo* electrochemical signals for NE release and for 5-HT release within CA<sub>1</sub> region of hippocampus in the freely moving and behaving animal were detected every five minutes; each five minute period included a two minute cell deposition time. Stable *in vivo* electrochemical signals for NE release and 5-HT release were evident before either adinazolam (10 mg/kg i.p., first hour, 2 mg/kg i.p., second hour) or diazepam (1 mg/kg i.p., first hour, 3 mg/kg i.p., second hour) was administered. Adinazolam (Pharmacia and Upjohn Co., Kalamazoo, MI) or diazepam (Sigma, St. Louis, MO) was dissolved in doubly distilled water; solutions were made fresh on the day of each study.

#### Behavior:

The behavioral chamber was novel to each animal, although each animal was habituated before either adinazolam or diazepam was injected, i.p. The behavioral chamber was equipped with side-by-side double doors (dimensions 15.75" x 23.5") to enable a facile injection procedure. A series of infrared photobeams was encased in aluminum frames around the chamber's perimeter. When activated with an IBM computerized circuit, the infrared photobeams detected the animal's position in the behavioral chamber on an x-y axes positional basis. Thus, multiple concurrent measures of the animal's activity were simultaneously assayed. The specific activities of each animal assayed were: (1.) Ambulations (running-forward locomotion interacting with the maintenance of a horizontal position of the head without lateral turning (Teitelbaum et al., 1990)), (2.) Rearing (maximal upward vertical movement of the head, without lateral turning (Teitelbaum et al., 1990)), (3.) Fine Movements (combined stereotypic movements of head bobbing, sniffing, and grooming), and (4.) Central Ambulations: [(locomotor activity in the central part of the chamber which indicates movements away from the walls.) (Central Ambulatory behavior is called anti-agoraphobic (anti-thigmotactic) behavior; the behavior indicates a reduced fear on the part of the animal (Geyer, 1990)]. Mesolimbic 5-HT release has been shown to be closely temporally allied with natural open-field movement (Broderick and Phelix, 1997). The status of the infrared photobeams was sampled every 100 msec. Our system is a modified version of an Activity



Pattern Monitor (San Diego Instruments, San Diego, CA). Data were computerized and calculated as measures of concurrent and separate activities every five minutes, for each five minute time period, during time course studies, for two hours.

#### Confirmation of Microelectrode Placement:

Placement of indicator microelectrodes in CA<sub>1</sub> hippocampus was confirmed by the potassium ferrocyanide in 10% formalin, blue dot method, with transcardial perfusion (80 ml saline). The precise electrical specifications for deposition of the blue dot in CA<sub>1</sub> hippocampus was 50  $\mu$ A current in a 30 second time period. Virtually no damage to brain tissue occurred.

#### Statistical Analyses:

Statistically significant differences in NE release and 5-HT release within CA<sub>1</sub> region of hippocampus in the freely moving and behaving animal, between drug treatment and same animal control, was determined by One Way Analysis of Variance (ANOVA) applying the post hoc tests, Dunnett's or Student-Newman-Keuls Multiple Comparison Methods for equal variances. Kruskal-Wallis One Way ANOVA on Ranks with post hoc test, Dunn's Multiple Comparison Method, was used in the event of unequal variances. Neurochemical results are expressed as % of control to minimize animal variations. Each animal was studied as its own control. Each component of open-field behavior [(1.) Ambulations (running-forward locomotion), (2.) Rearing, (3.) Fine Movements and (4.) Central Ambulations], was statistically analyzed, using the same statistical tests, described above. Behavioral results are expressed as frequency of events. Each animal was used as its own control. Statistics revealed the most dramatic differences between adinazolam and diazepam in the directional response occurred with the monoamine, 5-HT. Therefore, 5-HT results are presented first.

### Results

Adinazolam and 5-HT. Figure 1 shows the effect of adinazolam (10 mg/kg i.p., first hour, and an additional 2 mg/kg i.p., second hour) on synaptic concentrations of 5-HT within the CA<sub>1</sub> region of hippocampus of the freely moving and behaving, male, Sprague Dawley rat. Adinazolam significantly increased the *in vivo* electrochemical signal for 5-HT (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.0001$ ,  $H = 19.9$ ,  $df = 2$  ( $N = 4$ ) (unequal variance)). Post hoc analysis further showed that there were statistically significant differences from baseline in the second hour of study (Dunn's Method:  $p < 0.05$ ,

$q=4.06$  (second hr.)).

Serotonin release was increased to 115% over baseline (baseline=100%) within 10 minutes and was maximally increased to 209% above baseline within 90 minutes after adinazolam administration. Further post hoc analysis, which delineated one-half hour effects of adinazolam on 5-HT release in hippocampus, showed that each of the four one-half hour effects was statistically increased over baseline (Dunnett's Method:  $p<0.05$ ;  $q=4.55$ ,  $q=8.04$ ,  $q=10.02$ ,  $q=12.12$ : one-half hours 1 through 4 respectively) (equal variance). This post hoc analysis followed a One Way ANOVA:  $p<0.0001$ ,  $F=46.4$ ,  $df=4,23$  ( $N=4$ ) (equal variance). Dramatic increases in 5-HT release occurred at the 30 min. mark. Mean increases were 155.5% (first hour) and 197.5% (second hour).

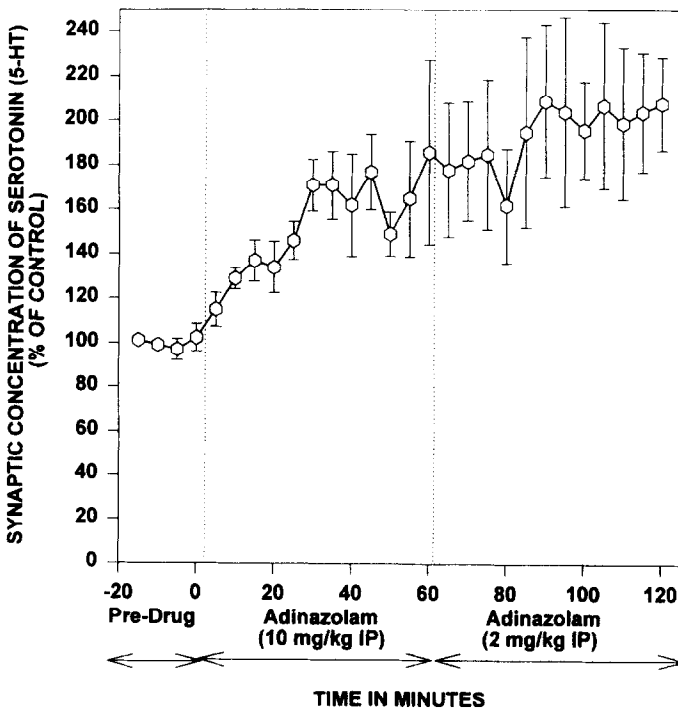


Fig 1. Time course studies showing the effect of adinazolam on synaptic concentrations of 5-HT within seconds of release, within CA<sub>1</sub> region of hippocampus in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of endogenous synaptic concentrations of 5-HT ( $N=4$ ) followed by mean  $\pm$  SE of changes in synaptic concentrations of 5-HT produced by adinazolam ( $N=4$ ; same animal control).

**Adinazolam and NE.** Figure 2 shows the effect of adinazolam (10 mg/kg i.p., first hour, and an additional 2 mg/kg i.p., second hour) on synaptic concentrations of NE within CA<sub>1</sub> region of hippocampus of the freely moving and behaving, male, Sprague Dawley rat. Adinazolam significantly decreased the *in vivo* electrochemical signal for NE (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.0001$ ,  $H = 18.7$ ,  $df = 2$  ( $N = 4$ ) (unequal variance)). Post hoc analysis showed that there was a statistically significant difference from baseline values in the second hour of the two hour time course studies (Dunn's Method:  $p < 0.05$ ,  $q = 3.492$ ). Initially, NE release was increased 114% over baseline (baseline = 100%) within 5 minutes, then remained increased for 15 minutes, returned to baseline at the 20 minute mark, was decreased 28% within 50 minutes and eventually decreased to 41% below baseline. Within 90 minutes after adinazolam administration, a maximal 47% decrease in NE release below baseline was exhibited. Due to the biphasic properties exhibited by adinazolam on hippocampal NE release, the mean decrease in the first hour was 8.2%, whereas the mean decrease in NE release observed in the second hour of study was 36.3% below baseline values.

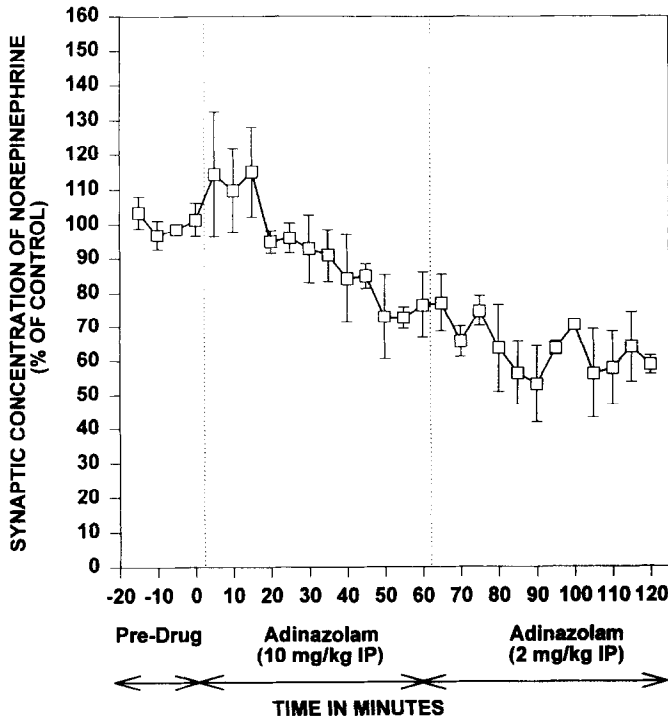


Fig 2. Time course studies showing the effect of adinazolam on synaptic concentrations of NE within seconds of release, within CA<sub>1</sub> region of hippocampus in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of endogenous synaptic concentrations of NE ( $N = 4$ ) followed by mean  $\pm$  SE of changes in concentrations of NE produced by adinazolam ( $N = 4$ ; same animal control).

Adinazolam and Frequency of Ambulations. Figure 3 shows the effect of adinazolam (10 mg/kg i.p. first hour, and an additional 2 mg/kg i.p., second hour) on the frequency (number) of Ambulations (running in forward locomotion) in each animal in which 5-HT release and NE release were detected *on-line* with *in vivo* microvoltammetry. Adinazolam exhibited biphasic behavioral properties. Adinazolam increased and then subsequently decreased the frequency of Ambulations; this biphasic effect was statistically significant (One Way ANOVA,  $p < 0.0046$ ,  $F = 5.05$ ,  $df = 4, 23$  ( $N = 4$ ) (equal variance)). Although adinazolam treated animals exhibited hyperactive behavior for 20 minutes, within

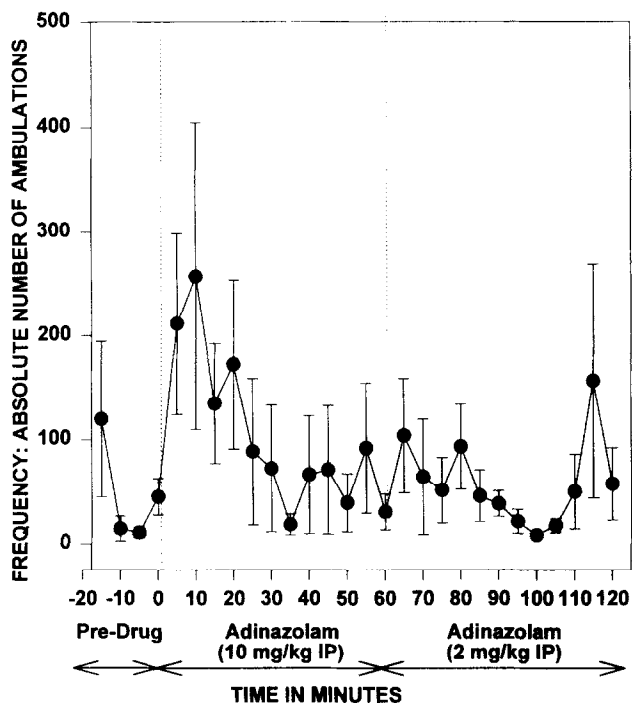


Fig 3. Time course studies showing the effect of adinazolam on Ambulatory behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Ambulatory behavior ( $N = 4$ ) followed by mean  $\pm$  SE of changes in Ambulatory behavior produced by adinazolam ( $N = 4$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before adinazolam injection.

30 minutes, the animals displayed sporadically reduced Ambulatory Movements by more than 50%. When adinazolam did not dramatically reduce Ambulations during the first hour of the time course studies, baseline conditions reappeared, actually followed by increases at the 40, 45, and 55 minute time points. After the second injection of adinazolam (2 mg/kg), although photobeam interruptions increased

from 31 to 104, statistical significance did not occur in this half-hour. At the 90 minute mark, another series of reductions occurred, eventually resulting in an increase of 10 photobeam interruptions at the end of the time course study. Indeed, 50% of the animals exhibited dramatic increases in Ambulatory activities at time points 15 and 115 and consequently larger standard errors occurred. Notably, initial increases in Ambulatory activity are consistent with the increases in 5-HT release and the initial increase in NE release. Post hoc analysis showed that adinazolam significantly increased the frequency of Ambulations (Dunnett's Method:  $p < 0.05$ ,  $q = 3.412$ ) in the first half-hour after adinazolam injection. The other three "q" values for the final three half-hours were not statistically significant over baseline;  $q = 0.168$ ,  $q = 0.585$ ,  $q = 0.134$ .

**Adinazolam and Frequency of Rearings.** Figure 4 shows the effect of adinazolam (10 mg/kg i.p., first hour, and an additional 2 mg/kg i.p., second hour) on the frequency of Rearings; each animal had

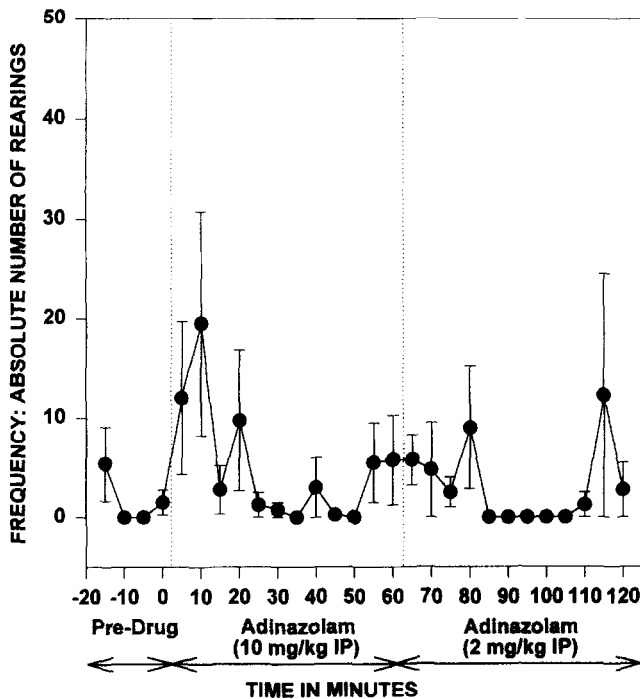


Fig 4. Time course studies showing the effect of adinazolam on Rearing behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Rearing (N=4) followed by mean  $\pm$  SE of changes in Rearing behavior produced by adinazolam (N=4; same animal control). Behavioral chamber was novel to each animal; animals were habituated before adinazolam injection.

5-HT release and NE release monitored *on line*, with *in vivo* microvoltammetry, and Ambulatory behavior was also concurrently recorded. Adinazolam did not significantly affect Rearing frequency (One Way ANOVA:  $p < 0.2605$ ,  $F = 1.42$ ,  $df = 4, 23$  ( $N = 4$ ) (equal variance)). However, Rearing frequency was increased in the first half-hour post-injection when Rearing events increased from a mean baseline value of 2 to a mean value of 8. Then, Rearing behavior dramatically decreased to mean values of 2, 4, and 3 respectively. However, similarly to the Ambulatory behavior exhibited post-adinazolam, there were spurts of increased Rearing behavior demonstrated throughout the 2 hour time course study, e.g. Rearing events numbered 12 at the 115 minute time point.

Adinazolam did not significantly increase the Rearing frequency when the first and second hour, post-adinazolam, are individually compared to the baseline (Kruskal-Wallis One Way ANOVA on Ranks:  $p = 0.3978$ ,  $H = 1.84$ ,  $df = 2$  ( $N = 4$ ) (unequal variance)). Further analysis showed that there was no statistically significant difference from baseline in the thirty minutes following each injection. (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.236$ ,  $H = 2.88$ ,  $df = 2$  ( $N = 4$ ) (unequal variance)).

**Adinazolam and Frequency of Fine Movements.** Figure 5 shows the concurrent effect of adinazolam (10 mg/kg i.p., first hour, and an additional 2 mg/kg i.p., second hour) on the frequency of Fine Movements in each animal in which 5-HT release and NE release were detected *on line*, with *in vivo* microvoltammetry, and in which Ambulations and Rearing frequency were monitored concurrently. Adinazolam significantly increased the frequency of Fine Movements (Stereotypy) (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.003$ ,  $H = 16.0$ ,  $df = 4$ , ( $N = 4$ ) (unequal variance)). Stereotypic fine movement events increased from a mean of 1 (baseline) to a mean of 7, 6, 4 and 0 in the first half-hour, second half-hour, third half-hour, and fourth half-hour of study respectively. Q numbers derived from the post hoc test (Dunn's Method) showed that only the first half-hour effect was significant ( $p < 0.05$ ,  $q = 2.619$ ).

Adinazolam's dramatic effects on increased stereotypy (Fine Movements) were nine-fold in the first five minutes post-injection period, as photobeam interruptions went from a baseline of 2 photobeam interruptions to 18 photobeam interruptions. Again, animals exhibited spurts of activity in the second hour at the 2 mg/kg supplemental doses. Sporadic increases were interspersed with sedative profiles.

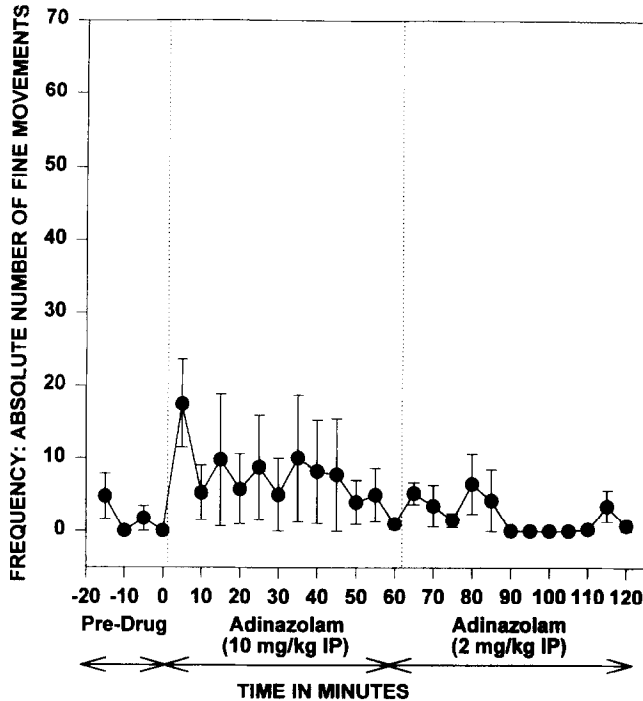
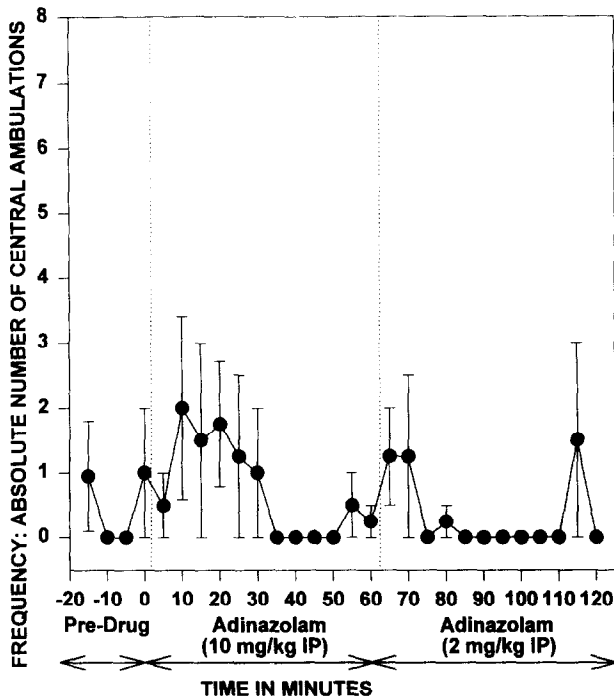


Fig 5. Time course studies showing the effect of adinazolam on Fine Movement behavior (Stereotypic Fine Movements of grooming and sniffing) in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Fine Movement behavior ( $N=4$ ) followed by mean  $\pm$  SE of changes in Fine Movement behavior produced by adinazolam ( $N=4$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before adinazolam injection.

Adinazolam and Frequency of Central Ambulations. Figure 6 shows the concurrent effect of adinazolam (10 mg/kg i.p., first hour, and an additional 2 mg/kg i.p., second hour) on the frequency of Central Ambulations (anti-agoraphobia) in each animal in which 5-HT release and NE release were detected *on line*, with *in vivo* microvoltammetry, and in which Ambulations, Rearing, and Fine Movement frequencies were monitored concurrently. Adinazolam increased and subsequently decreased the frequency of Central Ambulations; this biphasic effect was statistically significant (Kruskal-Wallis One Way ANOVA on Ranks,  $p < 0.0197$ ,  $H = 11.7$ ,  $df=4$  ( $N=4$ ) (unequal variance)). Although an increase in the Central Ambulatory behavior occurred in the first half-hour, the Dunn's Method "q" value was 2.0069 ( $p > 0.05$ ). Adinazolam began to exhibit anti-agoraphobia behavior ten minutes after injection, at which time, the increased response was approximately three-fold higher than the mean baseline for the Central Ambulatory response. Photobeam interruptions, which denote anti-agoraphobic behavior, are increased Ambulations (locomotor activity) into the center of the faradaic, behavioral

chamber. The increased Central Ambulatory response of adinazolam continued for thirty minutes post-injection at which time, Central Ambulatory movements decreased to zero. Five minutes after the second injection, Central Ambulatory activity increased five-fold over the last time point in the first hour. Moreover, the Central Ambulatory response diminished to zero at the twenty minute mark at the second dose of adinazolam. Again, at the 115 minute mark, Central Ambulatory behavior increased three-fold above baseline. Thus, adinazolam's anti-anxiety effect can be discerned in this paradigm. The transient increases and decreases in Central Ambulatory behavior are similar to the pattern of Ambulatory, Rearing, and stereotypic Fine Movement behavior, seen in Figs 3, 4, and 5.

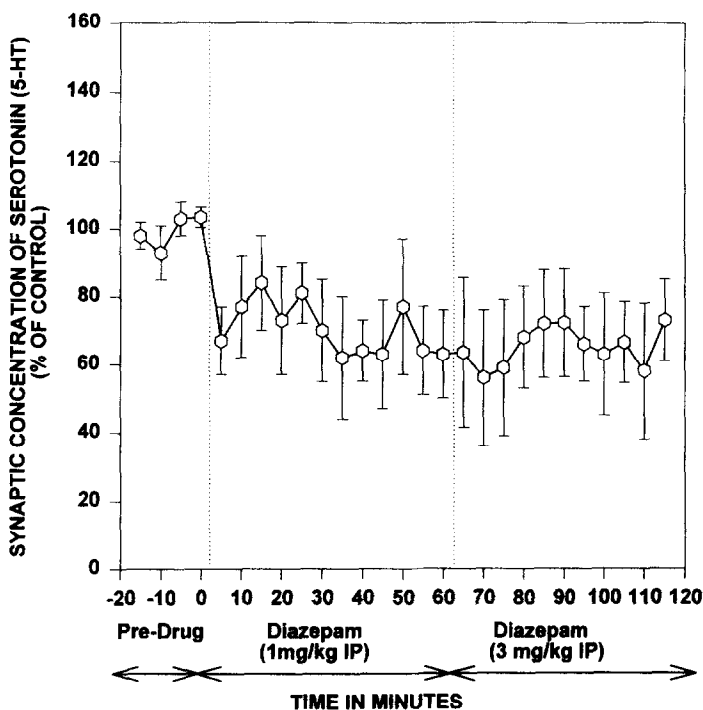


**Fig 6:** Time course studies showing the effect of adinazolam on Central Ambulatory behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Central Ambulatory behavior (N=4) followed by mean  $\pm$  SE of changes in Central Ambulatory behavior produced by adinazolam (N=4; same animal control). Behavioral chamber was novel to each animal; animals were habituated before adinazolam injection.

**Diazepam and 5-HT.** Figure 7 shows the effect of diazepam (1 mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on synaptic concentrations of 5-HT within CA<sub>1</sub> region of hippocampus of the freely moving and behaving, male, Sprague Dawley rat. Diazepam significantly decreased the *in vivo* electrochemical signal for 5-HT (One Way ANOVA:  $p < 0.0001$ ,  $F = 33.9$ ,  $df = 2, 25$  (N=5) (equal



variance)). Post hoc analysis showed that there was a statistically significant difference from baseline values in both the first and the second hours of the two hour time course study (Student-Newman-Keuls Method:  $p < 0.05$ ,  $q = 9.92$  (first hour), and  $q = 11.47$  (second hour)). The neurochemical profile for the effect of diazepam on hippocampal 5-HT release was interesting; the effect was not dose-dependent; the maximum decrease was approximately 45% below baseline values (baseline=100%). This occurred ten minutes after the second dose of diazepam (3 mg/kg i.p.) was administered. However, it is important to note that diazepam (at the 1 mg/kg dose) immediately decreased 5-HT release within CA<sub>1</sub> region of hippocampus to 32.5% below baseline values. No significant difference occurred between the first hour data (1 mg/kg i.p.) and the second hour data (3 mg/kg) (Student-Newman-Keuls Method:  $p > 0.05$ ,  $q = 2.19$ ). The mean decrease in 5-HT release after diazepam was 29% (first hour) and 34% (second hour). A significant return to baseline was not seen at the end of the 2 hour study.



**Fig. 7.** Time course studies showing the effect of diazepam on synaptic concentrations of 5-HT within seconds of release, within CA<sub>1</sub> region of hippocampus in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of endogenous synaptic concentrations of 5-HT (N=5) followed by mean  $\pm$  SE of changes in synaptic concentrations of 5-HT produced by diazepam (N=5; same animal control).

**Diazepam and NE.** Figure 8 shows the effect of diazepam (1 mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on synaptic concentrations of NE within CA<sub>1</sub> region of hippocampus of the

freely moving and behaving, male, Sprague Dawley rat. Diazepam significantly decreased the *in vivo* electrochemical signal for NE (One Way ANOVA:  $p < 0.0002$ ,  $F = 11.8$ ,  $df = 2, 25$  ( $N = 5$ ) (equal variance)). Post hoc analysis further revealed that the significant effect of diazepam on NE release in CA<sub>1</sub> region of hippocampus occurred at both doses of diazepam studied, i.e. the 1 mg/kg dose and the 3 mg/kg dose (Student-Newman-Keuls-Method:  $p < 0.05$ ,  $q = 5.05$  (first hour) and  $q = 6.88$  (second hour)). The effect of diazepam on hippocampal NE release exhibited a similar profile to the colocalized 5-HT release effects within the same synaptic terminal fields within hippocampus. A dose dependent effect did not occur; no significant effect between the first hour results (1mg/kg) and the second hour results (3mg/kg) were seen (Student-Newman-Keuls-Method:  $p > 0.05$ ;  $q = 2.59$ ). Immediately within 5 minutes of injection, diazepam decreased NE release within CA<sub>1</sub> region of hippocampus to 20% below baseline values; at the 25 minute mark, NE release began to show a return to baseline values but remained at about 5% below baseline at the end of the first hour of study. Again, an immediate decrease in NE release occurred,

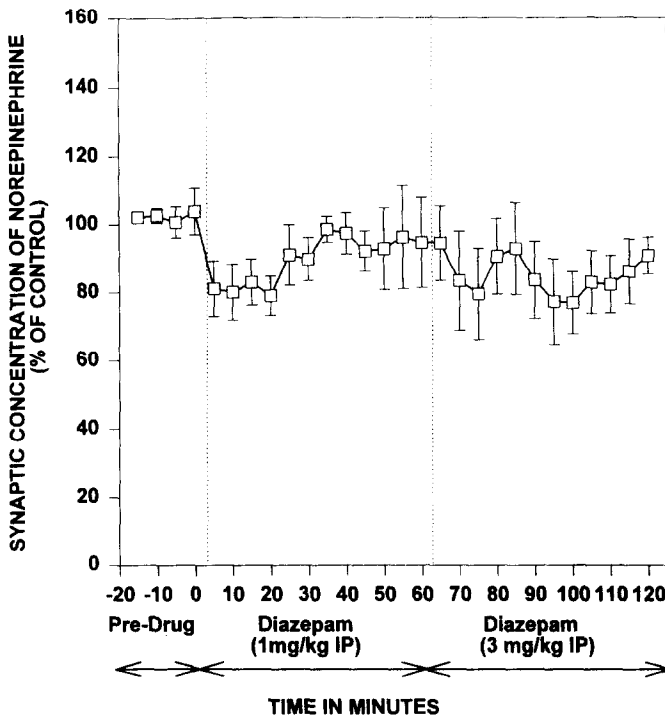


Fig 8. Time course studies showing the effect of diazepam on synaptic concentrations of NE within seconds of release, within CA<sub>1</sub> region of hippocampus in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of endogenous synaptic concentrations of NE ( $N = 5$ ) followed by mean  $\pm$  SE of changes in synaptic concentrations of NE produced by diazepam ( $N = 5$ ; same animal control).

to approximately 20% below baseline after the second dose of diazepam; the second dose produced a significant, more lasting, yet modest effect on hippocampal NE release. A trend for NE to return to baseline, after the 3 mg/kg dose of diazepam, was observed. The maximum decrease in NE release remained in the 20% below baseline range throughout the two hour time course of study. The mean decrease in NE release after diazepam was 10% in the first hour (first dose) and 15% in the second hour (second dose).

Diazepam and Frequency of Ambulations. Figure 9 shows the effect of diazepam (1mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on the frequency (number) of Ambulations (running in forward locomotion) (locomotor activity) in each animal in which 5-HT release and NE release were detected, *on-line* and concurrently. Diazepam significantly decreased the frequency of Ambulations in the 2 hour period of study (One Way ANOVA:  $p < 0.0032$ ,  $F = 7.31$ ,  $df = 2, 25$  ( $N = 5$ ) (equal variance)). Post hoc analysis showed that there were statistically significant differences from baseline in both hours

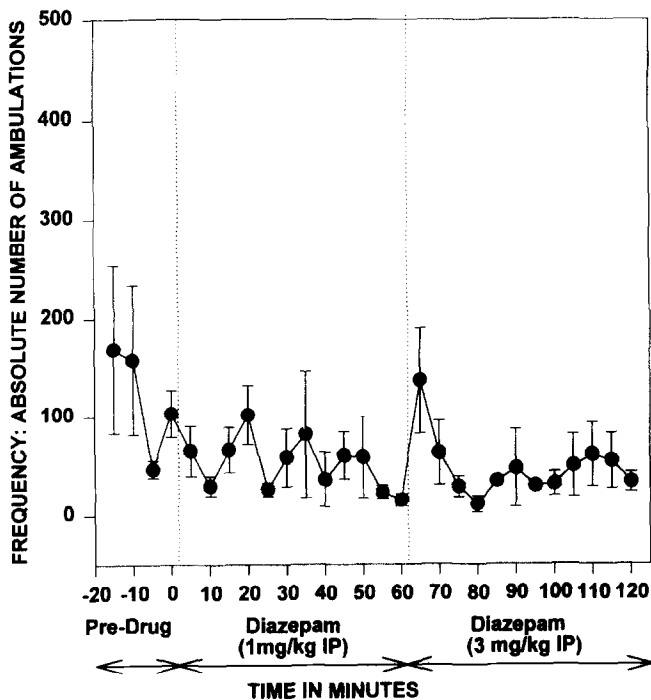


Fig 9. Time course studies showing the effect of diazepam on Ambulatory behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Ambulatory behavior ( $N = 5$ ) followed by mean  $\pm$  SE of changes in Ambulatory behavior produced by diazepam ( $N = 5$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before diazepam injection.

(i.e. at both doses), during the two hour time course of study (Student-Newman-Keuls-Method:  $p < 0.05$ ,  $q = 4.919$  (first hour) and  $q = 5.176$  (second hour)). The effect of diazepam on Ambulations exhibited a similar profile to that of its effect on synaptic concentrations of 5-HT release and NE release in hippocampus; there was no statistically significant difference seen, when first hour dose effects were compared with second hour second dose effects (Student-Newman-Keuls-Method:  $p > 0.05$ ,  $q = 0.363$ ). Ambulations remained below baseline values throughout the two hour period of study.

**Diazepam and Frequency of Rearings.** Figure 10 shows the effect of diazepam (1mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on the frequency of Rearings in each animal in which 5-HT release and NE release were detected, *on-line*, with *in vivo* microvoltammetry, and in which Ambulations activity patterns were monitored concurrently. Diazepam significantly decreased Rearing behavior (Kruskal-Wallis One Way Anova on Ranks:  $p < 0.0053$ ,  $H = 10.5$ ,  $df = 2$  ( $N = 5$ ) (unequal variance)). Post hoc analysis of the data further revealed a significant decrease in Rearing behavior after diazepam occurred in both hours, i.e. at the 1mg/kg dose and at the 3 mg/kg dose (Dunn's Method:

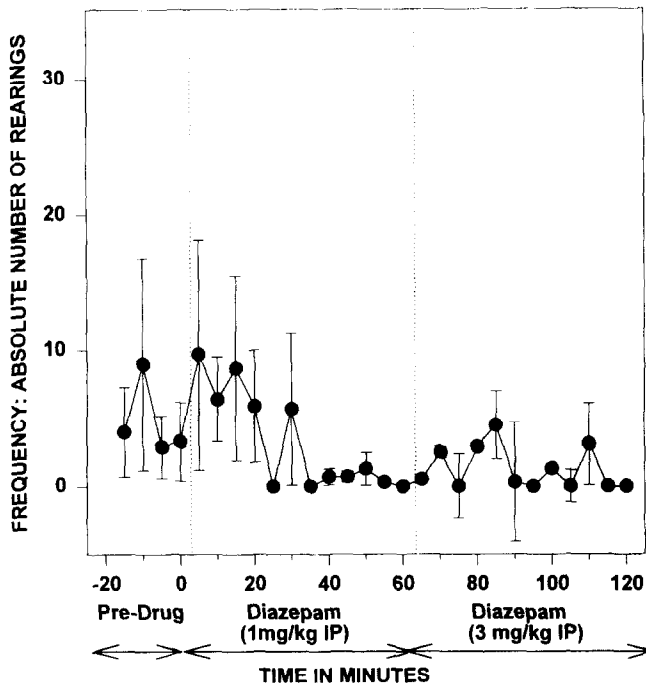


Fig 10. Time course studies showing the effect of diazepam on Rearing behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Rearing behavior ( $N = 5$ ) followed by mean  $\pm$  SE of changes in Rearing behavior produced by diazepam ( $N = 5$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before diazepam injection.

$p < 0.05$ ,  $q = 2.52$  (first hour and  $q = 3.24$  (second hour)). No statistically significant difference occurred between the two dose effects (Dunn's Method:  $p > 0.05$ ,  $q = 1.01$ ). Therefore, the effects of diazepam on Rearing behavior were not dose dependent at the doses studied. It is noteworthy that the initial effect, i.e. the first four time points after diazepam injection were similar to baseline values, but the standard errors were correspondingly high; the unusual response effect of one animal contributed to this effect.

**Diazepam and Frequency of Fine Movements.** Figure 11 shows the effect of diazepam (1 mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on the frequency (number) of Fine Movements in each animal in which 5-HT release and NE release were detected, *on-line*, with *in vivo* microvoltammetry, and in which ambulatory and rearing activity patterns were monitored concurrently, *on-line*, by infrared photobeams. Diazepam significantly decreased Fine Movement behavior (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.0095$ ,  $H = 9.30$ ,  $df = 2$  ( $N = 5$ ) (unequal variance)). Post hoc

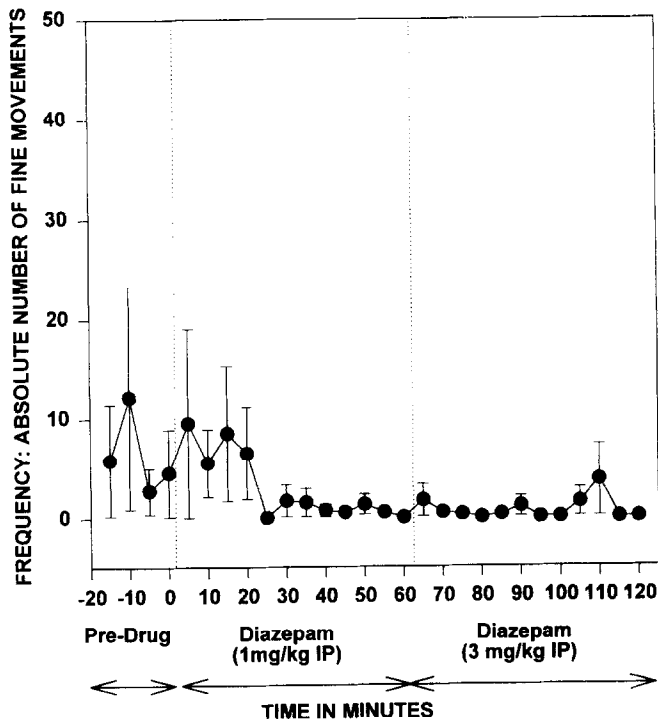


Fig 11. Time course studies showing the effect of diazepam on Fine Movement behavior (Stereotypic Fine Movements of grooming and sniffing) in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Fine Movement behavior ( $N = 5$ ) followed by mean  $\pm$  SE of changes in Fine Movement behavior produced by diazepam ( $N = 5$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before diazepam injection.

analysis revealed that the statistically significant decrease occurred at the 3 mg/kg dose of diazepam (Dunn's Method:  $p < 0.05$ ,  $q = 2.90$ ). There was no statistically significant difference in effects between the first hour dose (1 mg/kg) and the second dose (3 mg/kg) (Dunn's Method:  $p > 0.05$ ,  $q = 1.90$ ). The initial Fine Movement response to diazepam, at the 1 mg/kg dose, occurred similarly to the Rearing response to diazepam at the 1 mg/kg dose of diazepam. It was again, the unusual response of one animal which contributed to this behavioral effect; it was, in fact, the same animal who had provided the unusual Rearing response.

Diazepam and Frequency of Central Ambulations. Figure 12 shows the effect of diazepam (1mg/kg i.p., first hour, and an additional 3 mg/kg i.p., second hour) on the frequency (number) of Central Ambulations (anti-agoraphobia, anti-thigmotactic behavior) in each animal in which hippocampal 5-HT release and NE release were detected, *on-line*, with *in vivo* microvoltammetry, and in the same animals

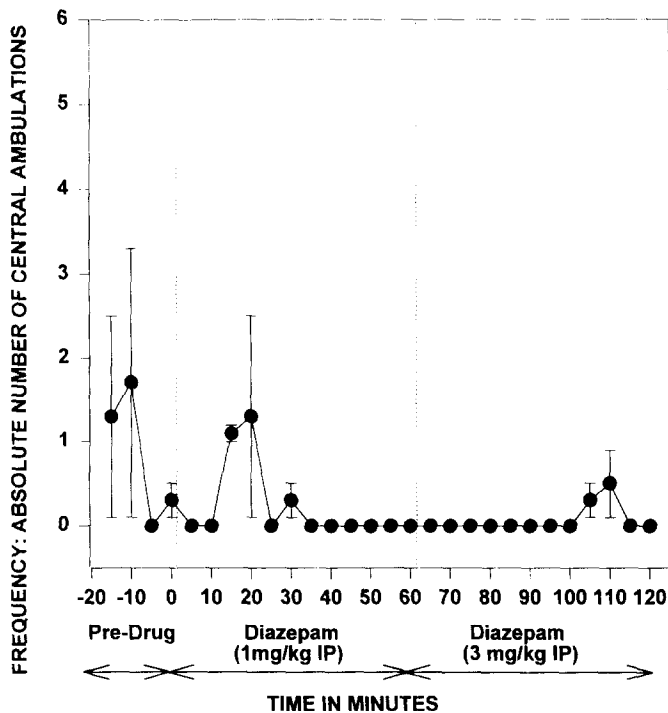


Fig 12. Time course studies showing the effect of diazepam on Central Ambulatory behavior in freely moving and behaving male, Sprague Dawley rats. Each data point represents the mean  $\pm$  SE of natural Central Ambulatory behavior ( $N=5$ ) followed by mean  $\pm$  SE of changes in Central Ambulatory behavior produced by diazepam ( $N=5$ ; same animal control). Behavioral chamber was novel to each animal; animals were habituated before diazepam injection.

in which Ambulatory, Rearing, and Fine Movement activity patterns were monitored concurrently, *on-line*, by infrared photobeam detection. Diazepam significantly enhanced the agoraphobic behavior (little or no entries into the center of the chamber) (Kruskal-Wallis One Way ANOVA on Ranks:  $p < 0.0344$ ,  $H = 6.74$ ,  $df = 2$  ( $N = 5$ ) (unequal variance)). The post hoc analysis showed a similar profile to the effect of diazepam on Fine Movement behavior, (3mg/kg effects) (Dunn's Method:  $p < 0.05$ ,  $q = 2.481$ ). Since increased Ambulations in the center of the chamber has been associated with a fear reduction response [anxiety reducing (anxiolytic) effect] of a drug (Geyer, 1990), it is noteworthy that diazepam produced a marked sedation which masked its anxiolytic properties in this Central Ambulatory paradigm.

### Discussion

#### Anxiety and Depression:

Always pertinacious has been the role for 5-HT in the mechanism of action of drugs classically used to treat depression (Coppen *et al.*, 1963; Walinder *et al.*, 1976; Menkes *et al.*, 1980; Wang and Aghajanian, 1980; van Praag, 1981; Broderick and Lynch, 1982; Charney *et al.*, 1984; de Montigny *et al.*, 1984; Asberg *et al.*, 1985; Goodwin, 1986; Price *et al.*, 1986; Blier *et al.*, 1987; Broderick *et al.*, 1989; Mann *et al.*, 1989; Broderick and Piercey, 1990, 1991; Broderick, 1991a; Post *et al.*, 1992; Mongeau *et al.*, 1993; Dubovsky, 1994; Preskorn *et al.*, 1994; Baldessarini, 1996; Broderick, 1997). Kline (1974) though, showed that measurable improvement after 5-HT drugs such as the TCAs, could take as long as two to three weeks to provide adequate pharmacotherapy for depression. Importantly, the TBZD, adinazolam, has been shown to produce a marked improvement within two to seven days post initiation of treatment (Pyke *et al.*, 1983). Therefore, deciphering the mechanism of action for the TBZDs is critical for assessing a tactical manipulation of the synapse, in order to determine strategies for improving antidepressant therapy.

#### Serotonin, Adinazolam and Diazepam:

This paper is the first report of an immediate action of the TBZD, adinazolam on 5-HT after acute (single) injection. The present results show that the mechanism of action of the TBZD, adinazolam is dramatically different from that of the classical BZD, diazepam. Adinazolam increased 5-HT release within the CA<sub>1</sub> region of hippocampus while the animal is freely moving and behaving. This response was not at all like the response of the classical BZD, diazepam, which decreased 5-HT release within CA<sub>1</sub> region of hippocampus using the same freely moving paradigm. The present data support previous

preclinical data which indicate that (a) the TBZD, adinazolam, acts through the monoamine, 5-HT (Turmel and de Montigny, 1984), (b) the BZD, diazepam, decreased hippocampal 5-HT release, which was increased by movement in the X-Maze in the freely moving rat (Wright et al., 1992) and (c) the TBZD, alprazolam also increased CA<sub>1</sub> hippocampal 5-HT release (Broderick, 1997). Furthermore, our results are consistent with the previous studies of Turmel and de Montigny (1984) in the sense that the TBZD, adinazolam, had different effects on 5-HT-ergic function than did the BZD, diazepam. In the Turmel and de Montigny studies, diazepam did not induce 5-HT sensitization in neuronal firing within the same 5-HT somatodendrites in which adinazolam did induce 5-HT sensitization in neuronal firing. The present results are also consistent with another previous study which reported that adinazolam inhibited aggressive behavior (Kostowski et al., 1986). Diminished aggressive behavior through an increase in central 5-HT levels, has been previously reported (Malick, 1978; Broderick and Lynch, 1982). Moreover, our hypothesis that a more sensitive technique such as *in vivo* microvoltammetry would better reveal 5-HT effects of adinazolam with better temporal and spatial resolution, is supported.

#### Norepinephrine, Adinazolam and Diazepam:

An overview of the scientific literature reveals that the TBZDs differ strikingly in pharmacological activity from the BZDs, in that the TBZDs show positive results in some assays used to test antidepressant activity (Hester et al., 1980; Lahti et al., 1983; Sethy et al., 1984). The present results agree with these positive results, e.g., the report of potentiation of NE-induced enhancement of blood pressure (Sethy and Hodges, 1982). The present data may lend explanation to previous discrepant preclinical data in the literature, about adinazolam, which directly conflict with the data from the clinical literature. To reiterate, the previous data reported that adinazolam was a weak inhibitor of NE reuptake at the synapse, *in vitro*, and adinazolam, when chronically administered, neither reduced rat cortex beta adrenergic receptor binding (Sethy and Harris, 1981), nor sensitized rat hippocampal pyramidal neurons to microiontophoretically applied NE (Turmel and de Montigny, 1984). Moreover, it appears that a notable difference occurs between the mechanism of action of the TBZD, adinazolam, versus that of the BZD, diazepam where NE is concerned. Whereas adinazolam initially increased NE release and then decreased NE release in CA<sub>1</sub> region of hippocampus in the freely moving and behaving rat, diazepam only decreased the *in vivo* electrochemical signal for NE. Moreover, the adinazolam-induced decrease in NE release was greater than the decrease in NE release produced by diazepam. Using the *in vivo* microvoltammetric biotechnology, with highly sensitive temporal and spatial resolution in the freely moving and behaving rat, a magnitude difference in NE effects between adinazolam and diazepam was revealed. Adinazolam produced a biphasic response on CA<sub>1</sub> hippocampal NE release whereas diazepam produced a monophasic response.



### Adinazolam: Specific Effects of Anesthesia:

The present results confirm the hypothesis that anesthesia could have been masking the effects of adinazolam on the monoamines, NE and 5-HT, as reported in the previous literature. Anesthetic effects of the TBZDs on 5-HT release are especially intriguing because in a similar series of studies from this laboratory, adinazolam did not produce increased 5-HT release in the CA<sub>1</sub> region of hippocampus in the chloral hydrate anesthetized animal after acute (single) injection (Broderick, 1991a). Although adinazolam has been shown to be well tolerated in patients who must undergo surgery under general anesthesia (Othmer and Othmer, 1988), the direct *on-line* effects of anesthesia on neurotransmitter function and release, during adinazolam treatment, deserves consideration. The lack of adinazolam's effect to modify dorsal raphe 5-HT neuronal firing after acute injection (Turmel and de Montigny, 1984), might be dependent, in part, on anesthesia, since Aghajanian showed in 1978 that administration of 5-HT in nerve terminals, should suppress DR neuronal firing. Moreover, BZDs did not alter the spontaneous firing rate of the DR, even though both systemic and iontophoretic administration of BZDs were found to potentiate the inhibitory effects produced by GABA on DR (Gallagher, 1978). Nerve cell firing rates have previously been shown to be affected by anesthesia, particularly in the case of catecholamines (Kelland *et al.*, 1989). Therefore, anesthetic effects may well alter nerve cell firing rates, *a priori*, thereby masking the effects of adinazolam on 5-HT.

This laboratory has studied the potency of chloral hydrate anesthesia to affect endogenous 5-HT release (Broderick, 1991b), using the neuronal impulse inhibitor and depolarization blocker, gamma-butyrolactone, (GBL), (Walters and Roth, 1972, 1974). In the presence of chloral hydrate, GBL, which is also an anesthetic, (Winters and Spooner, 1965), neuronal 5-HT depolarization was blocked, thereby, decreasing 5-HT release which is dependent on impulse flow. On the other hand, in the freely moving and behaving rat paradigm, when neuronal impulse flow was blocked by GBL, but chloral hydrate was not present, 5-HT release was not blocked (Broderick, 1992); these data suggest that GBL blocks inhibitory 5-HT autoreceptor mechanisms, which results in enhanced 5-HT release. These results indicate that chloral hydrate may act to maintain inhibitory presynaptic autoreceptors and that 5-HT neuronal impulses subsequently differ when anesthesia is present. Taken together, the data suggests that adinazolam's effects on 5-HT release in the chloral hydrate versus the freely moving rat paradigm, may depend on presynaptic 5-HT autoreceptors. Thus, we agree with Blier *et al.*, (1987), that critical mechanisms for antidepressant action reside in presynaptic 5-HT autoreceptors.

### Behavior: Sedative Effects:

The Behavioral Activity Patterns gleaned from adinazolam vs. diazepam administration, demonstrate that adinazolam inhibited, at least in part, the normal agoraphobia (anxiety) behavior, which is usually observed after an animal is placed in a novel environment (Geyer, 1990). In contrast, diazepam's known anti-anxiety effect was masked by its sedative properties. The sedative effect produced by diazepam, even at low doses, is clearly more potent than that observed during adinazolam administration. The Behavioral Activity Patterns, which were derived from the same animals and at the same time as the *in vivo* electrochemical signals for NE release and 5-HT release were detected *on-line*, showed that the TBZD, adinazolam, produced a significant reduction in three of the four open-field behavioral parameters, at least in certain specific time periods. Adinazolam did not significantly decrease the frequency on Rearing Behavior, for example. However, the adinazolam treated animals exhibited significant spurts of increased open-field behavioral activity distinct from the uninterrupted sedative behavior which was produced by diazepam.

That adinazolam produced some sedative properties in the clinical situation has been reported (O'Connor et al., 1985; Amsterdam et al., 1986; Feighner, 1986; Cohn et al., 1988). However, sedative properties produced by adinazolam have been delineated by clinical studies as "limited" (Kramer and Schoen, 1986) and as "rare" (Pyke et al., 1983). Our data are also consistent with preclinical studies which have shown that the TBZDs have less sedative properties than the classical BZDs (File and Pellow, 1985). It is widely known that the BZDs exhibit sedation, but, since adinazolam has little or no anticholinergic affects (Feighner, 1986), it is not surprising that adinazolam produced a lesser sedative effect than did diazepam in the freely moving and behaving rat animal model.

### Adinazolam: Comparison with High Affinity Typical (non-triazolo)BZD's:

Not to be ignored in the quest to reveal the mechanism of action of the TBZDs is a discussion of the comparison between a TBZD, such as adinazolam, and a high affinity GABA-A-TBZD, such as lorazepam or clonazepam. Controlled clinical studies have been performed with the expressed purpose of comparing the antidepressant effects of TBZDs with those of high affinity GABA-A-BZDs and the results have shown that similar antidepressant efficacy is produced by e.g. alprazolam vis-a vis lorazepam or alprazolam vis-a-vis clonazepam (Burrows et al., 1993, Pollack et al., 1993, Laakman et al., 1995, Romach et al., 1995, Davidson, 1997). Although adinazolam *per se*, has not been included in these controlled studies, it is likely that one could extrapolate a similar result for adinazolam as well.

But, that TBZDs have similar efficacy to lorazepam and clonazepam in clinical depression, begs the question: do both TBZDs and high affinity GABA-A-BZDs act through the exact same mechanism on

5-HT? The literature, for the most part, argues against this hypothesis (Gardner, 1986; Jefferson, 1996), e.g. one study showed that both lorazepam and diazepam were unable to reverse the helplessness syndrome, whereas the 5-HT reuptake inhibitor, fluvoxamine, produced a significant reversal of the helplessness syndrome (Martin *et al.*, 1996). Thus, further evidence is presented here that it is presumably either a direct effect of adinazolam to increase hippocampal 5-HT release, or some combined interactive NE/5-HT effects of adinazolam that may account for its unique clinical anxiolytic/antidepressant activity.

Nevertheless, there are considerations of other possible interactions of the triazolo for producing gradations of antidepressant, as well as anxiolytic activity. Some of these mechanisms could be e.g., (a) hetero-oligomeric GABA-A subunit control of GABA gated CL<sup>-</sup> channels (Mihic *et al.*, 1994, Costa and Guidotti, 1996), wherein potentiation of GABA-A responses could occur, (b) platelet activating factor (PAF) antagonism wherein blockade of calcium mobilization could reduce superoxide anion production in macrophages (Gardner *et al.*, 1993; Travers *et al.*, 1995) and/or (c) cytochrome P450-3A4 mechanisms, which could increase triazolo levels for enhanced activity (Nemeroff *et al.*, 1996). These remain as exciting adjunct and/or alternative mechanisms for exploration into the mechanism of action of the TBZDs.

#### 5-HT/NE Interactions:

A final note points to the interaction of 5-HT and NE as the mechanism of action for the dual antidepressant/anxiolytic effects of adinazolam. The recent development of the compound venlafaxine, a mixed 5-HT/NE transport antagonist speaks to the principle of mixing aminergic interactions, perhaps even potentiation in the treatment of anxiety and depression (Leonard, 1994). Drugs of the TBZD type and the “pyradolo” type, including alprazolam, adinazolam, and zometapine, truly represent a “borderland” therapeutic approach to mixed anxiety and depression (Baldessarini, 1996).

#### Conclusions

The data support our hypothesis that the separation of the *in vivo* hippocampal 5-HT release effects of the TBZDs provide a strong empirical explanation for the mechanism of action of the dual antidepressant and anti-anxiety action produced by the TBZDs. Specifically, in the case of the TBZD, adinazolam, the data indicates that the *modus operandi* which separates adinazolam's action from that of the BZD, diazepam, is adinazolam's increased 5-HT-ergic effects on release. Moreover, in the case of the TBZD, adinazolam, the hypothesis of a mixed aminergic action, i.e. an interactive 5-HT and NE synaptic mechanism for its dual antidepressant/anxiolytic effects, is likely.

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