BENZODIAZEPINES: POTENTIATION OF A GABA INHIBITORY RESPONSE IN THE DORSAL RAPHE NUCLEUS

DOROTHY W. GALLAGER *

Section on Biochemistry and Pharmacology, Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, Maryland 20014, U.S.A.

Received 3 November 1977, revised MS received 2 February 1978, accepted 7 February 1978

D.W. GALLAGER, *Benzodiazepines: potentiation of a GABA inhibitory response in the dorsal raphe nucleus*, European J. Pharmacol. 49 (1978) 133—143.

Based on evidence that the dorsal raphe nucleus (DR) has specific and independent receptors for 5HT, GABA and glycine (Gallager and Aghajanian, 1976; Wang and Aghajanian, 1977), alterations in the firing rate of DR neurons following the administration of benzodiazepines (BZ) were evaluated to determine whether they were the result of a direct interaction with 5HT receptors or due to interactions of these drugs with GABA and/or glycine. The effects of BZs after both direct and systemic application were tested in rats using microiontophoretic and single-cell recording techniques. Although the BZs did not alter the spontaneous firing rate of the DR, both the systemic and iontophoretic administration of these drugs were found to potentiate the inhibitory response produced by GABA. The data suggests that this potentiation is mediated postsynaptically. Since the effects of BZs on the spontaneous activity of the DR are only apparent following pretreatments with AOAA, it is speculated that these drugs may only have pronounced effects when GABAergic input is prominent.

Microiontophoresis Benzodiazepines 5HT GABA GLY Dorsal raphe nucleus

1. Introduction

Biochemical, behavioral and neurophysiological evidence suggests that the benzodiazepines (BZs) affect several neuronal systems within the CNS (review: Garattini et al., 1973; Costa and Greengard, 1975). The BZs have been shown to affect the turnover rate and/or tissue levels of most known neurotransmitters in the CNS including norepinephrine (NE), serotonin (5HT), dopamine (DA), acetylcholine (ACh), and γ-aminobutyric acid (GABA) (Costa and Greengard, 1975). In addition, the importance of the monoamine systems in the mediation of certain behaviors [e.g., NE in stress (Corrodi et al., 1968), 5HT in anxiety states (Wise et al., 1970), sleep processes (Jouvet, 1969), and control of seizures (Jenner et al., 1975)] has led to the suggestion that the antianxiety, sedating and anticonvulsant properties of the BZs are related to changes in these monoamine systems. However, recent studies suggest that at least part of the effects of the BZs result from a specific interaction with GABAergic transmission (Costa et al., 1975; Haefely et al., 1975). The mechanism of this interaction seems to be controversial since the facilitation of GABA-mediated transmission (Polc et al., 1974; Costa et al., 1975; Haefely et al., 1975; Curtis et al., 1976), synergistic actions with GABA (Kozhechkin and Ostrovskaya, 1977), direct activation of GABAergic receptors (Dray and Straughan, 1976) and antagonism

* Visiting Scientist (IPA: NTE 080778) on leave from Department of Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06508, U.S.A. Address correspondence to: D.W. Gallager, Ph.D., Clinical Center, Room 2N315, Section on Biochemistry and Pharmacology, Biological Psychiatry Branch, National Institute of Mental Health, 9000 Rockville Pike, Bethesda, Maryland 20014, U.S.A.

of GABA-mediated inhibition (Gähwiler, 1976; Steiner and Felix, 1976) have all been attributed to the BZs. Such discrepancies may be due to differences in GABAergic receptors in various species or brain regions (Curtis et al., 1976; Pericic et al., 1978) or to different actions of BZs at various levels of the nervous system (Soubrie et al., 1976). It has also been suggested that the effects of the BZs on other transmitter systems in the CNS may be secondary to their actions on the GABAergic system (Polc et al., 1974; Costa et al., 1975; Costa and Greengard, 1975). Because of the extensive GABAergic innervation of the monoamine and ACh systems (Davidson, 1976) it is apparent that any modification of the GABAergic system could result in extensive repercussions throughout the CNS.

Recently, Squires and Braestrup (1977) presented in vitro data demonstrating the existence of high affinity binding sites for BZs in brain tissue. Binding to these sites was not inhibited by the presence of even high concentrations of GABA. Therefore, their study suggests that GABA does not interfere directly with BZ binding sites. In vitro binding studies (Young et al., 1974) have also suggested that there is an interaction between the BZs and glycine binding sites in the CNS. However, to date, no evidence has been obtained for an action of BZs at glycine receptors in vivo (Curtis et al., 1976; Dray and Straughan, 1976; Steiner and Felix, 1976). Therefore, the relevance of in vitro binding studies to an in vivo mechanism of action of the BZs has not yet been established.

It has been shown that the dorsal raphe nucleus (DR), containing cell bodies for the principle 5HT projections to the forebrain (Dahlström and Fuxe, 1964), not only has presynaptic or "autoreceptors" for 5HT (Aghajanian et al., 1972) but also has specific and independent receptors for GABA and glycine (Gallager and Aghajanian, 1976; Wang and Aghajanian, 1977). All three of these putative neurotransmitters are inhibitory, the direct application of 5HT, GABA or glycine by microiontophoresis decreases the firing rate of DR neurons. Therefore, possible interactions of drugs (e.g., the BZs) with any one of these transmitters would result in similar behavioral and biochemical changes in this 5HT system. In the present study, both the direct and systemic effects of the BZs were tested on the neuronal activity of the dorsal raphe (DR) nucleus in the rat. Using microiontophoretic and single-cell recording techniques, alterations in the firing rate of DR neurons following the administration of BZs were evaluated to determine whether they were the result of a direct interaction with 5HT receptors or due to interactions of these drugs with GABA and/or glycine. The data presented here suggest that the BZs potentiate the inhibitory effects of GABA on DR neuronal firing rates.

2. Material and methods

2.1. Single-unit recording and microiontophoresis

75 male albino rats (Sprague-Dawley/ARS, 220—270 g) were used in this study. Data were obtained from two different animal preparations. In one group, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. Animals were kept anesthetized with additional injections of chloral hydrate as needed throughout the experiment. In the second type of preparation, unanesthetized, paralyzed, artificially respired animals were used. This preparation was used to avoid the possibility that anesthetic agents might interact with the BZs to potentiate or block their effects on neuronal activity. Animals were initially anesthetized with halothane and a tracheotomy was performed using a 13-gauge cannula to intubate the trachea. The cannula was then connected to a rodent respirator (Harvard Apparatus Co.) and a mixture of halothane and room air was administered until the completion of surgery. All incision sites and soft tissue pressure points, including

ear canals and nose bar contact area were infiltrated with a long-acting anesthetic (1% mepivacaine hydrochloride). After the infiltration with local anesthetic, gallamine triethiodide (Flaxedil, 16 mg/kg) was injected i.v. via a tail vein, resulting in the paralysis of skeletal muscle and cessation of spontaneous respiration. Animals were respired in room air at a rate adjusted to maintain an expired air CO_2 concentration of 3.5—4% as measured by an Infrared Industries CO_2 analyzer. Both types of animal preparations were mounted in a stereotaxic apparatus and a burr hole was made in the midline of the skull over the midbrain raphe area corresponding to the frontal planes from P400 μm to A650 μm of König and Klippel (1970).

In experiments where drugs were administered only by the systemic route, 2 mm glass micropipettes (tip diameters: 1—2 μm) were used. Micropipettes were filled with 2 M sodium chloride saturated with Fast Green FCF (Fisher) dye. Electrodes typically had an impedence of 3—6 MΩ when measured at 60 Hz. Micropipettes were lowered into the the midbrain raphe region with a hydraulic microdrive system. Electrode potentials were passed through a high input-impedence amplifier and monitored on a oscilloscope. Integrated firing rate was recorded by means of a rate meter. Body temperature was measured by rectal probe and was maintained between 36—37°C. Under these conditions, midbrain raphe cells displayed a regular rhythm and rate of 0.5—3.0 spikes/sec (mean = 1.6 ± 0.1 spikes/sec, n = 62) which has been described for histochemically identified 5HT units (Aghajanian and Haigler, 1974).

In microiontophoretic studies, 5-barrel micropipettes were used according to techniques previously described (Haigler and Aghajanian, 1974). The tips were broken back to 4—5 μm under microscopic control; solutions were then injected directly into the various barrels. The presence of fiberglass allowed the tips to become filled quickly by capillary action.

The central (recording) barrel was filled with a 2 M sodium chloride solution saturated with Fast Green dye. One side barrel, the "balance" channel, was filled with 4 M sodium chloride. The other three barrels were each filled with one of the following drugs: 0.05 M serotonin creatinine sulfate (5HT), pH 4.0, Sigma Chemical Co.; 0.01 M GABA in 0.2 M NaCl, pH 4.0, Calbiochem; 0.1 M glycine HCl, pH 4.0 (GLY), Sigma Chemical Co.; a saturated solution of picrotoxin, pH 4.0 (PIC), K & K Laboratories; 0.1 M Flurazepam Di HCl, pH 4.0 (FLU), Hoffmann—LaRoche, Inc.; 0.1 M chlordiazepoxide HCl, pH 4.0 (LIB), Hoffmann—LaRoche, Inc.; 0.1 M sodium nipecotate, pH 4.0 (NIP), Abbott Laboratories.

Drugs were ejected through micropipette barrels by turning the direct current source from —10 to —20 nA of "retaining" current to +1 to +20 nA of "ejecting" current. Circuits for drug ejection and current balance were similar to those described by Salmoiraghi and Weight (1967), except that a solid-state system was used. Impedences, measured in vitro at 60 Hz were 3—8 MΩ in the recording barrel, 15—20 MΩ in the balance channel and 80—150 MΩ in the drug barrels.

To obtain quantitatively reliable responses to agonists over the course of the microiontophoretic experiments (Bradshaw et al., 1973), the duration of microiontophoresis was standardized at 60 sec ejection periods. In addition, the time intervals and retaining currents (5HT, —12 nA; GLY, —12 nA; GABA, —20 nA) between successive applications of each agonist were also kept constant. When drugs (FLU, NIP, LIB) or antagonist (PIC) were iontophoresed, they were applied continuously while maintaining the regular series of agonist ejection. Application of drug or antagonist was then terminated (retaining currents: PIC, —10 nA; FLU, —10 nA; NIP, —10 nA; LIB, —10 nA), and a recovery of the agonist response was obtained when possible. When drugs or antagonists were injected intravenously, agonists were applied microiontophoretically both before and after the parenteral administration of the drugs.

2.2. *Identification of cells*

Recording sites were marked by passing a 16 μA cathodal current through the recording electrode/barrel for 10 min which resulted in the deposition of a discrete spot of Fast Green FCF dye. The precise location of the electrode tip was confirmed by histological examination and compared with the previously established location of 5HT containing neurons in the dorsal raphe nucleus (Aghajanian and Haigler, 1974).

2.3. *Systemic administration of drugs*

I.v. injections of drugs, expressed in terms of concentration of injecting solution, were administered via a tail vein. These drugs include: diazepam (DZ, 1 mg/ml and 5 mg/ml, Valium, Hoffmann—LaRoche, Inc.); picrotoxin (PIC, 3 mg/ml, K & K Laboratories); strychnine (STR, 1 mg/ml, Sigma Chemical Co.); Flurazepam Di HCl (FLU, 5 mg/ml, Hoffmann—LaRoche, Inc.); Chlordiazepoxide HCl (LIB, 10 mg/ml, Hoffmann—LaRoche, Inc.); d-lysergic acid diethylamide bitartrate (LSD, 50 μg/ml); clonidine HCl (10 μg/ml, Boehringer, Ingelheim). Amino-oxyacetic acid hemihydrochloride (AOAA, 20 mg/ml, Sigma Chemical Co.) was administered i.p. at least 50 min prior to recording.

3. Results

3.1. *Effect of intravenous BZs on the spontaneous firing rate of 5HT neurons*

The i.v. administration of BZs diazepam (up to 8 mg/kg, n = 11), chlordiazepoxide (up to 12 mg/kg, n = 3) and flurazepam (up to 30 mg/kg, n = 6) were found not to significantly affect the firing rate of raphe cells in chloral hydrate anesthetized animals, while low doses of LSD (10 μg/kg) totally inhibited raphe firing as previously reported (Aghajanian et al., 1972) (fig. 1). In addition, few if any effects on raphe cell firing were observed in unanesthetized, paralyzed rats using doses of diazepam up to 8 mg/kg (n = 5, not shown). However, in this latter preparation, when CO_2 levels dropped below 3.0% following injections of diazepam (suggesting a deteriorating condition in the rats, Julien, 1972), a simultaneous slowing of DR neurons was occasionally observed (n = 2).

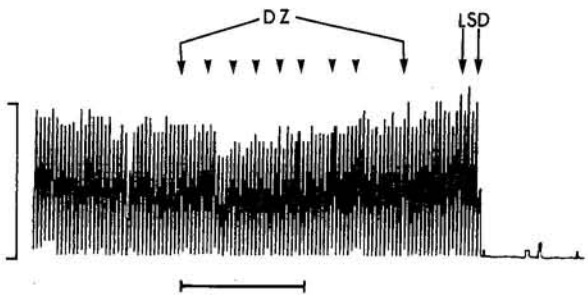

Fig. 1. Lack of effect of i.v. diazepam (DZ, cumulative dose of 8 mg/kg, 0.125, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg, at arrows) on the basal firing rate of a dorsal raphe neuron. LSD (10 + 10 μg/kg, i.v.) produced its usual depressant effect. *Ordinate:* spikes/10 sec, scale 0—25; *time marker:* 5 min.

3.2. *Ability of intravenous diazepam to interact with putative transmitters (5HT, GABA, GLY) in the dorsal raphe nucleus*

As previously reported (Gallager and Aghajanian, 1976) 5HT (10—16 nA), GABA (1—5 nA) and GLY (1—5 nA) all produced significant and specific inhibitions of raphe cell firing (n = 15 cells in 9 rats). Following the systemic administration of diazepam (DZ, 1 mg/kg, i.v.), the effect of a submaximal iontophoretic current of GABA (5 nA, producing approximately a 50% inhibition of DR firing) appeared to be increased (producing 100% inhibition of DR firing) without affecting either the spontaneous firing rate or the subsequent microiontophoretic response to 5HT (fig. 2). This potentiated response to iontophoresed GABA was specifically blocked by the simultaneous iontophoretic administration of the GABA antagonist, picrotoxin (10 nA) without altering the 5HT inhibition (fig. 2) (n = 5 cells in 4 animals). When submaximal

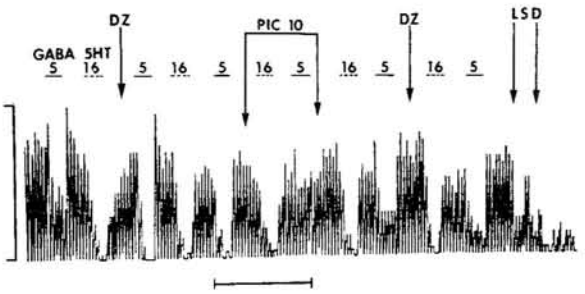

Fig. 2. The response of a dorsal raphe neuron to the microiontophoretic application of GABA and 5HT. GABA was ejected with a 5 nA current for 60 sec (solid line); 5HT was ejected with a 16 nA current for 60 sec (dotted line). Immediately following a 1 mg/kg i.v. dose of diazepam (arrow), a potentiated response to GABA (5 nA) but not to 5HT (16 nA) was observed. The ejection of PIC with a 10 nA current almost completely blocked the response to GABA but did not affect the 5HT response. An additional 1 mg/kg i.v. dose of diazepam potentiated the recovering GABA response. The cell was subsequently inhibited by LSD (5 + 5 μg/kg, i.v.). *Ordinate:* spikes/10 sec, scale 0—20; *time marker:* 5 min.

currents of three suspected transmitter substances in the DR were iontophoresed (5HT, 10 nA; GABA, 1 nA and GLY, 2 nA) only the GABA response was affected by the intravenous administration of DZ (1 mg/kg) (fig. 3). This potentiation of the GABA-induced inhibition of raphe cell firing, but not the GLY or 5HT inhibition was observed in 7 cells in 7 rats. Following an intravenous dose of the glycine antagonist, strychnine (0.6 mg/kg), a block of the inhibiting effects of iontophoretically applied glycine, but not 5HT or GABA on raphe cell firing was observed (fig. 3). In fact, the GABA response continued to be potentiated by an earlier dose of diazepam despite the presence of strychnine (fig. 3) (n = 3 cells in 2 rats).

3.3. Interactions with putative neurotransmitters in the dorsal raphe nucleus

The water-soluble BZs, chlordiazepoxide HCl (LIB) and Flurazepam (FLU) were applied via microiontophoresis to dorsal raphe cells in currents up to 12.5 nA (LIB) or 10 nA (FLU) (n = 14). Neither of these compounds was found to affect the spontaneous firing rates of DR cells at these currents. In our system, higher ejection currents resulted in blocking of drug channels. However, as seen with intravenous diazepam, the simultaneous iontophoretic administration of either LIB or FLU with a submaximal current of GABA,

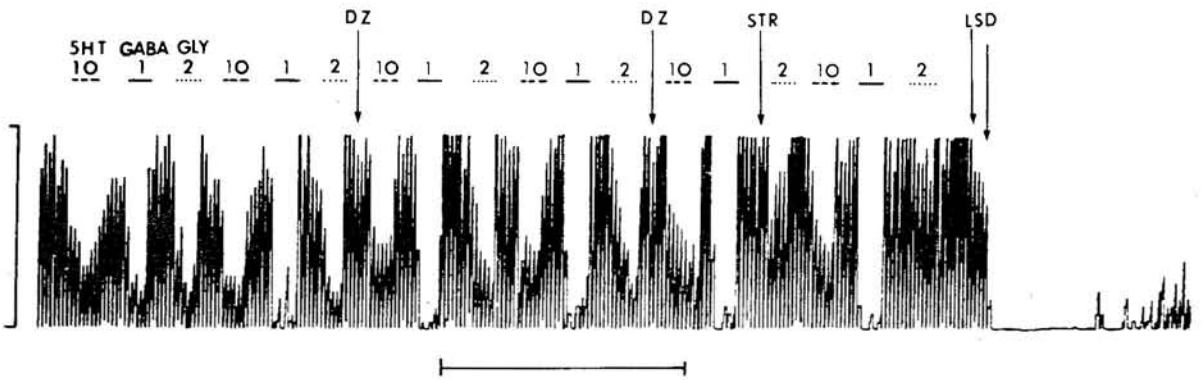

Fig. 3. The response of a dorsal raphe neuron to the microiontophoretic application of 5HT, GABA and GLY. A submaximal inhibition of firing was produced by the ejection of 5HT (10 nA) for 60 sec (dashed line), the ejection of GABA (1 nA) for 60 sec (solid line) and the ejection of GLY (2 nA) for 60 sec (dotted line). Following a 2 mg/kg i.v. dose of DZ (arrow) only the response to GABA was potentiated. An additional 2 mg/kg dose of DZ had no further effect, however, immediately following a 0.6 mg/kg i.v. dose of STR (arrow) a complete blockade of the GLY response was observed without affecting either the 5HT response or the potentiated GABA response. LSD (10 + 10 μg/kg, i.v.) produced its usual depressant effect. *Ordinate:* spikes/10 sec, scale 0—25; *time marker:* 10 min.

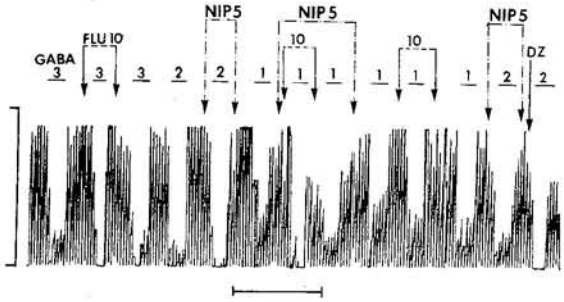

Fig. 4. Submaximal inhibition of dorsal raphe firing produced by various currents of GABA (1—3 nA) ejected for 60 sec (solid line). The simultaneous iontophoresis of FLU (10 nA) for 120 sec (dashed line) potentiated the response to GABA (3 nA). The iontophoresis of NIP (5 nA) for 120 sec (broken line) also potentiated the response to GABA (2 nA), although at this current NIP did not affect the spontaneous activity of the cell (not shown). The simultaneous ejection of both NIP (5 nA) for 240 sec (broken line) and FLU (10 nA) for 120 sec resulted in an additional potentiation of the GABA response (1 nA). A 1 mg/kg i.v. dose of DZ (arrow) also produced a potentiation of the GABA (2 nA) response. *Ordinate:* spikes/10 sec, scale 0—30; *time marker:* 5 min.

potentiated the inhibition normally produced by GABA in dorsal raphe firing (n = 14 cells in 9 rats, fig. 4). This effect was specific to GABA since the inhibitions produced by either 5HT or GLY were not affected by the simultaneous iontophoresis of one of the soluble BZs (not shown) (n = 10 cells in 8 animals).

A selective enhancement of the depressant effect of GABA was accomplished by the simultaneous iontophoresis of nipecotic acid, a GABA uptake blocker (Krogsgaard-Larson et al., 1975). The potentiation of GABA was maximal using an iontophoretic current of 5 nA of nipecotic acid (NIP) while ejection of up to 10 nA of NIP had no effect in the spontaneous activity of dorsal raphe cells (n = 5 cells in 3 rats). An additional potentiation of the GABAergic inhibition was produced by the simultaneous iontophoresis of both NIP and the BZ, Flurazepam (fig. 4, n = 6 cells in 4 rats).

3.4. Amino-oxyacetic acid (AOAA) pretreatment

At least 50 min after the intraperitoneal injection of AOAA (50 mg/kg, i.p.), a GABA catabolic inhibitor (Baxter and Roberts, 1961), the animal was anesthetized and prepared for unit recording as described in Materials and methods. Spontaneous firing rates of the 5HT cells appeared to be within normal (unpretreated) limits for this preparation (0.4—2.6/sec; mean = 1.3 ± 0.2 spikes/sec, n = 8). Microiontophoretic application of 5HT and GABA and potentiation of GABAergic inhibition by FLU were also observed (fig. 5). However, unlike previous untreated preparations, 1.0 mg/kg of intravenously administered DZ was able to either partially (n = 5) or totally (n = 3) inhibit spontaneous raphe unit activity. Following 0.6 mg/kg i.v. picrotoxin, recovery of activity was observed (fig. 5). This dose of picrotoxin was sufficient to partially block the inhibiting

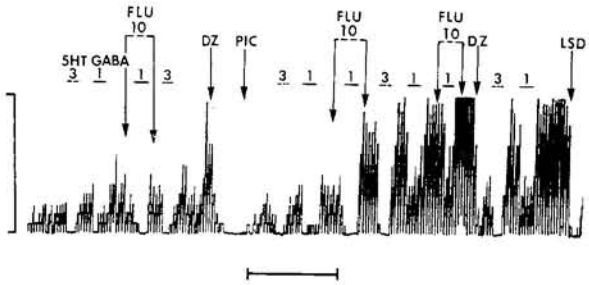

Fig. 5. Following pretreatment with AOAA (50 mg/kg, i.p.) for 50 min the response of a dorsal raphe neuron to the iontophoretic application of 5HT, GABA and FLU was tested. 5HT (3 nA) was ejected for 60 sec (dotted line) and GABA (1 nA) was ejected for 60 sec (solid line). FLU (10 nA) ejected for 120 sec potentiated the inhibition produced by GABA (1 nA). A 2 mg/kg i.v. dose of DZ (arrow) totally inhibited the spontaneous firing of the dorsal raphe neuron while a 0.6 mg/kg i.v. dose of PIC reversed this inhibitory effect. As the baseline firing rate increased, the ability of GABA (1 nA) to inhibit and of FLU (10 nA) to potentiate this effect decreased. An additional 2 mg/kg i.v. dose of DZ (arrow) only partially reversed this block. The cell was inhibited by LSD (10 μg/kg, i.v.). *Ordinate:* spikes/10 sec, scale 0—15; *time marker:* 5 min.

effects of microiontophoretically applied GABA, as well as the potentiation of this inhibition by both iontophoresed and systemically administered benzodiazepines (fig. 5). The inhibitory response of the dorsal raphe cell to iontophoretically applied 5HT or systemically administered LSD (10 μg/kg) was unaffected by the AOAA pretreatment.

3.5. Effect of intravenous diazepam on the spontaneous firing rates of other monoamine systems

In preliminary experiments, the effects of systemically administered diazepam on the spontaneous firing rates of norepinephrine-containing neurons in the locus coerulus and in the DA-containing cell bodies of the substantia nigra (zona compacta) were monitored. Single cells in the locus coeruleus (L.C., n = 4) were firing at a relatively slow rate (1—4/sec) and typically responded to noxious stimuli by a transient increase in firing rate (Korf et al., 1974; Cedarbaum and Aghajanian, 1976). The location of these L.C. cells were also subsequently confirmed by histological examination (Korf et al., 1974).

In the chloral hydrate anesthetized preparation, DZ in doses up to 20 mg/kg i.v. were found to be without significant effects in the spontaneous firing rate of the L.C. cells. These same cells were found to be subsequently inhibited by very low doses (7.5 ± 1.5 μg/kg) of clonidine as previously reported by Svensson et al. (1975) (n = 4, data not shown). However, a possibility of an interaction with one specific putative transmitter in the L.C. as demonstrated by the present study in the dorsal raphe nucleus has not yet been examined.

The spontaneous firing rates of dopaminergic cells in the substantia nigra (zona compacta) were also monitored for possible effects of intravenously administered diazepam. Cells located in this region by their characteristic firing patterns (Bunney et al., 1973) and histological localization (A9) appeared to have a more heterogeneous response to i.v. diazepam. In 6 animals, in doses up to 8 mg/kg, no significant change in spontaneous firing rate was observed. However, in 3 rats, up to a two-fold increase in firing rate was seen after an initial dose of 1 mg/kg of diazepam. Additional doses did not further increase this rate. Since in all cases the vehicle (containing propylene glycol, ethyl alcohol, benzoic acid and benzyl alcohol), which was administered prior to the first dose of DZ, was not found to affect DA firing, the increase did not seem to be due to the possible irritating properties of the solvents. However, additional systemic and microiontophoretic studies in this region should be done.

4. Discussion

The present study on dorsal raphe (DR) cells suggests that systemically and iontophoretically applied BZs enhance the inhibitory effects of GABA, in agreement with studies of Curtis et al. (1976) on Purkinje cells in the cerebellum, and of Kozhechkin and Ostrovskaya (1977) on cortical neurons in the sensorimotor cortex. No evidence was found in this study to support the suggestion that BZs either mimic (Dray and Straughan, 1976) or antagonize (Steiner and Felix, 1976; Gähwiler, 1976) the actions of GABA since the direct application of BZs via microiontophoresis did not produce inhibition of DR cell firing (like GABA), nor did they decrease the inhibitory effects of GABA (like picrotoxin). In addition, in agreement with electrophysiological studies in other brain regions (Curtis et al., 1976; Dray and Straughan, 1976; Steiner and Felix, 1976) no in vivo evidence for an interaction with glycine (Young et al., 1974) was found since the inhibitory response to glycine in the DR was unaffected by the presence of BZ. In addition, the glycine antagonist strychnine was able to block the glycine response completely while not affecting the GABA potentiation produced by the BZs.

Since the drugs which alter 5HT uptake or metabolism, or directly activate 5HT recep-

tors have been shown to affect the firing of DR neurons (Aghajanian and Haigler, 1973), the finding that systemically administered diazepam does not alter the spontaneous firing rate of DR neurons suggests that BZs do not directly affect 5HT. This is supported by the observation that the direct application of a BZ via microiontophoresis has no effect on the 5HT-induced inhibition of dorsal raphe firing. Thus, it appears from this study that changes in 5HT turnover (Lidbrink et al., 1974) and metabolism (Agarwal et al., 1977), as well as some proposed 5HT behavioral effects (Stein et al., 1973; Nakamura and Fukushima, 1976) may be secondary to the interaction of the BZ with GABAergic processes in the DR (Costa and Greengard, 1975). In addition, the data suggests that the GABAergic input to the DR (Wang et al., 1977; Wang and Aghajanian, 1977), if it is in fact modified by the BZs, is either not prominent or not tonically active as evidenced in a previous study by the lack of effects of the GABA antagonist picrotoxin on baseline firing rates in the DR (Gallager and Aghajanian, 1976).

However, following pretreatment of the animals with the GABA catabolic inhibitor amino-oxyacetic acid (AOAA), the systemic administration of the BZ, diazepam, resulted in a significant inhibition of the spontaneous firing of DR neurons in contrast to untreated control rats. Under these conditions, more GABA is available to interact with receptor sites (Iversen, 1971), further implicating the role of GABA in this potentiation. An accentuation by BZs of the pre- and postsynaptic inhibition produced by AOAA has also been reported in the cuneate nucleus by Polc and Haefely (1976).

Since the data presented in this study suggest that BZs potentiate GABAergic transmission in the DR, the question becomes "By what mechanism can this potentiation of the GABA response occur?" Potentiation can occur in at least four possible ways: (1) the BZs could directly stimulate the GABA receptor, causing at least an additive inhibition of DR firing, (2) the drugs could increase the presynaptic release of GABA, (3) the drugs could inhibit the removal of GABA from the receptor site, or (4) they could alter the postsynaptic response to GABA. Microiontophoretic data have been presented to show that in the DR, the BZs do not act directly to stimulate GABA receptors. In addition, since the inhibitory effects of GABA applied by iontophoresis to the vicinity of the dorsal raphe cells appear to be current-dependent, it is unlikely that the potentiation of the GABAergic response to iontophoretically or systemically applied BZs is the result of an increased presynaptic release of GABA. The lack of a GABA-releasing action is also supported by the data of Nelson-Kraus and Howard (1976) showing no change in the synaptosomal release of GABA in the presence of BZs. Thus, the potentiation in the DR does not occur through a direct stimulation of GABA receptors or indirectly through a presynaptic GABA-releasing action. An inhibition of the removal of GABA from the receptor site could occur by an inhibition of the metabolism of GABA, or an inhibition of GABA uptake. Following the inhibition of transaminase, the principle metabolic enzyme for GABA (Roberts, 1974), the BZs continue to potentiate GABAergic effects on the DR. Thus, the data support the suggestion that the BZs are not operating primarily as inhibitors of GABA transaminase (Sawaya et al., 1975). However, the presence of a high affinity uptake process for GABA located on the presynaptic terminals (Iversen et al., 1975) and the lack of an efficient enzymatic removal system suggest that reuptake provides the principle, if not the only mechanism for removal of GABA from the synaptic region (Krnjević, 1974). As previously reported by Krogsgaard-Larson et al. (1975) and Curtis et al. (1976), the simultaneous iontophoresis of (±)-nipecotic acid increased the inhibitory effect of GABA on DR cells. In the present study the inhibition of cell firing, produced by the simultaneous iontophoresis of GABA and nipecotic acid could be further potentiated by

the presence of a BZ. In vitro uptake studies by Iversen and Johnston (1971) also support the lack of prominent uptake effects of the benzodiazepines. Thus, the possibility of a decreased removal of GABA from the receptor site by decreased catabolism or uptake blockade does not seem to be a plausible explanation for the potentiation of GABA effects by the BZs. This leaves the possibility of a BZ-induced alteration in the postsynaptic response to GABA. Direct examination of this mechanism of action is not possible using microiontophoresis and single-cell recording techniques. However, direct binding studies using [^{3}H]diazepam support a direct interaction between GABA and BZ binding sites in rat brain. Pretreatment of animals in vivo with AOAA and GABA agonists have been shown to increase the affinity of [^{3}H]diazepam binding sites in rat cerebral cortex (Gallager et al., unpublished observations). In addition, the direct in vitro addition of GABA and GABA agonists have been shown to increase and GABA antagonists to decrease the affinity of [^{3}H]diazepam binding sites in rat brain cortex (Tallman et al., unpublished observations). Thus, these binding studies provide direct support for a postsynaptic site of action for the synergistic effects of GABA and the BZs.

As demonstrated in the present study, the effects of the BZs on the spontaneous firing rate of the DR are only apparent following pretreatment with AOAA. It is possible to speculate that this may be a more generalized phenomenon, such that changes in GABA levels may result in increased sensitivity of all neuronal systems innervated by GABA to the BZs. Further work on other neurotransmitter systems is necessary to test this possibility. Interestingly, certain behavioral states have also been reported to increase GABA levels. Sherman and Gebhart (1974) have reported that pain induced by the hot plate elevates GABA levels. If other painful or stressful conditions also increase GABA levels, then the BZs may have much more pronounced effects on neurotransmitter systems than is apparent in unperturbed, control animals. One may further speculate that in stress or anxiety states profound BZ effects may occur which do not occur under normal conditions. However, whether any of these effects are sufficient to account for the various pharmacological effects of the BZs in the CNS is not known.

Acknowledgements

The skillful technical assistance of Ms. Helen Liao and Ms. Dawn McBrien are gratefully acknowledged. The author would like to thank Dr. William Scott of Hoffmann—LaRoche, Inc., Nutley, N.J. for supplying the chlordiazepoxide and the flurazepam, Dr. Judith Walters, NINCDS for supplying the nipecotic acid and Boehringer, Ingelheim, G.F.R. for supplying the clonidine used in these experiments.

References

Agarwal, R.A., Y.D. LaPierre, R.B. Rastogi and R.L. Singhal, 1977, Alterations in brain 5-hydroxytryptamine metabolism during the "withdrawal" phase after chronic treatment with diazepam and bromazepam, Brit. J. Pharmacol. 60, 3.

Aghajanian, G.K. and H.J. Haigler, 1973, Studies on the physiological activity of 5HT neurons, in: Pharmacology and the Future of Man, Proc. 5th Intern. Congr. Pharmacology, Vol. 4, (S. Karger, Basel) p. 269.

Aghajanian, G. and H. Haigler, 1974, L-Tryptophan as a selective histochemical marker for serotonergic neurons in single-cell recording studies, Brain Res. 81, 364.

Aghajanian, G., H. Haigler and F. Bloom, 1972, Lysergic acid diethylamide and serotonin: direct actions in serotonin-containing neurons, Life Sci. (Part I), 11, 615.

Baxter, C.F. and E. Roberts, 1961, Elevation of γ-aminobutyric acid in brain: selective inhibition of γ-aminobutyric-α-ketoglutarate transaminase, J. Biol. Chem. 236, 3287.

Bradshaw, C., E. Szabadi and M. Roberts, 1973, The reflection of ejecting and retaining currents in the time-course of neuronal responses to microelectrophoretically applied drugs, J. Pharm. Pharmacol. 25, 513.

Bunney, B.S., J.R. Walters, R.H. Roth and G.K. Aghajanian, 1973, Dopaminergic neurons: effect of antipsychotic drugs and amphetamine on single cell activity, J. Pharmacol. Exptl. Therap. 185, 560.

Cedarbaum, J.M. and G.K. Aghajanian, 1976, Noradrenergic neurons of the locus coeruleus: inhibition by epinephrine and activation by the α-antagonist piperoxane, Brain Res. 112, 413.

Corrodi, H., K. Fuxe and T. Hökfelt, 1968, The effect of immobilization stress on the activity of central monoamine neurons, Life Sci. 7, 107.

Costa, E. and P. Greengard, 1975, Mechanism of action of benzodiazepines, in: Advances in Biochemical Psychopharmacology, Vol. 14 (Raven Press, New York).

Costa, E., A. Guidotti, C. Mao and A. Suria, 1975, New concepts on the mechanism of action of benzodiazepines, Life Sci. 17, 167.

Curtis, D.R., C.J.A. Game and D. Lodge, 1976, In vivo inactivation of GABA and other inhibitory amino acids in the cat nervous system, Exp. Brain Res. 25, 413.

Dahlström, A. and K. Fuxe, 1964, Evidence for the existence of monoamine containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons, Acta Physiol. Scand. 62, Suppl. 232, 1.

Davidson, N., 1976, Neurotransmitter Amino Acids (Academic Press, London) p. 179.

Dray, A. and D.W. Straughan, 1976, Benzodiazepines: GABA and glycine receptors on single neurons in the rat medulla, J. Pharm. Pharmacol. 28, 314.

Gähwiler, B.H., 1976, Diazepam and chlordiazepoxide: powerful GABA antagonists in explants of rat cerebellum, Brain Res. 107, 176.

Gallager, D.W. and G.K. Aghajanian, 1976, Effect of antipsychotic drugs on the firing of dorsal raphe cells. II. Reversal by picrotoxin, European J. Pharmacol. 39, 357.

Garattini, S., E. Mussini and L. Randall, 1973, The Benzodiazepines (Raven Press, New York).

Haefely, H., A. Kulscár, H. Möhler, L. Pieri, P. Polc and R. Schaffner, 1975, Possible involvement of GABA in the central actions of benzodiazepines, in: Advances in Biochemical Psychopharmacology, Vol. 12, eds. E. Costa and P. Greengard (Raven Press, New York) p. 131.

Iversen, L.L., 1971, Role of transmitter uptake mechanisms in synaptic neurotransmission, Brit. J. Pharmacol. 41, 571.

Iversen, L.L., F. Dick, J.S. Kelley and F. Schon, 1975, Uptake and localization of transmitter amino acids in the nervous system, in: Metabolic Compartmentation and Neurotransmission, ed. S. Berl, D. Clarke and D. Schneider (Plenum Publishing, New York) p. 65.

Iversen, L.L. and G.A.R. Johnston, 1971, GABA uptake in slices and homogenates and the effects of some inhibitors, J. Neurochem. 18, 1939.

Jenner, P., D. Chadwick, E.H. Reynolds and C.D. Marsden, 1975, Altered 5-HT metabolism with clonazepam, diazepam and diphenylhydantoin, J. Pharm. Pharmacol. 27, 707.

Jouvet, M., 1969, Biogenic amines and the states of sleep, Science 163, 32.

Julien, R., 1972, Cerebellar involvement in the antiepileptic action of diazepam, Neuropharmacol. 11, 683.

König, J. and R. Klippel, 1970, The rat brain: a stereotaxic atlas (Krieger, New York).

Korf, J., B.S. Bunney and G.K. Aghajanian, 1974, Noradrenergic neurons: morphine inhibition of spontaneous activity, European J. Pharmacol. 25, 165.

Kozhechkin, S.N. and R.U. Ostrovskaya, 1977, Are benzodiazepines GABA antagonists?, Nature 269, 72.

Krnjević, K., 1974, Chemical nature of synaptic transmission in vertebrates, Physiol. Rev. 54, 418.

Krogsgaard-Larson, P., G.A.R. Johnston, D.R. Curtis, C.J.A. Game and R.M. McCulloch, 1975, Structure and biological activity of a series of conformationally restricted analogues of GABA, J. Neurochem. 25, 803.

Lidbrink, P., H. Corrodi and K. Fuxe, 1974, Benzodiazepines and barbiturates: turnover charges in central 5-hydroxytryptamine pathways, European J. Pharmacol. 26, 35.

Nakamura, M. and H. Fukushima, 1976, Head twitches induced by benzodiazepines and the role of biogenic amines, Psychopharmacology 49, 259.

Nelson-Kraus, D.C. and B.D. Howard, 1976, Release of glycine and gamma-aminobutyric acid from synaptosomes prepared from rat central nervous tissue, Fed. Proc. Fed. Amer. Socs. Exp. Biol. 35, 543.

Pericic, D., J.R. Walters and T.N. Chase, 1978, Effect of diazepam and pentobarbital on amino-oxyacetic acid-induced accumulation of GABA, J. Neurochem. 29, 839.

Polc, P. and W. Haefely, 1976, Effects of two benzodiazepines, phenobarbitone and baclofen on synaptic transmission in the cat cuneate nucleus, Naunyn-Schmiedeb. Arch. Pharmacol. 294, 121.

Polc, P., H. Möhler and W. Haefely, 1974, The effect of diazepam on spinal cord activities: possible sites and mechanisms of action, Naunyn-Schmiedeb. Arch. Exptl. Pathol. Pharmakol. 284, 319.

Roberts, E., 1974, γ-Aminobutyric acid and nervous system function — a perspective, Biochem. Pharmacol. 23, 2637.

Salmoiraghi, G. and F. Weight, 1967, Micromethods in neuropharmacology: an approach to the study of anesthesia, Anesthesiology 28, 54.

Sawaya, M.C.B., R.W. Horton and B.S. Meldrum, 1975, Effects of anticonvulsant drugs on the cerebral enzymes metabolizing GABA, Epilepsia (Amst.) 16, 649.

Sherman, A. and G.F. Gebhart, 1974, Regional levels of GABA and glutamate in mousebrain following exposure to pain, Neuropharmacology 13, 673.

Soubrie, P., P. Simon and J.R. Boissier, 1976, Antagonism of diazepam agonist central anticholinergic drug-induced hyperactivity in mice: involvement of a GABA mechanism, Neuropharmacology 15, 773.

Squires, R.I. and C. Braestrup, 1977, Benzodiazepine receptors in rat brain, Nature, 266, 732.

Stein, L., C.D. Wise and B.D. Berger, 1973, Antianxiety action of benzodiazepines. Decrease in activity of serotonin neurons in the punishment system, in: The Benzodiazepines, eds. S. Garattini, E. Mussini and L.O. Randall (Raven Press, New York) p. 299.

Steiner, F.A. and D. Felix, 1976, Antagonistic effects of GABA and benzodiazepines on vestibular and cerebellar neurones, Nature 260, 346.

Svensson, T., B. Bunney and G. Aghajanian, 1975, Inhibition of both noradrenergic and serotonergic neurons in brain by the α-adrenergic agonist clonidine, Brain Res. 91, 291.

Wang, R.Y. and G.K. Aghajanian, 1977, Physiological evidence for habenula as major link between forebrain and midbrain raphe, Science 197, 89.

Wang, R.Y., D.W. Gallager and G.K. Aghajanian, 1977, Stimulation of pontine reticular formation suppresses firing of serotonergic neurones in the dorsal raphe, Nature 264, 365.

Wise, C.D., B.D. Berger and L. Stein, 1970, Serotonin: a possible mediator of behavioral suppression induced by anxiety, Dis. Nerv. Syst. Suppl. 31, 11, 34.

Young, A., S. Zukin and S. Snyder, 1974, Interaction of benzodiazepines with central nervous glycine receptors: possible mechanism of action, Proc. Nat. Acad. Sci. U.S.A. 71, 2246.