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Intra-Raphe Benzodiazepines Enhance Rat Locomotor Activity: Interactions with GABA

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SAINATI, S M AND S A LORENS *Intra-raphe benzodiazepines enhance rat locomotor activity Interactions with GABA* PHARMACOL BIOCHEM BEHAV **18**(3) 407–414, 1983 —Intracranial dose response relationships for the water-soluble benzodiazepines, chlordiazepoxide, flurazepam and midazolam, were performed by injecting the drugs through cannulae chronically indwelling in the median raphe nucleus of male albino rats Drugs were administered in doses of 0 0, 0 22, 0 44 0 88 and 1 75 nmole in 0 5 μl saline Both midazolam and flurazepam produced hyperactivity which was most prominent within the first 30 minutes post-injection Flurazepam, furthermore, proved twice as potent as midazolam Chlordiazepoxide, in contrast, was without effect at any of the doses tested This observation supports the view that chlordiazepoxide is a pro-drug which must be metabolized to form an active metabolite In another experiment animals received either saline or a sub-effective dose (0 22 nmole) of flurazepam or midazolam into the median raphe nucleus 5 minutes prior to either a subeffective dose of muscimol (0 22 nmole) or saline Only the combinations of a benzodiazepine plus muscimol produced hyperactivity These combinations, moreover, produced effects as robust as those of a 4-fold higher dose of muscimol alone (0 88 nmole) Other animals received either saline or bicuculline methiodide (0 88 nmole) Bicuculline did not affect activity level, but completely blocked the hyperkinetic effects of muscimol These data suggest that the hyperactivity effect of intra-raphe muscimol is due to activation of GABA receptors within the midbrain raphe, rather than at distant sites In addition the data suggest that the intra-raphe administration of certain benzodiazepines produces hyperactivity by facilitating GABA transmission

Chlordiazepoxide Flurazepam Midazolam GABA Muscimol Raphe Locomotor activity

THE benzodiazepines were introduced into clinical medicine in the early 1960's They quickly became the most commonly prescribed class of pharmaceuticals [28] Today, some 20 years after their introduction, the benzodiazepines are used widely as sedative-hypnotic, anti-convulsant, anti-anxiety, muscle-relaxant and pre-anesthetic drugs [4, 25, 28, 47]

There is a consensus that the benzodiazepines exert their behavioral and physiological effects by facilitating the post-synaptic action of the inhibitory amino acid neurotransmitter, *gamma*-aminobutyric acid (GABA) [12, 17, 19, 27, 31, 34, 41] For the most part, GABA-ergic cells constitute local circuit neurons [20], and are distributed throughout the neuraxis [36] Specific saturable high-affinity benzodiazepine binding sites of a type which occur exclusively in the central nervous system have been described [37, 56, 57] As facilitators of GABA neurotransmission, it is not surprising that the benzodiazepines can produce profound behavioral effects [27, 49, 52]

In a recent study we performed a series of experiments designed to investigate the extent to which GABA-ergic modulation of midbrain raphe serotonergic neurons influences locomotor activity in the rat [49,50] First, we replicated the earlier findings [46] that the acute microinjection of the GABA agonist [2, 3, 5, 18, 55], muscimol (100 ng), into the dorsal raphe nucleus produces hyperactivity Moreover, we found that the acute microinjection of muscimol (100 ng) into the median raphe nucleus produced increases in activity which were 4 times greater than those seen after similar injections into the dorsal raphe nucleus A dose-response analysis using animals with chronically-indwelling cannulae, and a complex Latin square design, showed that the median raphe nucleus indeed was more sensitive to the effects of muscimol [49,50] Thus, subsequent experiments utilized only cannulae implanted chronically in the median raphe nucleus

We were able to potentiate the effects of muscimol by administering intraperitoneally the benzodiazepine, chlordiazepoxide, and to block the effect with the GABA antagonist, bicuculline [2,3] These data support the view that the hyperkinetic effect of intra-raphe muscimol is due to the activation of GABA receptors

However, since GABA and benzodiazepine receptors are localized throughout the neuraxis, we cannot be certain that the effects of bicuculline and chlordiazepoxide are due to their binding allosterically to the same GABA receptors as those occupied following the intra-raphe injection of muscimol In order to determine whether the effects of peripherally-administered bicuculline and chlordiazepoxide are due to their interaction with muscimol at the same receptor sites, we performed the following series of experiments (1) an intra-raphe benzodiazepine dose-response analysis, (2) an intra-raphe bicuculline dose-response analysis, (3) a test

of the interactions between intra-raphe benzodiazepines and muscimol, and (4) a test of the interactions between intra-raphe bicuculline methiodide and muscimol

GENERAL METHOD

Animals

The experimental subjects were male Sprague-Dawley rats (King Animal Farms, Orange, WI), 90–120 days old and weighing 315–368 g at the time of surgery The animals were housed individually in a temperature (22 +/− 1 C), humidity (40–52%), and illumination (12 hour light-dark cycle) controlled room Food and water were available ad lib in the home cage

Surgery

Rats were anesthetized with penobarbital sodium (Butler Columbus, OH) 50 mg/kg, intraperitoneally, and placed in a Kopf stereotaxic instrument with the incisor bar set 3 2 mm above the interaural plane The scalps were incised and craniotomies performed A guide cannula (0 46 mm o d and 0 25 mm i d , Plastic Products Co , Roanoke, VA) was implanted in the median raphe nucleus A stylet (0 23 mm dia) then was inserted such that its tip was flush with that of the guide cannula This assembly then was cemented onto stainless steel anchoring screws embedded in the skull The coordinates were 2 5 mm lateral to the mid-line, and 9 0 mm ventral to the skull surface 1 5 mm rostral to lambda with the cannula angled 17 degrees lateral to the mid-sagittal plane At the time of surgery, each animal received 50 mg/kg ampicillin (Omni-pen-N, Wyeth) and 0 4 mg/kg atropine sulfate (Lilly), intramuscularly, and chloramphenicol (Chloromycetin 1% opthalmic ointment, Parke-Davis) topically around the wound All wounds were closed with autoclips (Clay Adams)

Drugs

All drugs were dissolved in 0 9% saline Muscimol (Sigma) was prepared and injected in concentrations of 0 22 and 0 88 nmole/0 5 μl (25 and 100 ng/0 5 μl) Bicuculline methiodide (Pierce Chemical Co Rockford, IL) was prepared in concentrations (as the base) of 0 22, 0 44 and 0 88 nmole/0 5 μl (81 161 and 323 ng/0 5 μl) Chlordiazepoxide hydrochloride (Roche), flurazepam dihydrochloride (Roche), and midazolam maleate (Roche) were prepared in concentrations of 0 11, 0 22, 0 44, 0 88 and 1 75 nmole/0 5 μl These concentrations, expressed in terms of nanograms of the base, are chlordiazepoxide—44, 88, 175, 350 and 700 ng, flurazepam—43, 85, 170, 340 and 680 ng, and midazolam—36, 71, 143, 286 and 571 ng

Apparatus

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

Procedure

Beginning 1 week post-operatively, the animals were adapted to the test apparatus and injection procedure [29] for 3 consecutive days (Wednesday–Friday) The subjects were placed in the photocell chambers for 30 minutes, removed and placed in a restraint for 30–60 seconds, then returned to the chambers for an additional 2 hours During the subsequent weeks, the animals were tested Monday through Friday, drug or vehicle injections being performed on Tuesdays and Thursdays The animals were placed in the apparatus for 30 minutes and their activity scores recorded at 15 minute intervals The rats then were removed, restrained, and injected (on drug days only) over 30 seconds with 0 5 μl of drug solution The animals then were placed back in the chambers for an additional 2 hours Activity counts were recorded 15 30, 60, 90, and 120 minutes post-injection

Histology

The animals were perfused transcardially with 100 ml of saline followed by 100 ml of phosphate-buffered formalin Their brains then were removed and post-fixed for at least one week prior to sectioning The tissue then was transferred to a solution containing 5% sucrose in 0 1 M phosphate buffer for 24–48 hours, frozen with dry ice and cut on a sliding microtome Every fourth section (50 μm) was retained, mounted on a gelatin coated slide and stained using the cresylecht violet procedure [45]

Statistical Analysis

Multivariate analyses were carried out using randomized block designs [11,33] The data from Experiment 1 were analyzed using an analysis of variance (ANOVA), three-factor mixed design with repeated measures on two factors (see [11], pp 73–84) Data from Experiment 2 was analyzed by an ANOVA, two-factor mixed design with repeated measures on one factor (*ibid*, pp 55–61) The results from Experiments 3 and 4 were analyzed using an ANOVA with a repeated measures—two factors design (*ibid* pp 48–54) Individual between-group comparisons were performed when merited, by Newman-Keuls' multiple range test [32,40] (see ref [11], pp 119–122), or by an F-test for simple effects (*ibid* pp 140–142) For within group comparisons a Newman-Keuls' test for related measures [32] (see [11], pp 137–138) was used Statistical analyses were performed on a Hewlett-Packard HP-85 minicomputer with a No 90053 statistical program package, as well as with Nos 300-0027, 300-0029 and 300-0035 ANOVA programs (Hewlitt-Packard Corp , Corvallis, OR)

RESULTS

EXPERIMENT 1 INTRA-RAPHE BENZODIAZEPINE DOSE-RESPONSE ANALYSIS

There is a substantial amount of evidence which indicates that GABA serves as a neurotransmitter in both the dorsal [22,39], and median [21] raphe nuclei The benzodiazepines, moreover, are though to act by facilitating GABA-ergic neurotransmission [12, 14, 22, 41, 43, 52, 54] We previously found [50] that injections of the GABA agonist, muscimol, directly into the median raphe nucleus produces dose-dependent increases in activity level We hypothesized, therefore, that intra-raphe injections of benzodiazepines also should produce hyperactivity by facilitating the effects of endogenously released GABA

Procedure

Three water soluble benzodiazepines (chlordiazepoxide, midazolam and flurazepam) were chosen for intra-raphe dose-response analysis Cannulae were implanted in the median raphe nucleus of 18 rats as described in the METHOD section Six of the rats were assigned to the chlordiazepoxide group, six to the midazolam group, and six to the flurazepam group The subjects were tested as described in the METHOD section On drug days, animals received saline or a dose of the appropriate benzodiazepine (0, 0 22, 0 44, 0 88 or 1 75 nmole) A three-factor split-plot ANOVA with repeated measures on two factors (see [11], pp 73–88) was used to analyze the results of this experiment

RESULTS

Histological Analysis

The behavioral data from 2 rats (one from the chlordiazepoxide group and one from the midazolam group) were eliminated prior to statistical analysis, since the cannula tips in these animals were localized 1 0–2 0 mm lateral to the median raphe nucleus The cannula tips in the accepted animals terminated in the rostral portion of the B-8 5-HT cell group [16], dorsal to the interpeduncular nucleus

Locomotor Activity

An ANOVA of the behavioral data demonstrated differences in the response to the three drugs, $F(2,15)=20\ 25$, $p<0\ 0002$ The effects of the different drug doses also were significant, $F(5,75)=13\ 79$, $p<0\ 0001$ The subjects' performances changed as a function of time after injection, $F(3,45)=668\ 26$, $p<0\ 00001$ In addition, the drug and dose, $F(10,75)=4\ 08$, $p<0\ 0005$, the drug and time, $F(6,45)=31\ 19$, $p<0\ 0001$, the dose and time, $F(15,225)=7\ 64$, $p<0\ 0001$, and the drug, dose and time, $F(30,225)=4\ 01$, $p<0\ 0001$, interactions all were significant Individual comparisons demonstrated that injections of flurazepam and midazolam into the median raphe nucleus produced a dose-dependent elevation in locomotor activity (Fig 1) Flurazepam was twice as potent as midazolam, the former having a peak effect at 0 44 nmole, the latter at 0 88 nmole Chloridazepoxide was without effect As shown in Fig 2, the benzodiazepine-induced hyperactivity was most prominent during the first 30 minutes post-injection

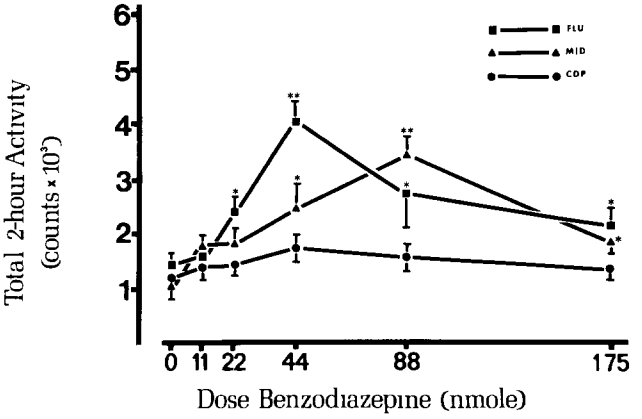

FIG 1 Intra-raphe benzodiazepine dose-response relationship Total activity scores (Mean±S E M) for the 2 hour period following injection of 0, 0 22, 0 44, 0 88 or 1 75 nmole of flurazepam (FLU, n=6), midazolam (MID n=5) or chlordiazepoxide (CDP, n=5) through cannulae chronically implanted in the median raphe (MR) nucleus Asterisks indicate significant elevation over the corresponding saline-injected control (dose=0) condition (*$p<0\ 05$, **$p<0\ 01$, Newman-Keuls' multiple range test)

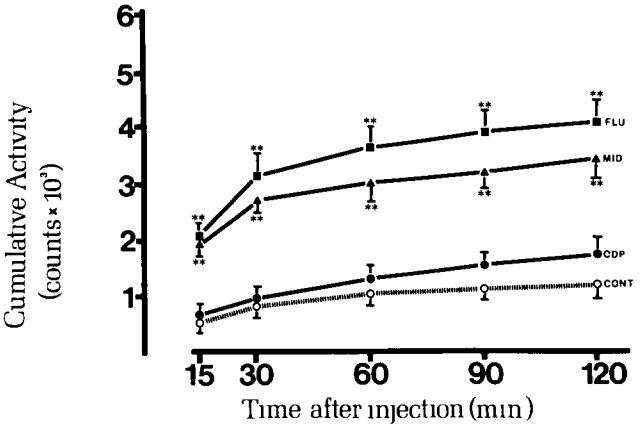

FIG 2 Temporal effects of intra-raphe benzodiazepines Cumulative activity scores (Mean±S E M) 15, 30, 60, 90 and 120 minutes following the injection of optimal doses of flurazepam (FLU, 0 44 nmole), midazolam (MID, 0 88 nmole), or chlordiazepoxide (CDP, 0 44 nmole) through cannulae chronically implanted in the median raphe nucleus For the sake of clarity, the post-saline control (CONT) scores for the 3 groups have been combined **significantly elevated over the corresponding saline-injected control condition ($p<0\ 01$, Newman-Keuls' test for related measures)

CONCLUSION

Facilitation of midbrain GABA neurotransmission by intra-raphe microinjections of the benzodiazepines, flurazepam and midazolam, led to an increase in locomotor activity Although qualitatively similar to the effects of muscimol injections into the median raphe nucleus, the magnitude and duration of the benzodiazepine effects were somewhat less [49,50] This is not surprising, however, since the effects of the benzodiazepines depend on the release of endogenous GABA, whereas muscimol, as a receptor agonist, does not The effective pharmacological doses of muscimol no doubt produce a greater concentration of receptor ligand than does the amount of GABA normally available for release

Interestingly, in contrast to midazolam and flurazepam, chlordiazepoxide failed to alter activity level following its injection into the median raphe nucleus This observation supports the view that chlordiazepoxide is a pro-drug, similar to prazepam and chlorazepate, which must be oxidized *in vivo* to produce a centrally active metabolite [9, 10, 15, 30, 48] Autoradiographic studies performed recently in our laboratory [51] support this conclusion

EXPERIMENT 2 INTRA-RAPHE BICUCULLINE DOSE-RESPONSE ANALYSIS

Bicuculline is reported to be a relatively specific GABA receptor antagonist [2, 3, 12] Since activation of GABA re-

ceptors by muscimol [49,50] and presumed facilitation of GABA-ergic transmission by benzodiazepines (Experiment 1) in the median raphe nucleus produces hyperkinesis we hypothesized that direct injection of the GABA antagonist bicuculline, should diminish locomotor activity Because the free base form of bicuculline must be dissolved in a vehicle too acidic for direct intracranial injections [42], this experiment was performed with the water-soluble salt bicuculline methiodide [38] We therefore, performed a dose-response analysis using intra-raphe microinjections of bicuculline methiodide

Procedure

Seven rats were outfitted with chronically indwelling cannulae in the median raphe nucleus Animals were tested as described in the Method section On drug days subjects received intracranial injections of either saline or bicuculline methiodide (0 22, 0 44 or 0 88 nmole, 81, 161 or 323 ng as the base) according to a randomized block design [33]

RESULTS

Histological Analysis

Of the seven rats tested, only 5 were found to have acceptable cannula placements The cannula tracts and tips in these rats were indistinguishable from those in the previous experiments, and terminated within the B-8, 5-HT cell group [16] dorsal to the interpeduncular nucleus

Locomotor Activity

A two-factor split-plot ANOVA with repeated measures on one factor (see [11], pp 55–61) failed to reveal a significant effect of bicuculline methiodide on locomotor activity Only the time after injection effect was significant $F(4,96)=22\ 24$, $p<0\ 0001$ Seizures were not observed in any of the animals following the administration of the bicuculline doses used

CONCLUSION

If direct activation of GABA receptors (such as produced by muscimol) or facilitation of GABA-ergic transmission (such as produced by the benzodiazepines) in the median raphe nucleus results in hyperactivity, then blockade of GABA receptors should have the opposite effect The data obtained in the present experiment, however, do not support this view Intra-raphe muscimol administration may acutely inhibit the firing rates of local 5-HT neurons Blockade of the inhibitory effects of endogenously released GABA at the same receptor sites nevertheless, may not result in the opposite condition Although it is possible that 5-HT perikarya in the region of the median raphe nucleus may increase their rate of discharge immediately following their release from a tonic GABA-ergic inhibition, this enhancement most likely would be followed by an almost immediate return to the baseline due to collateral feedback inhibition [1]

EXPERIMENT 3 INTERACTIONS OF INTRA-RAPHE BENZODIAZEPINES WITH MUSCIMOL

We reported previously [49,50] that intraperitoneal administration of a representative benzodiazepine, chlordiazepoxide, potentiated the hyperkinetic effect of intra-raphe muscimol injections To determine whether this potentiation was due to an action on benzodiazepine receptors locally in the region of the raphe or at distant sites, we combined a sub-effective dose of flurazepam or midazolam with a sub-effective dose of muscimol for injection into the median raphe nucleus

Procedure

Cannulae were implanted in the median raphe nuclei of 14 rats as described in the Method section Seven rats were assigned to the flurazepam-muscimol group and 7 to the midazolam-muscimol group In this experiment, animals received either saline vehicle, or a sub-effective dose (0 22 nmole) of flurazepam or midazolam into the median raphe nucleus 5 minutes prior to either saline (0 5 μl) or a sub-effective dose of muscimol (0 22 nmole) Otherwise, the procedure was the same as that in described in the Method section

A repeated measures-two factors ANOVA design was used for the statistical analysis of the behavioral data (see [11] pp 48–54) This is an analysis which can be used when all treatments in a two-factor experiment are administered to each subject The effects of each treatment can be discerned individually, and the interaction between the two can be ascertained

RESULTS

Histological Analysis

The behavioral data from 3 rats were eliminated prior to statistical analysis, since the cannula placements in these animals were localized 1 5–2 5 mm lateral to the median raphe nucleus The cannula tips terminated in the B-8 5-HT cell group [16] dorsal to the interpeduncular nucleus in the 5 rats comprising the flurazepam-muscimol group and in the 6 rats forming the midazolam-muscimol group

Locomotor Activity

Flurazepam-muscimol interaction An ANOVA of the total 2 hour post-injection activity scores demonstrated significant effects both of muscimol $F(1,26)=187\ 73$ $p<0\ 0001$, and of flurazepam, $F(1,26)=173\ 38$, $p<0\ 0001$ The effects of flurazepam and muscimol, moreover interacted, $F(1,26)=161\ 00$, $p<0\ 0001$ Individual comparisons however showed that only the combined injection of flurazepam and muscimol produced significant elevations over the vehicle-injected control condition (Table 1) This observation accounts for the significant effects of muscimol and flurazepam mentioned above

Midazolam-muscimol interaction An ANOVA of the total 2 hour post-injection activity scores demonstrated significant effects both of muscimol $F(1,27)=168\ 06$, $p<0\ 0001$ and of midazolam $F(1,27)=137\ 64$, $p<0\ 0001$ Similar to the results above, the effects of midazolam and muscimol interacted $F(1\ 27)=165\ 76$ $p<0\ 0001$ Individual comparisons showed that only the sequential injections of midazolam and muscimol together produced activity scores significantly higher than those after vehicle injections (Table 1) This observation accounts for the significant muscimol and midazolam effects reported above

CONCLUSION

Our previous dose-response analysis [49,50] demonstrated that muscimol in a dose of 0 22 nmole (25 ng) did not produce significant elevations in activity level The 0 22

TABLE 1

INTRA-RAPHE BENZODIAZEPINE INTERACTIONS WITH MUSCIMOL

Dose Benzodiazepine	Dose Muscimol: Vehicle	Dose Muscimol: 0 22 nmole
Flurazepam Vehicle	1848 ± 175	2251 ± 366
Flurazepam 0 22 nmole	2078 ± 174	5769 ± 392*
Midazolam Vehicle	1200 ± 182	1201 ± 165
Midazolam 0 22 nmole	1006 ± 95	5530 ± 126†

Total activity scores (Mean ± S E M) following injection of muscimol (0 22 nmole) or saline (0 5 μl) through cannulae chronically indwelling in the median raphe nucleus Five minutes prior to receiving muscimol, the animals were injected with flurazepam (0 22 nmole, n=5), midazolam (0 22 nmole, n=6) or saline (0 5 μl)

*Significantly different from muscimol vehicle plus flurazepam vehicle condition ($p<0$ 001), †Significantly different from muscimol vehicle plus midazolam vehicle condition ($p<0$ 001, Student s t-test, 2-tailed) [53]

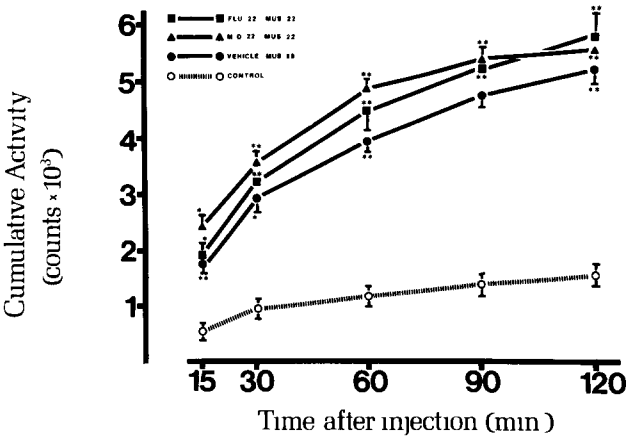

FIG 3 Interaction of intra-raphe benzodiazepines with muscimol Cumulative activity scores (Mean±S E M) 15, 30, 60, 90 and 120 minutes following injection of muscimol (0 22 nmole) or saline (0 5 μl) through cannulae chronically indwelling in the median raphe nucleus Five minutes prior to receiving muscimol, the animals were injected with flurazepam (0 22 nmole, n=5), midazolam (0 22 nmole, n=6) or saline (0 5 μl) The vehicle-muscimol 0 88 nmole data were obtained in Experiment 4d, which was conducted at the same time as the present experiment The control means (±S E M) were obtained in the same manner as reported in Fig 2 ** significantly elevated over the corresponding saline-injected control condition ($p<0$ 01 Newman-Keuls' test for related measures)

nmole dose of either flurazepam and midazolam also was below that required to induce hyperactivity (Experiment 1) However, when the subeffective dose (0 22 nmole) of muscimol was injected immediately following the intra-raphe administration of either benzodiazepine, a hyperkinetic effect was produced which was of the same magnitude as that of a four-fold higher dose (0 88 nmole) of muscimol alone, as illustrated in Fig 3 These observations support the conclusion that the benzodiazepines act by facilitating the postsynpatic effects of endogenously released GABA and of GABA agonists

EXPERIMENT 4 INTERACTIONS OF INTRA-RAPHE BICUCULLINE METHIODIDE AND MUSCIMOL

We have reported that peripheral administration of the GABA antagonist, bicuculline, blocked the hyperactivity-inducing effect of intra-raphe muscimol injections [49,50] We hypothesized, therefore, that pretreatment by intra-raphe administration of bicuculline methiodide should prevent the hyperkinesis induced by intra-raphe muscimol injections

Procedure

Cannulae were implanted in the median raphe nuclei of 7 rats as described in the Method section In this experiment, animals received either saline vehicle or bicuculline methiodide (0 88 nmole, 323 ng) into the median raphe nucleus 5 minutes prior to either muscimol (0 88 nmole, 100 ng) or saline injection The procedure otherwise was identical to that described in the Method section

RESULTS

Histological Analysis

Of the 7 rats studied 6 were found to have acceptable cannula placements in the rostral aspect of the median raphe nucleus, dorsal to the interpeduncular nucleus

Locomotor Activity

An ANOVA of the total 2 hour post-injection activity scores demonstrated that the effect of muscimol was significant, $F(1,27)=814$ 13, $p<0$ 0001 whereas that of bicuculline methiodide alone was not Furthermore, the effects of bicuculline methiodide and muscimol interacted, $F(1,27)=238$ 87, $p<0$ 0001 As shown in Table 2, intra-raphe bicuculline methiodide completely blocked the hyperactivity produced by a subsequent injection into the median raphe nucleus of an equimolar dose of muscimol

CONCLUSION

The results from these experiments suggest that the effects of peripheral injections of bicuculline on intra-raphe muscimol-induced hyperactivity probably are due to their interactions at the same GABA receptors within the midbrain

DISCUSSION

Evidence has been advanced which strongly suggests that GABA serves as a neurotransmitter in both the dorsal [6, 22, 23, 24, 35, 43, 44] and the median [21] raphe nucleus Benzodiazepine receptors also have been localized in the region of the median raphe nucleus [51, 56, 57] In a previous series of experiments [49,50], we found that direct intracranial injections of the GABA agonist, muscimol, into the dorsal or median raphe nucleus of rats produced a dose-dependent locomotor hyperactivity The median raphe nucleus,

TABLE 2
INTRA-RAPHE BICUCULLINE INTERACTIONS WITH MUSCIMOL

Dose Bicuculline Methiodide	Dose Muscimol	
	Vehicle	0 88 nmole
Vehicle	1638 ± 224	5210 ± 246*
0 88 nmole	1715 ± 235	1827 ± 103

Total activity scores (Mean ± S E M) for the 2 hour period following the injection of muscimol (0 88 nmole) or saline (0 5 μl) through cannulae chronically implanted in the median raphe nucleus (n=6) Five minutes prior to receiving muscimol or saline, the animals received bicuculline methiodide (0 88 nmole) or saline
*Significantly different from muscimol vehicle plus bicuculline vehicle condition ($p<0$ 001, Student's t-test, 2-tailed) [53]

moreover, was four times more sensitive to this effect of muscimol than was the dorsal raphe nucleus This hyperkinetic effect was potentiated by intraperitoneal administration of the benzodiazepine, chlordiazepoxide, and blocked by intraperitoneal injection of bicuculline In addition, depletion of forebrain 5-HT prevented the hyperactivity induced by intra-raphe muscimol

It remained to be determined, however, whether the effects of the peripherally administered bicuculline and chlordiazepoxide were due to their binding to the same neuronal membranes as the intra-raphe muscimol To investigate this problem we conducted the present study If benzodiazepine compounds do act to facilitate GABA-ergic transmission, it seemed plausible to hypothesize that intra-raphe administration of representative benzodiazepines might produce increases in locomotor activity We tested (Experiment 1) this hypothesis by injecting three different water-soluble benzodiazepines (chlordiazepoxide, flurazepam and midazolam) directly into the median raphe nucleus through chronically indwelling cannulae Water soluble compounds were chosen in order to avoid any potential behavioral effects that might ensue from the use of a non-aqueous vehicle The ethanol-propylene glycol vehicle commonly used to dissolve non-water soluble benzodiazepines most likely would have deleterious effects if injected directly into the brain We found that injections of flurazepam and midazolam directly into the median raphe nucleus both produced dose-dependent increases in locomotor activity (Fig 1) Flurazepam, moreover, produced a maximal hyperkinetic effect at a dose one-half as great as that required for midazolam This observation is consistent with the hypothesis that these compounds act on two different allosteric sites on a benzodiazepine-GABA-receptor complex [12, 41, 43, 44] The greater potency of flurazepam over midazolam in inducing hyperactivity also is in keeping with the relative order of potencies of these drugs in displacing radiolabeled benzodiazepines from binding sites *in vitro* [7, 8, 37] The weaker response to midazolam could also be due to the insolubility of this compound at physiological pH [26]

The failure of chlordiazepoxide both to induce hyperactivity when injected into the median raphe nucleus *in vivo* and to displace ^{3}H-flunitrazepam binding *in vitro* [51] is further evidence that chlordiazepoxide is a pro-drug which must be converted to active metabolites in order to have an effect in the central nervous system [9, 10, 15, 25, 28, 30, 48]

The benzodiazepines are thought to act post-synaptically by binding at a site on a large receptor-ionophore complex allosteric to the GABA binding site, and thereby increase the affinity of the latter for its ligand [12, 13, 14, 41, 42, 44] If so then the GABA agonist, muscimol, and the centrally active benzodiazepines should have additive hyperkinetic effects when injected one after the other directly into the median raphe nucleus We combined a sub-effective dose of muscimol with a sub-effective dose of either flurazepam or midazolam Both combinations produced hyperkinetic effects as robust as a fourfold higher dose (0 88 μmole) of muscimol alone (Fig 3) Inasmuch as specific benzodiazepine binding sites are known to exist in the central nervous system [37, 56, 57] it is possible that the hyperkinetic effects of intra-raphe benzodiazepines are independent of interactions at the GABA binding site The finding that the combinations of midazolam or flurazepam with muscimol produced supra-additive effects renders this latter interpretation unlikely

If facilitation of GABA-ergic neurotransmission in the median raphe nucleus produces hyperactivity, then blockade of GABA receptors might be expected to have the opposite effect However, intra-raphe bicuculline injections did not affect activity level This observation is not surprising, since release from tonic GABA-ergic inhibition [21] would result in an initial brief increase in the firing rates of 5-HT neurons, followed by an almost immediate return to baseline due to collateral feedback inhibition [1] If, on the other hand, the GABA-ergic inhibition in the median raphe is not tonic, then bicuculline methiodide would not be expected to produce the predicted hypoactivity The intra-raphe injection of bicuculline methiodide, however, did block the hyperkinetic effect of muscimol, providing additional evidence that the muscimol effect is due to a direct activation of GABA receptors within the raphe

The results from these experiments support the view that the intra-raphe injection of muscimol produces hyperkinesis via activiation of local GABA receptors In addition, they suggest that intra-raphe administration of certain benzodiazepines can produce hyperactivity by facilitating GABA transmission

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REFERENCES

1 Aghajanian, G K and R Y Wang Physiology and pharmacology of central serotonergic neurons In *Psychopharmacology A Generation of Progress* edited by M A Lipton, A Di Mascio and K F Killam New York Raven Press 1978, pp 171–183

2 Andrews P R and G A R Johnston GABA agonists and antagonists *Biochem Pharmacol* **28** 2697–2702 1979

3 Arnt, J and P Krogsgaard-Larsen GABA agonists and potential antagonists related to muscimol *Brain Res* **177** 395–400 1979

4 Baldessarini, R J Drugs and the treatment of psychiatric disorders In *The Pharmacological Basis of Therapeutics, 6th ed* edited by A G Gilman L S Goodman and A Gilman New York MacMillan Publishing Co , 1980, pp 436–447
5 Beaumont K , W S Chilton, H I Yamamura and S J Enna Muscimol binding in rat brain Association with synaptic GABA receptors *Brain Res* **148** 153–162, 1979
6 Belin, M F , M Aguera, M Tappaz, A MacRae-Degueurce, P Bobillier and J F Pujol GABA-accumulating neurons in the nucleus raphe dorsalis and periaqueductal gray in the rat a biochemical and radioautographic study *Brain Res* **170** 279–297, 1979
7 Braestrup, C and M Nielsen Benzodiazepine receptors *Arzneimittelforsch* **30** 852–857, 1980
8 Braestrup, C and R F Squires Brain specific benzodiazepine receptors *Brit J Psychiat* **133** 249–260, 1978
9 Breimer, D D Clinical pharmacokinetics of hypnotics *Clin Pharmacokinet* **2** 93–109, 1977
10 Breimer D D , R Jochemsen and H H von Albert Pharmacokinetics of benzodiazepines *Arzneimittelforsch* **30** 875–881, 1980
11 Bruning, J L and B L Kintz *Computational Handbook of Statistics, 2nd ed* Glenview, IL Scott Foresman 1977
12 Costa, E and A Guidotti Molecular mechanisms in the receptor action of benzodiazepines *Annu Rev Pharmacol Toxicol* **19** 531–546 1979
13 Costa, T D Rodbard and C Pert Is the benzodiazepine receptor coupled to a chloride anion channel? *Nature* **277** 315, 1979
14 Costa T , L Russel, C B Pert and D Rodbard Halide and *gamma*-aminobutyric acid induced enhancement of diazepam receptors in rat brain *Mol Pharmacol* **20** 470–476 1981
15 Curry S H and R Whelpton Pharmacokinetics of closely related benzodiazepines *Br J Clin Pharmacol* **8** 12S–15S 1979
16 Dahlstrom, A and K Fuxe 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) 1–55, 1964
17 De Feudis F V Amino acids as central neurotransmitters *Annu Rev Pharmacol* **15** 105–130 1975
18 Enna, S J and A Maggi Biochemical pharmacology of GABA-ergic agonists *Life Sci* **24** 1727–1738, 1979
19 Fonnum, F *Amino Acids as Chemical Transmitters NATO Advanced Study Series* Series A *Life Sci* New York Plenum Press 1978
20 Fonnum, F and J Storm-Mathisen Localization of GABA-ergic neurons In *Handbook of Psychopharmacology Vol 9* edited by L L Iversen S D Iversen and S H Snyder New York Plenum Press 1978 pp 357–401
21 Forchetti, C M and J L Meek Evidence for a tonic GABAergic control of serotonin neurons in the median raphe nucleus *Brain Res* **206** 208–212, 1981
22 Gallager D W Benzodiazepines potentiation of a GABA inhibitory response in the dorsal raphe nucleus *Eur J Pharmacol* **49** 133–143, 1978
23 Gallager D W and G K Aghajanian Effect of anti-psychotic drugs on the firing of dorsal raphe cells II reversal by picrotoxin *Eur J Pharmacol* **39** 357–364, 1976
24 Gamrani H , A Calas, M F Belin, M Aguera and J F Pujol High-resolution radioautographic identification of [3H]-GABA labeled neurons in the rat nucleus raphe dorsalis *Neurosci Lett* **15** 43–48 1979
25 Greenblatt D J and R I Shader *Benzodiazepines in Clinical Practice* New York Raven Press, 1974
26 Greenblatt D J R I Shader, M Divall and J S Harmatz Benzodiazepines a summary of pharmacokinetic properties *Br J Clin Pharmacol* **11** (Suppl 1) 11S–16S 1981
27 Haefely W , A Kulcsar, H Mohler, L Pieri, P Polc and R Schaffner Possible involvement of GABA in the central actions of benzodiazepines In *Mechanism of Action of Benzodiazepines* edited by E Costa and P Greengard New York Raven Press 1975, pp 131–149
28 Harvey, S C Hypnotics and sedatives In *The Pharmacological Basis of Therapeutics, 6th ed* , edited by A G Gilman, L S Goodman and A Gilman New York Macmillan Publishing Co , 1980, pp 339–375
29 Jacquet, Y F Intracerebral administration of opiates In *Methods in Narcotics Research*, edited by S Ehrenpreis and A Neidle New York Mercel-Dekker, Inc , 1975, pp 33–57
30 Johnson, P and P A Rising Absorption, distribution, metabolism and excretion of anxiolytics In *Anxiolytics*, edited by S Fielding and H Lal Mt Kisco, New York Futura Publishing Co , 1979, pp 211–246
31 Johnston, G A R Neuropharmacology of amino acid inhibitory transmitters *Annu Rev Pharmacol Toxicol* **18** 269–289, 1978
32 Keuls, M The use of studentized range in connection with an analysis of variance *Euphytica* **1** 112–122, 1952
33 Kirk, R *Experimental Design Procedures for the Behavioral Sciences* Belmont, CA Wadsworth Publ Co , 1968
34 Krogsgaard-Larsen, P and J Arnt Pharmacological studies of interactions between benzodiazepines and GABA receptors *Brain Res Bull* **5** (Suppl 2) 867–872, 1980
35 Massari, V J , Z Gottesfeld and D M Jacobowitz Distribution of glutamic acid decarboxylase in certain rhombencephalic and thalamic nuclei of the rat *Brain Res* **118** 147–151, 1976
36 Mc Geer, P L and E G Mc Geer Amino acid neurotransmitters In *Basic Neurochemistry 3rd ed* , edited by G J Siegel, R W Albers, B W Agranoff and R Katzman Boston Little, Brown and Co , 1981, pp 233–253
37 Mohler H and T Okada Benzodiazepine receptor demonstration in the central nervous system *Science* **198** 849–851, 1977
38 Mohler, H and T Okada GABA receptor binding with 3H(+)-bicuculline methiodide in rat CNS *Nature* **267** 65–67, 1977
39 Nanopoulos, D , M F Belin, M Maitre and J F Pujol Immunocythochimie de la glutamate decarboxylase mise en evidence d'elements neuronaux GABAergiques dans le noyau raphe dorsalis du rat *C R Seances Acad Sci Paris* **290** (Ser D) 1153–1156, 1980
40 Newman, D The distribution of the range in samples from a normal population, expressed in terms of an independent estimate of standard deviation *Biometrika* **31** 20–30, 1939
41 Olsen, R W Drug interactions at the GABA receptor-ionophore complex *Annu Rev Pharmacol Toxicol* **22** 245–277, 1982
42 Olsen, R W , M Ban, T Miller and G A R Johnston Chemical instability of the GABA antagonist, bicuculline, under physiological conditions *Brain Res* **98** 383–387, 1975
43 Paul, S M , P J Marangos and P Skolnick The benzodiazepine-GABA-chloride ionophore receptor complex common site of minor tranquilizer action *Biol Psychiatry* **16** 213–229, 1981
44 Paul, S M and P Skolnick Benzodiazepine receptors and psychopathological states toward a neurobiology of anxiety In *Anxiety New Research and Changing Concepts* edited by D F Klein and J Rabkin New York Raven Press, 1981
45 Powers, M M and G Clark An evaluation of cresyl echt violet acetate as a nissl stain *Stain Technol* **30** 83–88 1955
46 Przewocka, B , L Stala and J Scheel-Kruger Evidence that GABA in the nucleus dorsalis raphe induces stimulation of locomotor activity and eating behavior *Life Sci* **25** 937–946, 1979
47 Rall, T W and L S Schleifer Drugs effective in the therapy of the epilepsies In *The Pharmacological Basis of Therapeutics 6th ed* edited by A G Gilman, L S Goodman and A Gilman New York MacMillan Publishing Co 1980, pp 466–474
48 Randall, L O and B Kappell Pharmacological activity of some benzodiazepines and their metabolites In *The Benzodiazepines* edited by S Garattini, E Mussini and L O Randall New York Raven Press, 1973, pp 27–51
49 Sainati, S M Midbrain benzodiazepine-GABA-serotonin interactions effects on locomotor activity in the rat Doctoral Dissertation Loyola University of Chicago, 1982

50 Sainati, S M and S A Lorens Intra-raphe muscimol-induced hyperactivity depends on ascending serotonin projections *Pharmacol Biochem Behav* **17** 973–986, 1982
51 Sainati, S M , H K Kulmala and S A Lorens Further evidence that chlordizaepoxide must be metabolized before producing behavioral effects *Fed Proc* **41** 1067, 1982
52 Sepinwall, J and L Cook Mechanism of action of the benzodiazepines behavioral aspect *Fed Proc* **39** 3024–3031, 1980
53 Student Errors of routine analysis *Biometrika* **19** 151–164, 1927
54 Tallman, J F , J W Thomas and D W Gallager GABAergic modulation of benzodiazepine binding site sensitivity *Nature* **274** 383–385, 1978
55 Worms, P , H Depourtere and K G Lloyd Neuropharmacological spectrum of muscimol *Life Sci* **25** 607–614 1979
56 Young, W S and M J Kuhar Autoradiographic localisation of benzodiazepine receptors in the brains of humans and animals *Nature* **280** 393–394, 1979
57 Young, W S and M J Kuhar Radiohistochemical localization of benzodiazepine receptors in rat brain *J Pharmacol Exp Ther* **212** 337–346, 1980