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Diazepam-Treated Female Rats: Flumazenil- and PK 11195-Induced Withdrawal in the Hippocampus CA1

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SLOAN, J. W., E. P. WALA, X. JING, J. R. HOLTMAN, JR. AND B. MILLIKEN. *Diazepam-treated female rats: Flumazenil- and PK 11195-induced withdrawal in the hippocampus CA1.* PHARMACOL BIOCHEM BEHAV **61**(1) 121–130, 1998.—Six female rats had a loading dose of 180 mg of diazepam (DZ) contained in two Silastic capsules implanted in their backs. Thereafter, a single 90-mg capsule was implanted weekly for 4 weeks prior to weekly microinjections of 1 μl of flumazenil (6.25, 12.5, or 25 μg) and PK 11195 (3.125, 6.25, or 12.5 μg) or vehicle into the CA1. Three control rats had empty capsules implanted but received only the high dose of flumazenil after 5 weeks. The time of DZ exposure spanned 8 weeks. Mean steady-state plasma levels of DZ were 1.06 ± 0.11, and the mean total (DZ + metabolites) was 2.46 μg/ml ± 0.37. Flumazenil elicited a dose-related precipitated withdrawal score (PAS) in DZ-treated rats (but not in controls) characterized by dose-related increases in convulsive (twitches and jerks), motor and autonomic signs, dose-related increases in the percent of total power in the low frequency (1–4 Hz), and decreases in the high-frequency (18–26 Hz) bands of the EEG recorded from the dentate and the amygdala. PK 11195 produced a dose-related increase in the 4–12 Hz band of the EEG recorded from the CA1, whereas the PAS was mild and not dose-related. However, the 6.25 and 12.5-μg doses elicited a significant PAS that tended to increase with dose. These data indicate that chronic DZ produces dependence, and that in the CA1 it involves the participation of central and possibly peripheral benzodiazepine (BZ) receptors located within this structure. © 1998 Elsevier Science Inc.

Withdrawal Hippocampus Hippocampus CA1 Flumazenil PK 11195 Benzodiazepine antagonists Central benzodiazepine receptors Peripheral benzodiazepine receptors Focal injection EEG

THE benzodiazepines (BZs) continue to be among the most commonly prescribed of any class of drugs, mostly for emotional disturbances, sedation, sleep, somatic disorders, and epilepsy (28,34). Approximately 12.5% of the population (among the elderly, twice as many women as men) use an anxiolytic, most of which are BZs (68). BZs do, however, produce unwanted side effects (especially with high doses and long-term use) such as tolerance, dependence (8,28,35), alterations in steroidogenesis (10,11,66), and alterations in the immune response (14), to name a few. A single class of BZ receptors consisting of two types of central BZ receptors (CBR) were originally identified (CBR-1 and CBR-2, localized post- and presynaptically, respectively) (53). The CBR are thought to be responsible for the therapeutic actions of the BZs, although current evidence suggests that the peripheral BZ receptors (PBR) are also involved, and, in addition, may play a role in the emergence of many unwanted side effects, particularly during chronic use. The CBR are restricted to the central nervous system (CNS) where they have a heterogenous distribution and, in general, are part of the macromolecular $GABA_A$ receptor complex, which contains an integral binding site for the CBR where the BZs bind and potentiate the actions of GABA at GABA-dependent chloride channels. The PBR distribution belies the name because they are found in the CNS as well as in peripheral tissues. They are located in large part on the outer mitochondrial membrane and are

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thought not to be directly connected to any GABA-regulated chloride channel; however, the PBR can alter the rate of steroidogenesis and several steroid metabolites alter $GABA_A$, suggesting a possible link between the CBR/$GABA_A$ complex and the PBR (12,44). Within the brain, the PBR are found mainly on glial cells but have also been identified on neuronal cells in low density (3,4).

The CBR specific antagonist, flumazenil, precipitates diverse signs of withdrawal in a variety of BZ-dependent animals (35,69) which led us to the hypothesis that different brain structures produce different signs of precipitated withdrawal. In male rats chronically exposed to DZ continuously released from subcutaneously implanted silastic capsules, flumazenil was demonstrated to evoke an intense precipitated withdrawal that included clonic and tonic–clonic convulsions (20,67). This procedure for producing BZ dependence was confirmed in female rats (36). Experiments were, therefore, conducted to determine whether the flumazenil-induced withdrawal syndrome in the rat treated chronically with DZ differed between brain structures. Over 20 brain areas were explored, and it was found that different brain structures produce different types of dependence, as revealed by differences in the precipitated withdrawal syndrome. The PBR antagonist, PK 11195, also evoked signs of precipitated withdrawal in some, but not all, brain areas that differed qualitatively and quantitatively from the withdrawal syndrome evoked by flumazenil. Preliminary data collected from one of the structures examined, the hippocampus, showed regional variations in the signs and intensity of flumazenil-induced withdrawal with the CA1 of the dorsal hippocampus showing more intense signs of withdrawal than the other three areas examined (body of hippocampus, CA1 of Ammons Horn, and the dentate gyrus) (56). The hippocampus has been shown to contain not only CBR, for which flumazenil shows specificity, but low concentrations of PBR, for which PK 11195 shows specificity (27). Several BZs bind both to the CBR and the PBR, among them are DZ and flunitrazepam, whereas other CBR ligands such as nordiazepam, clonazepam, zolpidem, and the CBR antagonist, flumazenil, do not bind, or show very low affinity for the PBR. In contrast, the BZ, RO5-4864, shows low-affinity binding at CBR sites but is active in the nanomolar range at peripheral sites (27).

Several lines of evidence have shown that flumazenil and PK 11195 differ in their binding specificities (4,27,53), pharmacological activity (35,43), and, in spite of the fact that they produce some of the same signs of precipitated withdrawal, there are qualitative and quantitative differences in the these effects (37,63). Both antagonists attenuate the withdrawal syndrome when administered chronically with the agonist (19,30,41,42), and both have been shown to produce intrinsic effects of their own when administered in high doses (16,17). The finding that flumazenil potentiates the proconflict effect of RO5-4864 and the anticonflict effect of PK 11195 in the rat led to the conclusion that these antagonists can act in opposing ways. This hypothesis was supported by the observation that bulbectomy, an area rich in PBR in the rat, suppressed the deterrent effect of punishment (43). It has been shown that the PBR are involved in tolerance and/or dependence to lorazepam in mice and to DZ in rats (37,39,41,65). The concurrent administration of PK 11195 with lorazepam prevented lorazepam-elicited behavioral tolerance, attenuated the behavioral discontinuation syndrome, effects that were antagonized by RO5-4864 (41,42), and its coadministration with DZ prevented the development of tolerance to DZ's effects on the EEG (39). A significant precipitated withdrawal syndrome was induced in male rats treated subcutaneously (SC) with DZ (15 mg/kg/per day) for 8 days by the intraperitoneal (IP) administration of either flumazenil (15 and 20 mg/kg) or PK 11195 (5 and 10 mg/kg). Diarrhea, decreased spontaneous motor activity, arched back, and tail erection were more marked in PK 11195-treated rats, whereas tremors and some behavioral signs such as the startle response were more pronounced in flumazenil-treated rats (37). Further, it was shown that in DZ-dependent rats, flumazenil induced a phase of strong irregular spiking activity that remained located in the duodenum in contrast to PK 11195, which induced a period of propagated myoelectric complexes and accelerated the intestinal transit more than did flumazenil (38).

In an attempt to better understand the dependence-producing properties of DZ, the study reported herein was undertaken as one part of a larger study to test the hypothesis that different BZs produce unique types of dependence that differ between brain structures. The current study measures the ability of graded doses of flumazenil and PK 11195 to induce a dose-related precipitated withdrawal in the CA1 of the hippocampus of noncastrated female rats treated chronically with DZ.

METHOD

Animals

Experiments, approved by the Institutional Animal Care and Use Committee of the University of Kentucky, were performed in nine (six DZ-treated and three controls) female Sprague–Dawley rats (~250 g) exposed to a 12 L:12 D cycle (lights on 0600 h) with free access to food and water. They were housed individually in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. All surgeries were performed using sterile conditions and ketamine anesthesia (80 mg/kg/IP). The rats were euthanatized by brain removal during deep pentobarbital anesthesia (120 mg/kg/IP).

Surgical Implantation of Guide Cannulae and EEG Electrodes

The rats were mounted in a Kopf stereotaxic instrument and a unilateral indwelling stainless steel guide cannula-recording electrode (22 gauge, insulated, except at the tip, with epoxylite 6001M) was implanted into the occipital cortex directed toward the CA1 of the dorsal hippocampus with the following intraural coordinates: AP = 3.8 mm, L = 4.5 mm, V = 8.0 mm] (49). Intracranial stainless steel recording electrodes (Plastic Products Co.) were implanted into the dentate (AP = 4.7 mm, L = 2 mm, V = 6.2 mm); entorhinal cortex (AP = 3.7 mm, L = 6.8 mm, V = 2.2 mm); basolateral amygdala (AP = 6.44 mm, L = 5 mm, V = 1.6 mm), and into the frontal cortex (AP = 10.2 mm, L = 2.0 mm, V = 8.2 mm) along with an indifferent electrode embedded in the skull behind the lambda. The electrodes were connected to a pedestal and secured to the bone with acrylic cement. Electrodes were not placed into the entorhinal cortex and amygdala of control rats.

Chronic Administration of DZ

After recovery from surgery, two Silastic capsules (Medical Grade Silastic tubing, i.d. 0.147 cm × o.d. 0.195 cm × 7 cm long) containing 90 mg of DZ each and sealed at both ends with Silicon Type A Silastic Medical Adhesive were implanted SC into the backs of rats. Thereafter, one 90-mg capsule of DZ (or empty capsule for controls) was implanted at

weekly intervals as previously described (20) with minor modifications (36).

Drugs

DZ and flumazenil were generous gifts from Hoffmann–LaRoche (Nutley, NJ) and PK 11195 was purchased from Research Biochemicals International. Flumazenil and PK 11195 were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO).

Precipitated Withdrawal

After 5 weeks of DZ or empty-capsule stabilization, the rats were placed in a round Faraday observation arena with grounded stainless steel walls, 18 inches i.d. × 9 inches high, with a stainless steel screen top and a Plexiglas floor covered with sawdust. The pedestal of the rat headset was connected to a Grass 78D EEG/polygraph through a commutator (SL6C) and concentric mercury swivel (Plastics One, Roanoke, VA). The EEG and signs and symptoms of precipitated withdrawal were recorded simultaneously for 10 min prior to (baseline) and 20 min after focal injections in the freely moving rats. The withdrawal signs were recorded in 5-min epochs on the standardized observation forms and on the EEG tracing. One microliter of DMSO, flumazenil (6.25, 12.5, and 25 μg), or PK 11195 (3.125, 6.25 or 12.5 μg) dissolved in DMSO was microinjected unilaterally via a chemotrode (28 gauge) into the CA1 region of the dorsal hippocampus (V = 6.8 mm). Injections were made without handling the rat by using a Hamilton syringe connected to the chemotrode with polyethylene tubing (PE-20). Each rat served as its own control with respect to the vehicle. The doses of flumazenil were selected on the basis of preliminary results obtained from microinjection studies into the CA1 of the DZ-dependent rat (56). The doses of PK 11195 were chosen from preliminary experiments in our laboratory and from others (37–39). The three doses of flumazenil and the three doses of PK 11195 were administered on Monday and Thursday of each week, respectively, with each drug administered in a Latin square replicate-block design. DMSO was administered on Monday after the last dose of PK 11195. Controls (empty capsule-implanted rats) received only the highest dose of flumazenil (25 μg) and the vehicle (DMSO) administered on Monday and Thursday of week 5, and therefore, were in the experiment for a shorter time (5 weeks) than the DZ-dependent animals (8 weeks).

The signs of precipitated withdrawal, identified by one observer (blinded as to treatment) and recorded by another, included signs used to calculate the precipitated withdrawal score (PAS) such as convulsive phenomena (clonic and tonic–clonic convulsions, twitches, and jerks); motor signs (turning, backing, jumping, head, and body tremors); dyskinesias (writhing); autonomic signs (respiratory rate), and affective signs (arched back and vocalization). The frequency of occurrence of each of these signs (except respiration rate, which was counted one time each epoch) was recorded for each epoch (36). The behavioral score (BS) was calculated as previously described (36,40,46) from items related to activity such as flaccid, loss of righting reflex, prone, sitting, curled posture, walking, standing, preening, exploring, digging, wet dog or head shakes, and poker tail (stiff tail held parallel to the floor of the cage). Postures such as flaccid, loss of righting reflex, and prone were given a negative weight ranging from −16 for flaccid to a −2 for prone, whereas all other signs were given positive scores ranging from +1 for sitting to +7.2 for poker tail. Each sign was counted one time per epoch, if present (with the exception of wet dog or headshakes and digging, where each occurrence was counted). If more than one type of negative sign occurred, the one most negative was used because, for example, a flaccid rat has lost its righting reflex and is prone; therefore, only the flaccid sign is scored. A positive total score indicates behavioral activation, whereas a negative score indicates a depressant effect. The frequency of occurrence of other unscored signs were also documented and analyzed (stretching, rearing, chewing, ear twitches, head bobbing, hot foot walking, blinking, rearing, rigid walking, and scratching). The weighted data for calculating the PAS, BS as well as the unweighted individual signs were normalized by subtracting the average preantagonist or preDMSO (vehicle) score from each postantagonist or postvehicle score for each DZ-treated or control rat. The normalized DMSO value was then subtracted from each normalized postantagonist value for each epoch for each rat for each sign and a mean value and estimation of its variance for each epoch was calculated [as well as an average $AUC_{(0–20min)}$] and used to determine time action and dose–response curves. No maximum attainable score is assigned for either the PAS or the BS.

EEG Recording and Analysis

The baseline EEG signals were recorded from the site of injection in the CA1 and from the indwelling electrodes for 10 min prior to the injection of antagonists or vehicle followed by a 20-min postinjection period in DZ-treated and control rats. All EEG signals were digitized continuously at 256 samples/s and stored on a data acquisition board for off-line analysis by Fast Fourier transform (FFT) on 4-s epochs after filtering the signals with a bandpass filter (1–32 Hz). The power spectrum was averaged for 5-min intervals, and the total distribution across frequency ranges was determined. The frequency range was divided into five frequency bands: delta (1–4); theta and alpha (4–12); beta 1 (12–18); beta 2 (18–26), and fast beta (26–30 Hz). A mean value and its standard error was obtained using nonnormalized data for the percentage of the total power in the individual bands in a given site and for a given epoch or for the $AUC_{(0–20\ min)}$ after injecting graded doses of flumazenil, PK 11195, or the vehicle into the CA1.

Plasma Levels of DZ and Its Metabolites

Blood was collected via the tail vein at weekly intervals for determining plasma levels of DZ and its metabolites by HPLC according to previously described procedures (36,62).

Histology

At the end of the study, brains were removed from the rats while they were deeply anesthetized with pentobarbital, and immediately fixed in 10% formalin for 2–7 days. The brain was then placed in 10% sucrose solution prior to dissecting and freezing the areas interest with CO_2. Sections were cut at 32 μ with a cryostat, thaw mounted on gelatin-coated slides, dried, stained with neutral red, and examined using a Nikon Ophiphat microscope and the atlas of Paxinos and Watson (49) to verify the correct placement of cannulae and electrodes.

Data Analysis

Prior to the use of parametric statistics, the source population was determined to have normal distribution and homoscedasticity ($p < 0.05$) by the Kolmogorov-Smirmov normality test and the Levene Median equal variance tests. In

some instances where the data failed the normality and/or equal variance test, the nonparametric Mann–Whitney rank sum *t*-statistic was used to calculate *t* and its *p*-value. Other analyses included linear regression, paired and unpaired *t*-tests, one- and two-way repeated measures analyses of variance (ANOVA) with the Bonferroni, Student–Newman–Keuls or Dunnet's tests used when appropriate for post hoc comparisons. Sigma Stat software for windows was used for these calculations, and a probability level of 0.05 or less was required for significance.

RESULTS

Body Weight

The DZ-treated rats ($n = 6$) gained weight with time and at the end of the experiment were 38.12 ± 4.58 g heavier than their precapsule implant weight (Fig. 1), whereas the empty capsule-implant controls ($n = 3$) neither gained nor lost significant weight (paired *t*-test, data not shown). The DZ-treated rats gained weight rapidly from week 6 to week 8.

Plasma Levels of DZ and Its Metabolites

The mean steady-state plasma levels for DZ and its metabolites (week 5 through week 8) remained relatively stable for the DZ-treated rats (Fig. 2). DZ was the major BZ detected in plasma, while temazepam and nordiazepam were the metabolites found in the lowest concentrations. Oxazepam was the only BZ that had a significant regression on time during this period ($y = -1.53 + 0.287$ x, $p < 0.040$). It should be pointed out, however, that the assumption of accumulation should be made with caution because single IV doses of DZ were not administered, a factor that precludes calculation of the accumulation index.

Precipitated Withdrawal

Due to space limitations, some findings are not presented in figure or table form, which include the following observations: 1) nine of nine rats (six DZ-treated and three controls) in this study were verified histologically to have correct placement of electrodes and cannulae. 2) The repeated weekly challenge with flumazenil or PK 11195 did not alter the intensity of the PAS, BS, or the signs comprising the two scores as determined by one-way repeated measures ANOVAs of the data. 3) In control rats, only DMSO and the highest dose of flumazenil (25 μg) were administered into the CA1. A two-way repeated measures ANOVA comparing the PAS induced in the two treatment groups (DZ-treated and controls) with doses (0 and 25 μg) as the repeated factor, showed that there was a significant between-treatments, $F(1, 7) = 10.73$, $p < 0.0136$, and between-doses $F(1, 7) = 0.48$, $p < 0.0143$ effect and a significant interaction between treatments and dose, $F(1, 7) = 8.83$, $p < 0.0208$. The DZ-treated rats had a higher PAS than the control rats and the flumazenil-treated rats had a higher PAS than DMSO-treated rats, $p < 0.05$ (Bonferroni method of all pairwise multiple post-hoc comparisons). DMSO produced no statistically significant effect on the PAS in either DZ-treated or in control rats.

Figure 3A and B show the time action curves for the PAS elicited by flumazenil and PK 11195, respectively, after injection into the CA1 of DZ-treated rats. Each dose of flumazenil evoked a PAS significantly greater than the vehicle (DMSO) during the first two postinjection epochs. Further, the 25 μg dose of flumazenil induced a more intense PAS in DZ-treated rats than in controls during these epochs. The peak effect on the PAS was observed within 5 min and then declined during the remaining 20-min postinjection period for both flumazenil (A) and PK 11195 (B), but only the lowest (3.125 μg) and highest doses (12.5 μg) of PK 11195 were significantly greater than DMSO during the 5-min epoch postinjection. The 12.5 μg dose of PK 11195 was significantly greater than DMSO for each epoch of the 20-min of observation. There was no evidence for a dose-related effect of PK 11195 during any epoch of the time action curve (B). Part C shows the highly significant dose–response relationship for the PAS on dose of flumazenil but not for the PAS induced by graded doses of PK 11195. Note that the configuration of the PAS dose–response curve for PK 11195 is "U-shaped." The PAS evoked by the

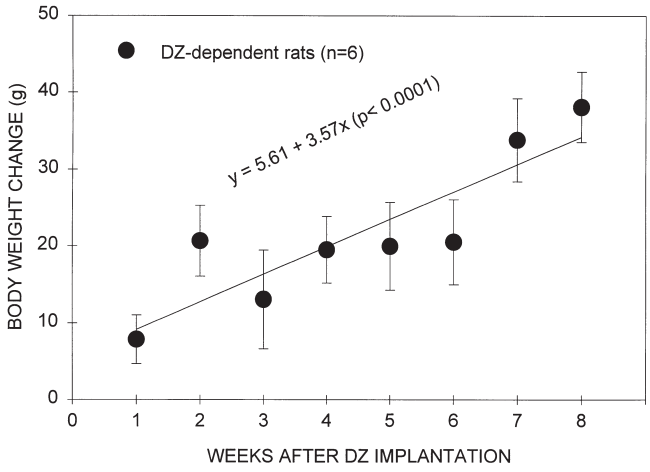

FIG. 1. The regression line for body weight change on time (shown with its equation) in rats with Silastic capsules containing 90 mg of DZ implanted subcutaneously in their backs each week, which were not removed during the course of the study. The rats were weighed each week just before the capsule was implanted. Each value represents the mean weekly weight change from the preimplant weight (±SEM), $n = 6$ rats.

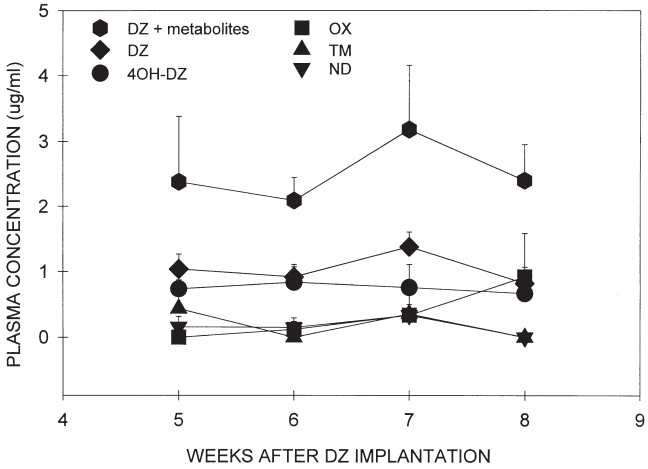

FIG. 2. Plasma levels for DZ and its metabolites [4-hydroxy diazepam (4-OHDZ); oxazepam (OX); temazepam (TM); and nordiazepam (ND)] measured weekly during the course of the precipitated withdrawal experiments. Values are the mean + SEM, $n = 5$ rats.

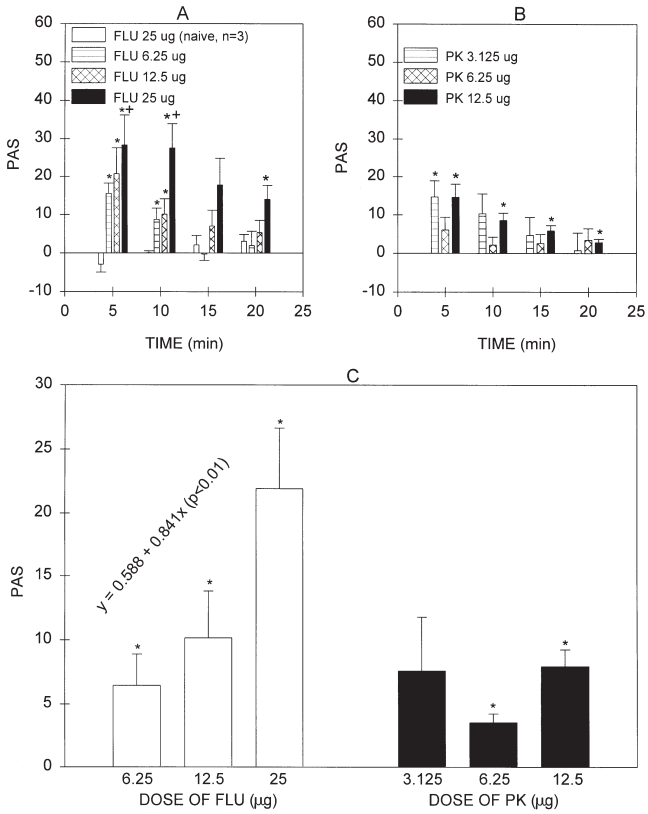

FIG. 3. The time action course of the normalized PAS induced by flumazenil (FLU) [25 μg in 1 μl)] in empty capsule-implanted naive controls (empty bars) (n = 3) and by graded doses of flumazenil (6.25, 12.5, and 25 μg in 1 μl) in DZ-treated (90 mg/week) (n = 6) female rats. Values are the mean + or − SEM (A). B shows the time course of the normalized mean PAS + SEM (n = same six rats shown in A) evoked by the microinjection of 1 μl of graded doses of PK 11195 (PK) (3.125, 6.25, and 12.5 μg) into the CA1 of the dorsal hippocampus. C shows dose–response curves for the PAS presented as the normalized $AUC_{(0–20min)}$, mean + SEM (n = 6) induced by flumazenil (calculated from data in A) and PK 11195 (calculated from data in B). The equation for the regression line for the PAS on dose of flumazenil is shown over the bar graph for flumazenil. *Denotes significantly greater than the solvent, DMSO ($p < 0.05$). +Significantly greater than the control rats.

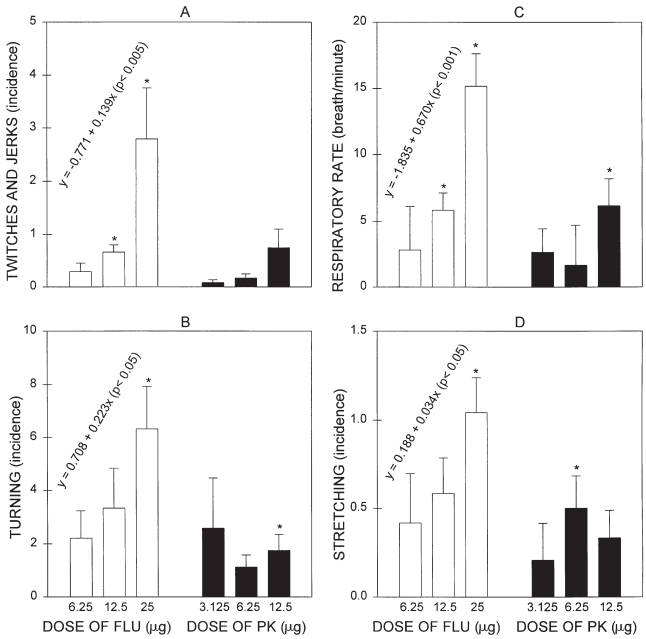

FIG. 4. Comparison of dose–response effects of graded doses of flumazenil (open bars) and PK 11195 (filled bars) microinjected into the CA1 of female rats treated chronically with DZ on some precipitated withdrawal signs: (A) twitches and jerks; (B) turning; (C) respiratory rate (signs used to estimate the PAS); and stretching (a sign not used in estimating the PAS) (D). The unweighted data represent the frequency of occurrence of each sign and are presented as the normalized $AUC_{(0–20\ min)}$, mean + SEM, n = 6 rats. Respiration was counted once each epoch and represents the average normalized rate/min. The equations for the significant regression of a given sign on dose is shown above the bar graphs for flumazenil. *Denotes significantly greater than DMSO ($p < 0.05$).

two highest doses of PK 11195, however, appeared to be dose-related. Flumazenil (25 μg) induced a more intense PAS than each of the doses of PK 11195 (3.125, 6.25, and 12.5 μg) ($p < 0.05$), whereas neither the 6.25 nor the 12.5 μg dose of flumazenil differed statistically from any of the three doses of PK 11195. Flumazenil elicited a significant dose–response relationship for some signs comprising the PAS (Fig. 4) such as increased twitches and jerks (A); turning (B); respiratory rate (C), as well as for the unscored sign, stretching (D). Unlike flumazenil, PK 11195 produced no significant linear regression of any abstinence sign on dose, although there was a tendency in that direction for twitches and jerks (Fig. 4A). However, significantly more turning (B) and tachypnea (C) were elicited by the 12.5 μg dose, and significantly more stretching (D) by the 6.25 μg dose of PK 11195 than by DMSO. The configuration of the dose–response curves for PK 11195-evoked turning (B) and respiratory rate (C) were "U-shaped," while stretching presented with an inverted "U-shaped" curve (D).

Table 1 shows that neither twitches and jerks nor clonic or tonic–clonic convulsions were induced by DMSO in any DZ-treated rat or in any empty capsule-implanted control rat. Note also that no convulsive sign was evoked in any control rat by 25 μg of flumazenil. In DZ-treated rats, flumazenil produced increasingly more twitches and jerks with increasing dose. The nonparametric Friedman repeated measures ANOVA on ranks for flumazenil (doses = 6.25, 12.5, and 25 μg) revealed a chi-square of 8.33 with 2 degrees of freedom (p = 0.0155). Each dose of flumazenil differed from the other two doses by an all pairwise comparison ($p < 0.05$). A one-way repeated measures ANOVA of the twitches and jerks induced by graded doses of PK 11195 (3.125, 6.25, and 12.5 μg) showed no significant between doses difference, $F(2, 10) = 3.52$, $p < 0.07$. Only the highest doses of flumazenil (25 μg) and PK 11195 (12.5 μg) induced convulsions (one clonic) and (one clonic and one tonic–clonic), respectively, but in different DZ-treated rats.

Flumazenil induced a dose-related increase in the BS in the DZ-treated rats during the 15-min postinjection epoch (Fig. 5). The 25 μg dose of flumazenil, on the other hand, was not different to DMSO microinjected into the CA1 of the empty capsule-implanted control rats, calculated as either the $AUC_{(0–20min)}$ or for the 15-min postinjection epoch (data not shown). PK 11195, unlike flumazenil, produced a "U-shaped" BS dose–response curve during the 15-min postinjection epoch, and no dose-related effect was seen for the BS or the signs comprising it for any other time period.

TABLE 1

THE EFFECT OF DMSO, GRADED DOSES OF FLUMAZENIL AND PK 11195 IN DIAZEPAM-TREATED RATS (90 mg/WEEK) AND THE EFFECT OF DMSO AND FLUMAZENIL (25 μg) IN CONTROL RATS (EMPTY CAPSULE IMPLANTED) ON CONVULSIVE SIGNS AFTER FOCAL ADMINISTRATION INTO THE CA1 OF THE HIPPOCAMPUS

	Treatment						
		Dose of FLU (μg)			Dose of PK (μg)		
Measure	DMSO	6.25	12.5	25	3.125	6.25	12.5
	Diazepam-Treated rats						
Twitches and jerks	0*(0/6)†	7(3/6)	17‡(6/6)	67§(6/6)	2(2/6)	4(3/6)	18(4/6)
Clonic convulsions	0(0/6)	0(0/6)	0(0/6)	1(1/6)	0(0/6)	0(0/6)	2(1/6)
Tonic–clonic convulsions	0(0/6)	0(0/6)	0(0/6)	0(0/6)	0(0/6)	0(0/6)	1(1/6)
	Control rats						
Twitches and jerks	0(0/3)	—	—	0(0/3)	—	—	—
Clonic convulsions	0(0/3)	—	—	0(0/3)	—	—	—
Tonic–clonic convulsions	0(0/3)	—	—	0(0/3)	—	—	—

*Number of convulsive signs observed.
†Number of rats that exhibited convulsive signs/number of rats in study.
‡Different from DMSO, 6.25 and 25 μg of flumazenil, Student–Newman–Keuls Test, $p < 0.05$.
§Different from DMSO, 6.25 and 12.5 μg of flumazenil, Student–Newman–Keuls Test, $p < 0.05$.

EEG Activity

Prior to the injection of antagonists, DZ-treated rats did not differ from empty capsule-implanted rats after 5 weeks with regard to the percent of total power in the low-frequency band (1–4 Hz). There was, however, a decrease in the percent of total power in the 4–12 Hz band (theta waves), whereas all other higher frequency bands tended to increase (data not shown). Observation of the EEG tracings showed that obvious motor convulsions did not always accompany EEG evidence of convulsive episodes evoked by the focal injection of flumazenil in the DZ-treated rat. Moreover, the motor manifestation of convulsive activity (clonic and tonic–clonic convulsions, twitches, and jerks) frequently occurred slightly later than the onset of EEG changes such as high-voltage, low-frequency spike complexes. EEG data recorded from sites that showed significant positive or negative regression of the percent of total power on dose of flumazenil or PK 11195 in various EEG frequency bands are shown in Fig. 6. Significant changes in EEG frequency bands occurred with a dose of the antagonist in three of the five brain structures from which the EEG was recorded. An ANOVA of the regression line revealed that graded doses of PK 11195 evoked a dose-related increase in the percent of total power in the 4–12 Hz band of the EEG recorded from the CA1, $F(1, 20) = 5.55$, $p < 0.029$ (A). Flumazenil, on the other hand, did not produce dose-related effects on the percent of total power in any of the EEG bands recorded form the CA1. Recordings from the dentate showed a significant increase in the percent of total power in the low frequency delta waves (1–4 Hz) on the dose of flumazenil, $F(1, 19) = 5.30$, $p < 0.033$ (Fig. 6B), and a significant dose-related decrease in beta 2 waves (18–26 Hz), $F(1, 19) = 6.03$, $p < 0.024$ (Fig. 6C) along with a significant decrease in this band recorded from the amygdala (Fig. 6D). PK 11195 produced no significant dose-related effect on these bands in either the dentate or the amygdala (data not shown). There were no significant dose-related effects with regard to changes induced by graded doses of flumazenil or PK 11195 on the percent of total power in any band of the EEG recorded from the entorhinal and frontal cortices (not shown).

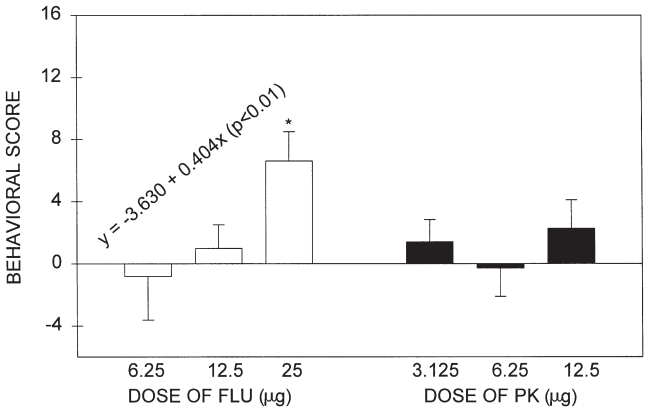

FIG. 5. Comparison of dose–response effects of graded doses of flumazenil and PK 11195 on the BS after microinjection into the CA1 of the dorsal hippocampus of DZ-treated rats. The data are normalized, weighted, and calculated as described in the Method section. The equation for the significant regression of the BS on dose of flumazenil is shown above the bar graph. Values are presented as the $AUC_{(0–20\ min)}$, mean + or − SEM ($n = 6$) for the 15-min postinjection epoch. *Denotes significantly greater than DMSO ($p < 0.05$).

DISCUSSION

The present study examined the precipitated withdrawal syndrome induced by graded doses of the CBR antagonist, flumazenil, and the PBR antagonist, PK 11195, administered into the CA1 of female rats chronically exposed to DZ. All rats appeared to be in good health during the study with no detectable neurologic or histologic changes resulting from re-

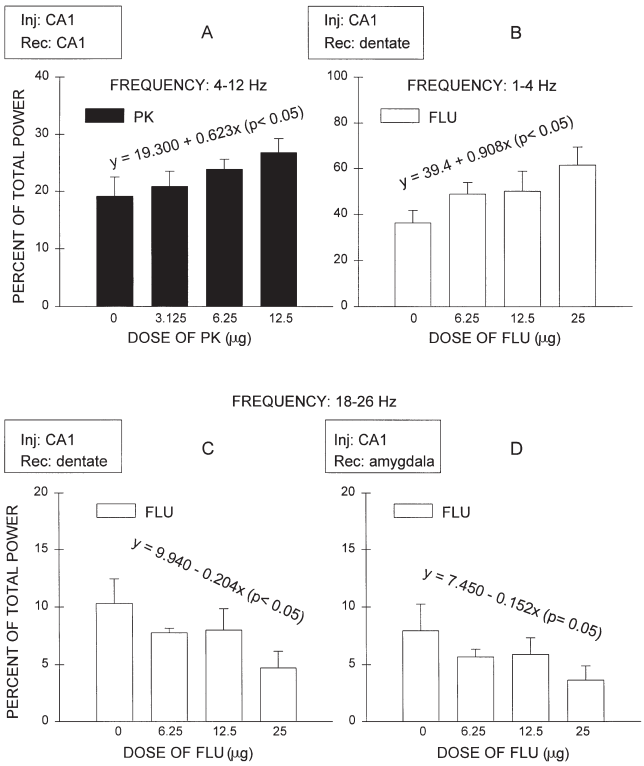

FIG. 6. Dose–response effects of PK 11195 (PK) [0 μg (DMSO), 3.125, 6.25, 12.5 μg] and flumazenil (FLU) [0 μg (DMSO), 6.25, 12.5, 25 μg] microinjected into the CA1 of the dorsal hippocampus on the percent of total power in the 4–12 Hz (theta and alpha), 1–4 Hz, and 18–26 Hz frequency bands of the EEG during the 15-min postinjection epoch recorded from the brain sites indicated (A–D, respectively) in rats chronically exposed to DZ (90 mg/week). The equation above the bar graphs represents the significant regression of the percent of total power on dose of PK or FLU. Values are expressed as the mean + SEM (n = 6 rats for all doses except for the 6.25 μg dose of FLU where n = 5).

peated focal injections. The failure of control rats to gain significant weight across time, unlike other control groups [(36); unpublished observations], may have been related to the fact that they were observed for a shorter period (5 weeks) than the DZ-treated rats (8 weeks).

Flumazenil reverses all the CBR- but not the PBR-mediated effects of an acute dose of a BZ agonist (34), and does not precipitate withdrawal after an acute dose of DZ (32,67). Less is known about the interaction of PK 11195 with an acute dose of DZ. PK 11195 has a high affinity for the PBR, and, in low doses, acts as an antagonist of the convulsant and proconflict actions of the PBR agonist, Ro 5-4864, whereas in high doses it may have agonistic actions (15,43). The binding of [^{3}H] PK 11195 to rat cerebral cortex is displaced by DZ (2), with an order of potency of PK 11195 > Ro 5-4864 > DZ > clonazepam (5).

In the present study, all doses of flumazenil and the two highest doses of PK 11195 (6.25 and 12.5 μg) (but not DMSO) elicited a significant precipitated withdrawal syndrome in rats treated chronically with DZ, whereas in controls neither flumazenil (25 μg) nor DMSO induced a significant response. Although PK 11195 controls were not included in the present study, no significant withdrawal effects were seen after the focal injection of graded doses of either flumazenil or PK 11195 into the CA1 of male control rats (unpublished observations); after IP injection into control male rats (38), or after intrathecal injection into control female rats (65). With the conditions and doses used in the present and the above-cited studies, no significant intrinsic effect of either antagonist was apparent. In other situations, neither antagonist is totally devoid of intrinsic activity and can show both antagonistic and apparent agonistic actions, depending upon the dose and effects measured (16,17,55). The flumazenil-induced PAS reported herein was dose-related, suggesting a receptor-related event, whereas the PK 11195-induced PAS was not. The PAS elicited on dose of PK 11195 was mild and had a "U-shaped" configuration. One reason for the lack of a robust PK 11195-induced PAS may be related to a sparse population of PBR within the CA1. Bilateral instead of unilateral injections of antagonists would likely produce a more intense PAS. In preliminary studies in male and female rats treated chronically with DZ (90 mg/week), IV doses of PK 11195 (2.5, 5, 10, 20, and 30 mg/kg) elicited a dose-related $PAS_{(0–20min)}$ in males and in females a dose-related $PAS_{(MAX)}$ in doses of 5, 10, and 20 mg/kg. A significant regression of the PAS on chronic dose of DZ (0, 90, and 540 mg/week) was also produced in female rats by a single IV dose of PK 11195 (20 mg/kg) [(58); unpublished observations]. The above findings and the tendency reported herein for the PAS induced by the two highest doses of PK 11195 to increase with dose suggests that the dose range was too restricted. There are probably other explanations for the lack of a robust PK 11195-induced PAS, such as the high variance seen with the 3.125 μg dose; possible hormonal effects [in DZ-treated male rats (90 mg/kg/week) the microinjection of PK 11195 (0, 3.125, 6,25, and 12.5 μg) into the CA1 elicited a dose-related PAS—preliminary data, (57)], or the possibility that PK 11195 acts on multiple receptors within the CA1. It has been suggested that at doses higher than needed to saturate the PBR, PK 11195 may act on non-BZ sites on the GABA–BZ receptor complex (15) or even on classical BZ binding sites (17). Many of the signs produced by flumazenil were also produced by PK 11195, but they were fewer in number and presented a less clearly defined dose–response relationship.

Using methods that differed from the current studies in gender, route and duration of DZ administration (15 mg/SC for 8 days), dose and route of antagonist administration (IP), others have previously reported, in agreement with the current data, that both flumazenil and PK 11195 produce a significant precipitated withdrawal syndrome. The precipitated withdrawal scores increased only slightly with increased doses of flumazenil (15 to 20 mg/kg) and of PK 11195 (5 to 10 mg/kg) and the scores did not differ between drugs, although some signs were different between drugs (7,37).

In the present study, PK 11195, in the same doses as flumazenil (6.25 and 12.5 μg), tended to induce a less intense PAS for each dose than flumazenil. Similarly, the PAS induced in female DZ-treated rats (90 mg/week for 5 weeks) by equal IV doses of flumazenil and PK 11195 (10 and 20 mg/kg) was also less for PK 11195 than for flumazenil (63). In contrast, the PAS elicited by 12.5 μg of PK 11195 was about equal in intensity to that induced by 25 μg of flumazenil administered intrathecally into the spinal cord (an area enriched in PBR) of female DZ-treated rats (540 mg/week for 3 weeks) (65). Interestingly, the focal injection of flumazenil (25 μg) into the CA1 induced a PAS (21.5 ± 4.82) three times higher than that induced in the substantia nigra (7.77 ± 1.81) and about 20 times higher than the PAS elicited in the dorsal raphe of female

DZ-treated rats (540 mg/week) for 3 weeks (64). These data support the concept that the CBR and PBR are heterogeneously distributed within the CNS, and that the signs of flumazenil and PK 11195-precipitated withdrawal will therefore vary between CNS structures in DZ-dependent rats.

In acute doses, DZ elicits the appearance of 7–12 Hz spindle bursts in the EEG, a reduction in the amplitude of hippocampal theta rhythm, and the presence of 15–30 Hz beta-like activity. With repeated doses of DZ, the fast-wave activity decreases while increased periods of high frequency waves occur (31,39,64). Whereas, to the best of our knowledge, BZ antagonist-induced precipitated withdrawal effects on the EEG of rats have not been reported, the abrupt withdrawal of BZs in humans is associated with decreased beta activity of the EEG (24,33). While the effects on the EEG in the present study were not robust, mild but dose-related EEG changes were seen in some but not all brain sites. Flumazenil elicited a significant dose-related increase in the delta band (1–4 Hz) and a decrease in the beta 2 band (18–26 Hz) of the EEG recorded from the dentate and from the amygdala in the DZ-treated rats. This is in general agreement with the above-cited human studies. Both the dentate and the amygdala have neuronal connections with the CA1 (1,48), are enriched in CBR-2 subtypes as is the CA1 (45,50), and are areas where the current and preliminary data show that flumazenil elicits convulsive signs of precipitated withdrawal (56). Activation of the CBR-2 subtype is thought to be responsible for the EEG and behavioral stimulatory signs elicited by chronic BZs, and the change in EEG beta-like activity is thought to be due to repeated and concurrent activation of CBR 2 subtypes and PBR, although clarification requires further study. Interestingly, PK 11195 (in contrast to flumazenil, which tended to induce a dose-related decrease), elicited an increase in the percent of total power in the theta and alpha waves (4–12 Hz) recorded from the CA1, an area that contains PBR.

The chronic administration of DZ, as well as some other BZs, produce molecular changes in the CBR/$GABA_A$ complex, which some investigators have equated with tolerance and/or dependence. Subunit variants of the $GABA_A$ receptor are heterogeneously distributed throughout the brain and are found in high concentration in the hippocampus, particularly the α_2 β_3, and γ_2 subunits in the CA1–3 pyramidal cells and in the dentate. The α_1 subunit, expressed together with the β_1, γ_2 subunits, mimics the native CBR-1 receptor, whereas the CBR-2 shows a specificity for the α_2 and α_3 subunits; α_2, α_3, and α_5 subunits may produce subvariants of CBR-2 (18,47,50). Chronic DZ produces regional differences in the α_1 mRNA of the $GABA_A$ receptor with decreased levels occurring in some sites, but not in the hippocampus of rats after 21 days (26). Levels of α_1 and β_2 mRNAs were also decreased in some sites but not in the hippocampus of lorazepam-dependent mice (29). Different drugs appear to produce different effects in this regard and in BZ binding density (54,60) as well as in GABA-mediated feedforward and recurrent inhibition in the CA1 of the hippocampus and in GABA-mediated inhibitory postsynaptic potentials [cf. (60)]. These findings suggest that receptor up- and/or downregulation and $GABA_A$ subunit expression varies with the BZ, dose, duration of treatment, species, and brain structure. Chronic DZ and other BZs produce molecular changes that can alter CBR function such as a decrease in the bicuculline-induced seizure threshold, subsensitivity to GABA in regional brain sites, uncoupling at $GABA_A$/CBR subtypes, and alterations in $GABA_A$ receptor subunit mRNA. The magnitude of changes in $GABA_A$ receptor function by chronic treatment in the rat varies between brain structures, and is affected by the efficacy of the ligand, with full agonists such as DZ producing the biggest change. Modifications in the $GABA_A$ receptor complex appear to be involved in the expression of tolerance and physical dependence by poorly understood mechanisms (21,52,61).

It should be emphasized that the current studies were conducted in female rats, whereas most of the studies cited herein were conducted in male rats. In this regard, it has been reported that in drug naive rats, susceptibility to seizures is highest during proestrus and estrus (23), and that after graded acute doses of DZ, male but not female rats show dose-related conditioned defensive burying behavior induced by constant current shock, and proestrus rats are more sensitive than metestrus or male rats to the lower doses of DZ (13). Further, in spite of the fact that the hippocampus shows a sex-specific difference in GABAergic stimulation of the BZ sites (males more than females) and that progesterone increases the BZ receptors in very specific regions of this structure, it has been reported that $GABA_A$ receptors do not vary over the rat estrous cycle, and no significant correlations are seen between circulating levels of estradiol or progesterone and BZ binding parameters in cycling female rats. Moreover, both intact male and female rats chronically exposed to DZ, show tolerance to the anticonvulsant effects of DZ (66). It should also be pointed out that in DZ-dependent mice decreased testosterone levels increased the number of flumazenil-evoked convulsions in males, whereas a change in estrogen levels was without effect in females (51). In this regard, there is a large body of evidence that the PBR are the critical factor in the rate-limiting step of steoidogenesis; that the PBR as well as the CBR are tonically and physically regulated by neural and hormonal mechanisms, both in the periphery and within the brain of both sexes; that stress can modify both CBR and PBR and that DZ can alleviate some of these stress-induced changes (6,12,14,22,25). These data suggest that there are ongoing hormonal changes in both sexes that have the potential for altering the intensity of either the precipitated or abrupt BZ withdrawal response. In this laboratory, the variance within a group of female rats is generally not different from the variance within a group of male rats for most measures of precipitated withdrawal in that the data passes the equal variance test in a two-way ANOVA of the groups. Thus, in the presence of possible fluctuating hormonal influences, preliminary data have shown that there are gender differences for some measures of precipitated withdrawal in intact DZ-dependent rats after either flumazenil or PK 11195 [(58,59); and unpublished observations]. The present data show that regardless of molecular or hormonal changes, flumazenil elicits a relatively mild but significant PAS after microinjection into the CA1 of noncastrated female rats treated chronically with DZ. The dose-related effects of flumazenil suggests that DZ dependence is produced in the CA1 that involves the CBR, whereas the role of the PBR in this regard is less convincing in view of the failure of PK 11195 to produce dose-related changes in the PAS. Interestingly, preliminary data have shown that PK 11195 elicits a dose-related PAS in the CA1 of male rats treated chronically with DZ (90 mg/week) (58), suggesting that gender could have played a role in the failure to obtain a dose–response effect in the CA1 in the present study. In spite of the fact that steroid hormone fluctuations could have played a role in the present study, the fact that the two highest doses (6.25 and 12.5 μg) were elevated in a dose-related way and that a dose-related $PAS_{(MAX)}$ on IV dose of PK 11195 was elicited in DZ-dependent female rats [(58); unpublished observations] suggest that some of the chronic effects of DZ are

probably also mediated by the PBR located within the CA1. This concept is strengthened by previously cited studies where systemically administered PK 11195 elicited CNS signs of withdrawal in DZ-dependent male rats (37); where it antagonized the development of tolerance to the EEG effects of DZ in rats (66); when coadministered with lorazepam, it prevented the development of tolerance and attenuated withdrawal in male mice; when given 4 days postlorazepam, it attenuated the increase in BZ receptor binding in vivo in the hippocampus but not in the cortex, and reduced the seizure threshold after abrupt withdrawal, an effect that was blocked by Ro5-4864 (9,41,42).

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