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Precipitated Withdrawal in the Substantia Nigra in Diazepam-Dependent Female Rats

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WALA, E. P., J. W. SLOAN, X. JING AND J. R. HOLTMAN, JR. *Precipitated withdrawal in the substantia nigra in diazepam-dependent female rats.* PHARMACOL BIOCHEM BEHAV **64**(4) 857–868, 1999.—Female rats were exposed to diazepam (DZ) implants (90 mg/week) or to empty capsules (controls) for 5 weeks. Rats were focally injected (1 μl) into the substantia nigra (SNR) with the central (CBR) and peripheral (PBR) benzodiazepine receptor antagonists, flumazenil [(FLU} 6.25, 12.5,or 25 μg], and PK 11195 [(PK) 3.125, 6.25, 12.5, or 25 μg], respectively. Rats were observed for behavioral and EEG manifestation of withdrawal syndrome. In female rats, both FLU and PK induced a dose-related precipitated abstinence score (PAS), tachypnea, and head bobbing. Twitches and jerks tended to increase with increasing dose of both FLU and PK. Furthermore, FLU evoked dose-related turning and head and body tremors. The FLU- and the PK-induced PAS were accompanied by an increase in total power of the EEG in the SNR. The involvement of the CBR and PBR in physical dependence on DZ in the SNR is suggested. The present data in female rats are discussed with regard to similarities and differences with previous studies in male rats. © 1999 Elsevier Science Inc.

Central benzodiazepine receptors Peripheral benzodiazepine receptors Intranigral injection Flumazenil PK 11195 EEG Gender

PROLONGED administration of benzodiazepines (BZs) results in physical dependence, as indicated by a withdrawal syndrome upon abrupt termination of the treatment or administration of BZ antagonist(s) in human and laboratory animals [for review, see (96)]. Furthermore, chronic treatment with some BZs affects the electroencephalogram (EEG), as indicated by a reduction of the spindle bursts and increase of the high frequency waves, an effect thought to be associated with a decrease of the sedative action of the drug (25). Flumazenil, the specific antagonist of the central BZ receptors (CBR; localized in the CNS), inhibits the central effects of BZs, and can unmask all signs of withdrawal that are mediated through the gamma-aminobutyric acid ($GABA_A$)/CBR/ionophore complex [for review, see (96)] and reverses the BZ-altered EEG (35). Recent data indicate (38, 64, 66, 75, 79) that in diazepam-treated rats, a withdrawal syndrome can be also precipitated by PK 11195 [1-(2-chlorophenyl)-*N*-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide], an antagonist of the peripheral BZ receptors (PBR; localized both in various peripheral organs and in the CNS, mainly on glial cells, but also on neurons). The PBR are thought not to be directly related to the GABA-regulated anion channels, and BZ binding to this site is not enhanced by GABA [see (21), for references]. The mechanism of action of PK 11195, structurally different from BZs, is not clear. Although PK 11195 binds with high affinity to the PBR but not to the CBR, it possesses intrinsic action at a higher concentration than is required to saturate the PBR [see (60) for references). There is a line of evidence suggesting that some actions of PK 11195 are mediated by an incompletely characterized site at the chloride channel of the $GABA_A$ receptor (7,12,15,16,42).

It has been reported that, in rats, neuronal responses to chronic BZ treatment can be altered by the hormonal milieu and that gonad-related factors affect the BZ-induced changes in the $GABA_A$ receptors in a regionally specific manner (89,90). Gender-related differences in precipitated withdrawal were observed both after peripheral administration of the CBR antagonist, flumazenil, [IV infusion in male (93) and female rats (37); IP injection in male and female mice (54)] and after peripheral administration of the PBR antagonist, PK 11195 [IV bolus injection in male and female rats (63,65)]. It is not known, however, whether sex-related differences in the quantitative and qualitative aspects of the precipitated withdrawal syndrome are pronounced after direct application of flumazenil and PK 11195 into the brain.

The substantia nigra (SNR) is a useful brain site to study local actions of the CBR and PBR antagonists. This brain area, which has an intensive GABAergic innervation and is

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tonically inhibited by GABA input, has a high density of the CBR (40,46,97) and a low population of the PBR (9,50). Because diazepam binds to the CBR and PBR, it is likely that both types of receptors mediate antagonist(s)-induced withdrawal in the SNR in diazepam-dependent rats. In male rats, the SNR has been well characterized with respect to its role in the anticonvulsant effect of BZs (31,59,81,100), different aspects of BZ tolerance (55,58,70,72), modulation of $GABA_A$ sensitivity and firing rate by acute and chronic BZ treatments (92,94,95), and receptor binding (71). Although local BZ enhancement of GABAergic responses in the SNR was not affected by sex, there is a body of electrophysiological data suggesting gender-related reactivity of the SNR in the rat. Accordingly, the basal firing rate of nigral neurons was higher in male than in female rats; in comparison to male rats, female rats showed attenuated inhibition of the firing rate of the SNR neurons after systemic administration of BZ, and the systemic administration of flumazenil induced a compensatory increase in spontaneous nigral firing in male but not in ovariectomized female rats chronically exposed to BZ (86–88). Thus, it is likely that the gender differences in flumazenil-induced enhancement of the firing rate of reticulata neurons are manifested by differences in a flumazenil-precipitated withdrawal in the SNR in female and male diazepam-dependent rats.

The aim of the present work was to characterize the withdrawal syndrome (manifested both by behavioral and EEG changes) induced by focal administration of flumazenil and PK11195 in the SNR in female rats chronically exposed to diazepam-filled implants, and to compare the results with those in male rats subjected to identical treatment (78).

METHODS

Rats

Experiments were performed in noncastrated female Sprague–Dawley rats (approximately 90 days old, weighing about 250 g). Rats were housed in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985) in a humidity- and temperature-controlled facility with 12 L:12 D cycles with a light onset at 0600 h. Each rat was kept separately in a transparent cage with a sawdust-covered floor. Rats had free access to standard laboratory chow and tap water. The body weights were recorded weekly (before implantation of the successive capsule). All surgeries were performed under sterile conditions and ketamine chloride anesthesia (80 and 40 mg/kg IP for brain surgery and subcutaneous implantation of capsules, respectively). At the end of the study the rats were euthanized with pentobarbital sodium (120 mg/kg IP). The experiment was conducted according to a protocol approved by the University of Kentucky Animal Care and Use Committee.

Drugs and Chemicals

Silastic capsules were made out of Medical Grade Silastic Tubing (i.d. 0.147 cm $\times$ o.d. 0.195 cm; 7 cm long; sealed on both ends with Silicon Type A Silastic Medical Adhesive) (Dow Corning; Midland, MI). The capsules were filled with 90 mg of crystallized diazepam [recystalization of diazepam in ethyl alcohol increased its in vitro release from silastic tubing (37)], or were empty (control). The capsules were conditioned by soaking for 15 min in ethyl alcohol and bovine albumin (3%), consecutively. Solutions of flumazenil, and PK 11195 in dimethylsulfoxide (DMSO) were freshly prepared on the day of the experiment, and were protected from light. Diazepam and flumazenil were gifts from Hoffmann–LaRoche (Nutley, NJ); PK 11195 was purchased from Research Biochemical International; DMSO and HPLC standard were from Sigma.

Injecting Cannulae-Recording Electrode

Anesthetized rats were mounted in a Kopf stereotaxic instrument, and their skulls were exposed. An indwelling guide cannulae (GC)-recording electrode (20-mm long, stainless steel G-22 tubing with an attached electrode, insulated except at the tip with epoxilite 6001 M) was directed toward the SNR. The intraural coordinates (mm) were as follows: GC: anterior posterior = 3.7; lateral = 2.4; vertical = 5; injecting chemotrode (stainless steel tubing, G-28): vertical = 1.8 mm (49). An indifferent electrode was imbedded in the skull behind the lambda. The electrodes were connected to a pedestal and secured to the bone with acrylic cement. The patency of the GC was kept with the help of close-fitting obturator. After completion of surgery the rats were injected (IM) with sterile saline and kept under the heating lamp for about 24 h. Saline (1 ml) was injected daily for 3–5 days until the body weights returned to presurgery levels. The brain implants were well tolerated, and no pathologic behaviors were observed after recovery from the surgery.

Silastic Capsules

About 7 days after the surgery the rats were subcutaneously implanted in the back with diazepam-containing (90 mg) or empty silastic capsules according to the protocol of Gallager (14) with minor modification (37). The rats were initially implanted with two capsules (2×90 mg of diazepam or empty), and thereafter, an additional capsule was implanted at weekly intervals for 7 consecutive weeks (duration of chronic treatment: 4 weeks prior to and 4 weeks during focal injections). The implantation procedure was simple and fast (about 5 min). Previously implanted capsules were not removed to avoid complicated surgery.

Focal Injections

Focal injections were initiated 3 days after the fifth implantation of capsules (4 weeks of treatment with diazepam or empty capsules), and were continued for 4 weeks.The experiments started approximately at 0900 h, and each day the rats were tested in the same order. The rat was placed in an observation chamber (a round Faraday cage with a grounded solid metal wall and a sawdust-covered floor) and connected to a Grass 78 D EEG/polygraph through SL6C commutator and a concentric mercury swivel (Plastic One). After acclimatization (about 10 min), the baseline EEG was recorded for 10 min, and simultaneously the rat was observed (in two 5-min epochs) for baseline values. All rats were observed by the same observer (J.W.S.), who was blind to the treatment. The signs called by the observer were recorded by a second investigator on the standardized observation forms and by the third investigator on the EEG tracing.

Flumazenil [6.25, 12.5, or 25 μg ($2.06–8.2 \times 10^{-8}$ M)]; PK 11195 [3.125, 6.25, 12.5, or 25 μg ($0.88–7.1 \times 10^{-8}$ M)], and DMSO-vehicle were rapidly (about 5 s or less) injected into the SNR in a volume equal to 1 μl [10% of approximate target tissue volume (11)]. The choice of the lowest doses of antagonists was based on approximately two times lower K_d for PK 11195 [0.64 ± 0.03 nM (3)] than for flumazenil [1.0 nM (43)] in the rat cerebral cortex. The doses increased in the order of 1:2:4 and 1:2:4:8 for flumazenil and PK 11195, respectively.

The highest doses of both antagonists were the same, and were limited by solubility in DMSO (25 mg/ml). Flumazenil and PK 11195 were injected into the substantia nigra 3 days apart at weekly intervals [there was no significant order effect when the CBR and PBR antagonists were systemically administered 3 days apart (64)]. The order of doses was balanced by a replicate block Latin square design (4 × 4). By the end of the experiment each rat had received three doses of flumazenil, four doses of PK 11195, and the vehicle. A week later all rats were injected with a mixture of flumazenil and PK 11195 (12.5 μg of each). All injections were made without handling the rat by using the chemotrode inserted into the GC and connected to a Hamilton syringe with PE-20 polyethylene tubing. The tight fit between the GC and the chemotrode reduced the reflux of injected solution. After injection, the syringe was gently disconnected while the chemotrode was left in place for the duration of the experiment.

After focal injections, the EEG was recorded for 20 min and simultaneously the rat was observed (in 5-min epochs) for signs of withdrawal. Each rat served as self-control with respect to a preinjection baseline and the effect induced by DMSO-vehicle.

Signs of Withdrawal

As previously described in detail (37), the scored withdrawal signs included clonic convulsions (symmetrical clonic spasm of bilateral limbs); tonic–clonic convulsions (tonic spasm, clonic jerking, full extension in some cases); twitches and jerks (isolated spasms of head, body, or limbs); head and body tremors; jumping (violent episodic movement); backing (backward walking); turning (rapid change of direction); writhing; vocalization; arched back and change in respiratory rate (breath/min). For each rat, the number of episodes of the above signs was summed (except respiration, which was counted only once per epoch), weighted, and added together for each 5-min observation period to generate the precipitated abstinence score (PAS; an indication of the overall intensity of the withdrawal syndrome). The other withdrawal signs (counted and recorded in 5-min epochs but not included in the calculation of the PAS) were as follows: stiff tail (which was usually parallel to cage floor), stretching of the body, head bobbing (short quick up-and-down movement), hot-foot behavior, digging in the sawdust bedding, wet dog shakes, ataxia, head and body scratching, rearing (upright position), chewing, blinking, and ear twitching. Some signs related to activity such as fixed posture (sitting, standing), walking, exploring the cage, or curled position were also recorded. All of the above-listed withdrawal signs were frequently observed after a bolus IV injection of flumazenil in rats chronically exposed to diazepam implants (37,75).

To normalize the data for between-subjects differences in baselines values, for each rat, individually derived preinjection scores (average of baseline observations) were subtracted at each time point from the postinjection scores. The areas under the normalized time action curves ($AUC_{0–20min}$) were calculated by the trapezoidal rule, and used to construct dose–response curves. The data are presented both as the PAS and scores for individual withdrawal signs.

EEG

The EEG was recorded from the site of injection for 10 min prior to and 20 min after focal injection. The EEG signals were sampled at 256 samples/s using a data acquisition board, and were stored for off-line analysis. Signals were filtered with the bandpass set at 1–32 Hz. Fast Fourier transform analysis was performed on 4-s samples. The polygraph records were visually inspected, and epochs containing artifacts were discarded. The power spectrum was averaged across 5-min epochs, and the total distribution across frequency ranges was determined. To normalized data for differences in the EEG baselines for each rat the postinjection change in total power (TP_{EEG}) of the EEG (expressed as μV^2) was calculated with respect to an individually derived baseline, and presented as the percentage of increment or decrement of (1–32 Hz) TP_{EEG} over the individually derived baseline. The frequencies were grouped into the following bands: 1–4, 4–12, 12–18, 18–26, and 26–32 Hz. The absolute power was determined in each frequency band, and the relative band power of the EEG power spectrum was calculated (percent of the TP_{EEG}).

Plasma Levels

Blood samples (about 0.5 ml) were collected at weekly intervals (before capsule implantation) into EDTA tubes by venipuncture of the tail vein. Steady-state plasma levels of diazepam and its metabolites nordiazepam, oxazepam, temazepam, and 4-hydroxydiazepam were determined by HPLC as previously described (37).

Estrous Cycle

Vaginal smears were done at the day of focal precipitation (about 1500 h) to determine the stage of the estrous cycle. A moistened Q-tip was gently inserted and rotated within the vagina. The swab was pressed in a drop of saline on a slide and vaginal fluid was examined under the light microscope. Appearance of the superficial genitalia was observed.

Histology

At the end of the studies rats were anesthetized with pentobarbital sodium and infused with formalin. The brains were removed and fixed in formalin. Frozen sections were cut at 32 μm, and every other section was mounted on gelatin-coated slides, stained with neutral red, coverslipped, and examined under the light microscope to localize the GC electrode. Rats used in the data presented here had chemotrode tips placed within the border of the SNR. Some brain sections showed signs of damage presumably resulting from the multiple injections (the tissue lost was not quantitatively evaluated). However, rats did not show neurological impairments nor did the time factor significantly affect the intensity of withdrawal. One diazepam-dependent and one control rat were excluded from the study, and thus, data from seven diazepam-exposed and three control female rats are presented herein.

Statistics

The use of parametric statistics was verified by normal distribution and equal variance (Kolmogorov-Smirnov normality test, and Levine median equal variance test). The effects induced by different doses of flumazenil and PK 11195 on the PAS, the individual signs of precipitated withdrawal (data normalized for baseline) and the EEG (changes in TP_{EEG}) were analyzed by one-way repeated-measures (RM) ANOVA, with dose used as a factor. The between-dose difference were inferred with post hoc all pairwise Student–Newman–Keuls test. The dose–response curves for normalized data were subjected to regression analysis. Differences in the PAS and TP_{EEG} produced in diazepam-exposed and control rats by different doses of flumazenil or PK11195 were analyzed by a two-way

ANOVA with treatment (diazepam vs. empty capsules) and dose taken as the factors. Gender differences were compared by two-way RM ANOVA (with sex and dose taken as factors). Spectral content of the EEG at preinjection (baseline) and postinjection (5 min) was compared by paired *t*-test, while differences in the spectral content of the EEG in control and diazepam-dependent rats were evaluated by unpaired *t*-test. Across time, changes in flumazenil- or PK11195-induced withdrawal scores as well as changes in body weights and plasma levels were analyzed by a one-way ANOVA, with time (weeks) taken as the factor. If the data failed normality and/or equal variance tests, they were analyzed with the appropriate nonparametric tests (Friedman RM ANOVA on ranks; Mann–Whitney rank test; Wilcoxon signed rank test). A probability level of 0.05 or less was considered significant. All statistical calculations were made with the help of SigmaStat statistical software.

RESULTS

Body Weight

There was a trend toward less weight gain (mean values for 8 weeks treatment) in female rats chronically exposed to diazepam-filled implants (14.0 ± 6.5 g) than in control rats (31.1 ± 9.9 g); however, the between-groups difference was below the level of statistical significance. The increase in body weight with time was of statistical significance both in diazepam-dependent and in control rats, $\chi^2(7) = 18.9$ and 15.7, $p = 0.015$ and 0.028, respectively; RM ANOVA on ranks. Focal injections of flumazenil and PK 11195 did not significantly affect body weight gain either in diazepam-dependent (13.3 ± 4.3 vs. 14.7 ± 10.66 g) or in control rats (32.0 ± 4.39 vs. 33.2 ± 4.39 g) for weeks 1–4 vs. weeks 5–8 of chronic treatment, respectively.

Plasma Levels

Chronic exposure to diazepam slowly released from silastic implants (90 mg/cap/week) generated in female rats the average steady state plasma levels of diazepam, nordiazepam, oxazepam, temazepam and 4-hydroxydiazepam equal to 1.55 ± 0.14, 0.43 ± 0.19, 0.65 ± 0.23, 0.39 ± 0.17, and 1.22 ± 0.19 μg/ml, respectively. Plasma levels did not significantly change across 8 weeks of chronic treatment. Focal injections of flumazenil and PK 11195 did not affect steady-state plasma levels (mean levels of diazepam were equal to 1.53 ± 0.14 and 1.61 ± 1.44 μg/ml for weeks 1–4 and 5–8, respectively).

Precipitated Abstinence Score (PAS)

Figure 1 shows time curves for the PAS after focal injections of flumazenil, PK 11195, and DMSO-vehicle in female diazepam-dependent and control rats. As can be seen, both antagonists induced a rapid (within 5 min) but transient (lasting about 20 min) precipitated withdrawal syndrome in diazepam-dependent rats. The withdrawal syndrome was not evoked by flumazenil and PK 11195 in control rats nor by DMSO in diazepam-treated rats. Both flumazenil and PK 11195 induced a significantly higher PAS ($AUC_{0-20min}$; corrected for preinjection baseline) in diazepam-dependent than in empty capsule control rats, $F(2, 32) = 18.9$, $p < 0.0025$ and $F(3, 43) = 16.9$, $p < 0.005$; two-way RM ANOVA, for flumazenil and PK 11195, respectively. In diazepam-dependent rats,

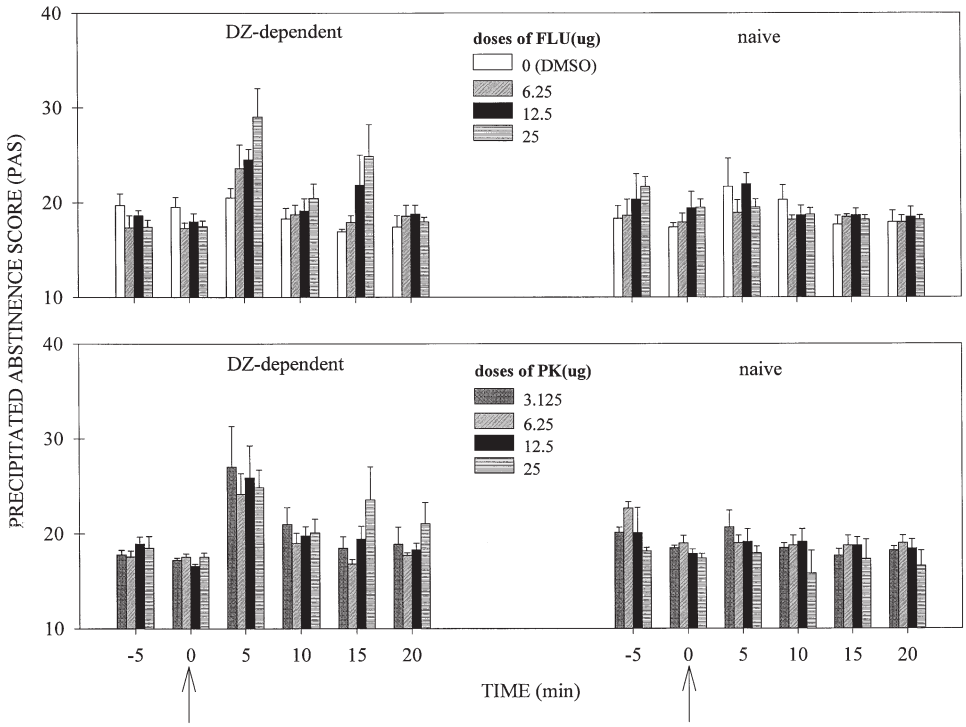

FIG. 1. Time courses of the precipitated abstinence score (PAS) prior to and after focal administration (1 μl) of flumazenil (FLU; 6.25, 12.5, or 25 μg), PK 11195 (PK; 3.125, 6.25, 12.5, or 25 μg) and vehicle (DMSO) into the substantia nigra in diazepam (DZ)-dependent (90 mg/cap/week; $n = 7$) and empty capsule control (naive; $n = 3$) female rats. Data are mean + SEM of "n" rats. ↑ indicates time of focal injection of FLU or PK.

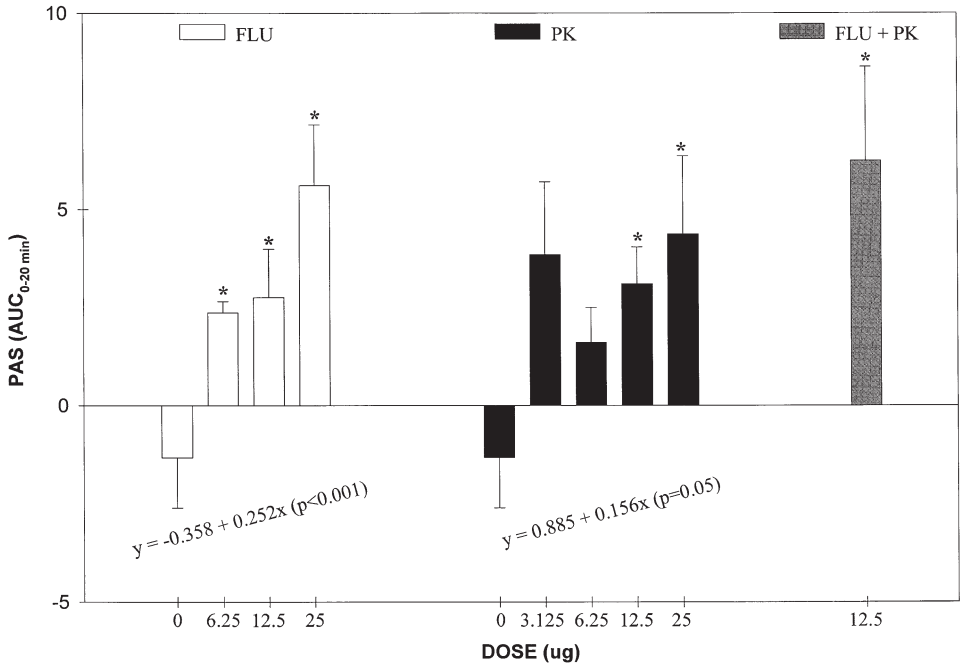

FIG. 2. Dose–response curves for the precipitated abstinence score (PAS) induced by focal injections (1 μl) of flumazenil (FLU; 6.25, 12.5, or 25 μg); PK 11195 (PK; 3.125, 6.25, 12.5, or 25 μ), and FLU + PK (12.5 μg of each) into the substantia nigra in female rats chronically exposed to diazepam-filled implants (90 mg/cap/week). Data are presented as mean + SEM (n = 7) area under the PAS time course ($AUC_{0–20min}$) corrected for baseline (postinjection-average preinjection). Vehicle (DMSO) = 0 dose. * Significantly different from DMSO ($p < 0.05$; paired t-test).

the PAS significantly increased with increasing focal doses of both antagonists (Fig. 2). Differences between the flumazenil- and PK 11195-induced PAS were below the level of statistical significance (for common doses of antagonists: 6.25–25 μg). Concomitant focal injection of flumazenil and PK 11195 (12.5 μg of each antagonists) produced a higher PAS than observed after administration of either antagonist given alone [flumazenil (12.5 μg) or PK 11195 (12.5 μg)], however, differences were below the level of statistical significance. The average ($AUC_{0–20min}$) and maximum (5 min) PAS did not significantly change across time (4 weeks) of focal injections of flumazenil and PK 11195. Further, there was a lack of a statistically significant relationship between the PAS and the stage of the estrous cycle.

Twitches and Jerks

In female diazepam-dependent rats unilateral injections of flumazenil and PK 11195 in the SNR did not evoke behavioral manifestation of clonic no tonic–clonic convulsions. However, both antagonists precipitated twitches and jerks that emerged rapidly(within 5 min) and then progressively declined. With few exceptions twitches and jerks were accompanied by spikes and high amplitude low-frequency waves on the EEG tracing. Table 1 indicates that 1) the number of twitches and jerks tended to increase with increasing dose of both flumazenil and PK 11195; 2) flumazenil and PK 11195 had an additive effect on twitches and jerks; and 3) twitches and jerks were not evoked by the vehicle in diazepam-treated rats nor by flumazenil and PK 11195 in control rats. Scores for flumazenil- and PK 11195-evoked twitches and jerks ($AUC_{0–20min}$) significantly differed between doses, $\chi^2(3) = 9.05$, $p = 0.05$; and $\chi^2(4) = 13.5$, $p = 0.01$; one-way RM ANOVA on ranks; for flumazenil and PK 11195, respectively. In this regard: 1) Flumazenil (25 μg) and PK 11195 (3.125–25 μg) evoked significantly more twitches and jerks than DMSO-vehicle (0 score) ($p < 0.05$; post hoc Student–Newman–Keuls test). 2) In comparison to flumazenil, PK 11195 evoked significantly more twitches and jerks, $F(1, 41) = 9.9$, $p = 0.025$; two-way RM ANOVA; for common doses of antagonists (6.25–25 μg). 3) The dose–response relationships for twitches and jerks ($AUC_{0–20min}$) were below the level of statistical significance both for flumazenil and PK 11195 (analysis of regression line). Table 2 summarizes the number of focally evoked twitches and jerks in relation to the stage of the estrous cycle in diazepam-dependent rats. Although there was a trend toward a higher incidence of twitches and jerks evoked by flumazenil and PK 11195 in the SNR at estrous than at the other stages of the estrous cycle, the observed differences were below the level of statistical significance.

Respiratory Rate

In female diazepam-dependent rats focal injection of either flumazenil or PK 11195 in the SNR caused dose-related changes in respiratory rate, $F(1, 27) = 4.9$, $p = 0.05$, and $F(1, 34) = 8.6$, $p = 0.01$, for flumazenil and PK 11195, respectively. There was a lack of statistically significant difference between the CBR and PBR antagonists on this measure. Flumazenil (6.25 –25 μg) as well as the highest dose of PK 11195 (25 μg), induced tachypnea. After focal injection of DMSO-vehicle, respiratory rate was less than preinjection baseline, while increasing doses of PK 11195 (3.125–12.5 μg) progressively enhanced respiration in the rat.

TABLE 1

NUMBER OF TWITCHES AND JERKS EVOKED BY FLUMAZENIL, PK 11195, AND DMSO-VEHICLE IN THE SUBSTANTIA NIGRA IN FEMALE DIAZEPAM-DEPENDENT AND CONTROL RATS

	Focal Injection								
		Flumazenil (FLU)			PK 11195 (PK)				FLU + PK
Chronic Treatment	DMSO	6.25 μg	12.5 μg	25 μg	3.125 μg	6.25 μg	12.5 μg	25 μg	12.5 + 12.5 μg
Dizepam	0* (0/7)†	14 (4/7)	14 (5/7)	48 (5/7)	15 (6/7)	13 (5/7)	23 (7/7)	65 (5/7)	51 (6/7)
Control	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	0 (0/3)	—

*Number of twitches and jerks.
†Number of rats that had twitches and jerks/number of rats in the group.

Other Signs of Withdrawal

Both flumazenil and PK 11195 induced head bobbing that significantly increased with increasing dose, $F(1, 27) = 4.9$, $p = 0.05$, and $F(1, 34) = 14$, $p = 0.001$, for flumazenil and PK 11195, respectively. Scores ($AUC_{0–20min}$) for head bobbing were similar for both antagonists. On the other hand, flumazenil, but not PK 11195, induced dose-related increase in head and body tremors and turning, $F(1, 27) = 5.1$ and 5.4, $p = 0.05$, respectively. In female control rats, neither flumazenil nor PK 11195 precipitated dose-related signs of abstinence.

EEG

In diazepam-dependent female rats the flumazenil- and the PK11195-induced PAS were accompanied by an increase in total power of the EEG (TP_{EEG}) in the SNR; however, the between-dose differences and dose–response curves for TP_{EEG} were lacking statistical significance. In control rats, focal injections of antagonists did not affect the EEG. Figure 3 shows that, in comparison to the prediazepam baseline (experimentally naive rats), chronic exposure to diazepam implants significantly reduced 1–4 Hz and enhanced 4–32 Hz frequency bands in the TP_{EEG}. There was a trend for a shift toward the low-frequency band after focal injections of flumazenil and PK 11195.

DISCUSSION

The present data show that in female rats chronically exposed to diazepam-filled implants both flumazenil and PK 11195 precipitated a dose-related withdrawal syndrome (PAS) in the SNR. Thus, the involvement of the CBR and PBR in dependency on diazepam in the SNR is suggested. It must be mentioned that the PAS induced by the lowest dose of PK 11195 (3.125 μg) was higher than expected. The same phenomena were observed after systemic (75), central [the CA1 area of the hippocampus (66), and spinal subarachnoid space (79) administration of PK 11195 in female rats chronically treated with diazepam, and thus, the involvement of different subsets of the PBR is possible. The existence of the PBR subtypes has been previously suggested [see (24) for references]. The intensities of flumazenil- and PK 11195-induced (6.25–25 μg) withdrawal syndromes (PAS) were similar. This is a puzzling observation in view of the high population of the CBR (40,46,97) and low population of the PBR (9,50) in the SNR.

In the SNR, focally injected flumazenil and PK 11195 seemed to have an additive effect on the PAS. It is well established that flumazenil has a high affinity for the CBR (mainly localized to the symptosomal fraction of neurons), but does not bind to the PBR (23). PK 11195 acts as an antagonist with specificity for the PBR (localized on the outer mitochondrial membrane, mainly in the peripheral tissues, but also found within the CNS, on glial cells and neurons) [3,29];however, at the higher concentrations, it seems to act at a novel binding site functionally coupled to $GABA_A$ and [^{35}S]TBPS-labeled chloride ionophore (15). There is a volume of data suggesting that the effect of PK 11195 in the presence of a BZ is partially mediated through a direct modulation of the function of a chloride channel rather than by indirect action on the PBR [see (42) for references]. At the present time it is not possible to conclude whether the additive effect of flumazenil and PK 11195 in the SNR is mediated through an interaction with a common site or through action on the CBR and PBR.

The repeated weekly focal injections of flumazenil or PK 11195 did not alter the PAS across the 4-week period of precipitation experiments. This is consistent with previous data when flumazenil or PK 11195 was administered in 4–7-day intervals in several animal species (17,28,36,62). It is noteworthy, however, that the intensity of precipitated withdrawal decreased when flumazenil was administered in 1–3-day intervals (13,27), and thus, it has been postulated that exposure to flumazenil resets GABAergic responsiveness to a pre-BZ state (1,2,13). Interestingly, recent data from our laboratory indicate that the PAS decreased when PK 11195 was systemically administered in daily but not weekly intervals in diazepam-dependent rats (64).

The flumazenil- and PK 11195-induced PAS peaked within 5 min and then rapidly declined toward preinjection baseline (about 20 min). The transient effect of flumazenil

TABLE 2

TWITCHES AND JERKS EVOKED BY FOCAL INJECTIONS OF FLUMAZENIL (FLU) AND PK 11195 (PK) IN THE SUBSTANTIA NIGRA IN RELATION TO STAGE OF ESTROUS CYCLE IN DIAZEPAM-DEPENDENT RATS

	Stage of Estrous Cycle			
Injected Antagonist	Proestrus	Estrus	Late Estrus	Diestrus
Flumazenil	5* (1/3)†	62 (7/14)	4 (2/4)	6 (4/7)
PK 11195	0 (0.2)	88 (9/15)	8 (6/7)	20 (8/11)

Data are for pooled doses of FLU (6/25–25 μg) and PK (3.125–25 μg).
*Number of twitches and jerks.
†Rats that expressed twitches and jerks/number of rats in the group.

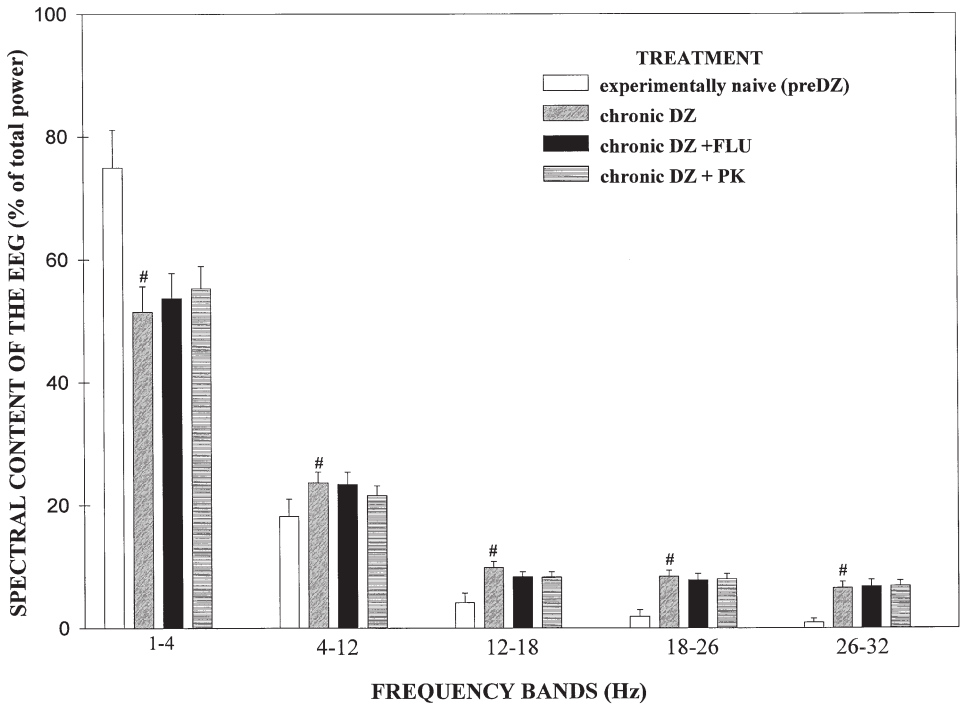

FIG. 3. Spectral content of the EEG [% of total power of the EEG (1–32 Hz)] in the substantia nigra in female rats at the time when rats were: 1) experimentally naive [prior to diazepam (DZ) treatment (pre-DZ)]; 2) chronically exposed to DZ (90 mg/cap/week) [mean preinjection baselines across 4 weeks of DZ treatment (chronic DZ)]; and 3) focally injected with flumazenil (FLU) and PK 11195 (PK) [5 min postinjection; mean values for pooled doses (6.25–25 μg) of antagonists (chronic DZ + FLU), and (chronic DZ + PK), respectively]. Data are mean + SEM of seven rats. # Significantly different from experimentally naive rats (pre-DZ vs. chronic DZ; $p < 0.5$; paired *t*-test).

can be explained by its rapid pharmacokinetics in the rat brain [$T_{0.5}$ = 16 min after IV flumazenil (30)]. Maximum total radioactivity was found in the brain within 1 min after IV administration of [^{3}H] PK 11195 in rats (4). However, a lack of significant difference in the levels of PK 11195 in brain at 5 and 30 min after IV injection (77) suggests that in comparison to flumazenil, PK 11195 is eliminated slower from the brain in the rat.

In female rats chronically exposed to diazepam (90 mg/week) focal unilateral injections of flumazenil and PK 11195 into the SNR did not induce a motor manifestation of convulsions. This is consistent with a lack of convulsions after unilateral intranigral injections of flumazenil in female rats exposed to a high dose of diazepam (540 mg/week) (76), and suggests that either bilateral injections are required to evoke convulsions, or that precipitated convulsions do not originate in the SNR. It has been well documented that systemic administration of flumazenil (3,93) and PK 11195 (63–65,75) precipitate dose-related clonic and tonic–clonic convulsions in diazepam-dependent rats. Furthermore, flumazenil and PK11195 reversed the convulsant action of the PBR agonist, RO5-4864 [see (12) for references].

In the SNR, twitches and jerks (convulsive signs of precipitated withdrawal) showed a trend toward increase with increasing dose of both flumazenil and PK 11195, and were the sole sign of withdrawal, which had a higher score for PK 11195 in comparison to flumazenil (for pooled 6.25–25 μg doses of both antagonists). Furthermore, flumazenil and PK 11195 seemed to have an additive effect on twitches and jerks in the SNR. The latter was less pronounced after concomitant IV injections of antagonists (64). Taken together, the involvement of the CBR and the PBR in the mediation of twitches and jerks in the SNR is suggested. It is also possible that in the SNR flumazenil and PK 11195 have a common site of action with regard to twitches and jerks.

The data suggest that at estrous flumazenil and PK 11195 evoked more twitches and jerks in the SNR in diazepam-dependent rats. This is in agreement with reports that in rats susceptibility to seizures was highest during estrus when the levels of neuroactive steroid [metabolite of progesteron: tetrahydroprogesterone (3α-5α-THP)] that modulate GABAergic transmission was lowest in the brain [see (16) for references], and that elevated levels of progesteron as well as administration of 3α-5α-THP have an anticonvulsant effect (85). Further, it has been shown that an increased incidence of seizures is associated with declining levels of progesteron across the menstrual cycle, and that the proconvulsant withdrawal properties of progesteron are due to declining levels of 3α-5α-THP (44,45).

A growing body of evidence suggests that the mechanism of action of BZ is affected by sexual hormones. Gender differences were reported in modulation of the $GABA_A$/CBR/ionophore complex [see (89) for references], NMDA receptor functions (22), and in some behavioral (6,52,74) but not in the anticonvulsant effect of BZs (51,89), nor in changes in anxiety-related behaviors during flumazenil-induced withdrawal (68). Comparison between the present data in diazepam-treated female rats and previous data (78) from age-matched male (Sprague–Dawley, approximately 90 days old, weighing about 350 g) rats subjected to identical experimental protocol pointed out marked differences between sexes in several measures.

Pharmacokinetics

In comparison to male rats, in female rats identical chronic treatment with diazepam implants (90 mg/cap/week) resulted in significantly higher ($p < 0.05$; unpaired *t*-test) steady-state plasma levels of diazepam (1.55 ± 0.14 vs. 0.71 ± 0.05 μg/ml) and its metabolite, 4-hydroxydiazepam (1.22 ± 0.19 vs. 0.13 ± 0.041 μg/ml). Levels of other metabolites such a nordiazepam, temazepam, and oxazepam were also higher in female than in male rats; however, differences were not of statistical significance. In comparison to male rats, female rats were exposed to about a 40% greater dose of diazepam (90 mg/week ≈360 and 257 mg/kg/week in female and male rats, respectively), whereas plasma levels of diazepam was about 100% higher in female than in male rats. Further, chronic exposure to an approximately equal dose (mg/kg) of diazepam (90 and 120 mg/cap/week for female and male rats, respectively) generated higher brain levels of diazepam in female (4.89 ± 2.39 μg/g) than in male (1.64 ± 0.082 μg/g) rats (unpublished data from our laboratory). This is in line with reports that sex affects the pharmacokinetics of BZs. In this regard, the clearance of oxazepam was greater in men than in woman (18); oxydation and *N*-demethylation of chlordiazepoxide was impaired with age in men but not in woman (19). Further, female rodents had higher plasma levels of oxazepam (98) and PK 11195 (77) than males. These data are in agreement with reports that C3-hydroxylation of diazepam was more rapid in male than in female liver preparations (56); that the hepatic cytochrome P450 activity was significantly higher in male rats than in female rats at any stage of the estrous cycle (82); that the CYP3A9 gene had a higher expression in the liver in female than in male rats (80), and that there was a sexual dimorphism of P450 2D in the brain (69). In male rats, half-lives for diazepam, flumazenil, and PK 11195 in plasma are equal to about 1.15 h (10); 8.5 min (34), and 5 h (77), respectively. To the best of our knowledge, pharmacokinetics of flumazenil has not been compared between sexes, whereas distribution but not elimination of PK 11195 was greater in female than in male rats (77).

Intensity of Precipitated Withdrawal

The intensity of precipitated withdrawal is a function of concentrations of BZs as indicated by significant relationships between the flumazenil(oral)-induced PAS and the steady-state brain or plasma levels of diazepam and its metabolites in diazepam (oral)-dependent female dogs (61). The present data indicate, however, that, despite relatively higher plasma levels of BZs in the female than in the male rat, the latter showed a significantly higher flumazenil-induced PAS in the SNR, $F(1, 55) = 9.8$, $p = 0.05$; two-way ANOVA. This suggests greater sensitivity in male than in female rats. Distribution of the CBR in the human brain did not differ between gender (99); however, to the best of our knowledge, it is not known whether the population of $GABA_A$/CBR in the SNR is similar in female and male rats. Anatomical differences between male and female rats are also possible. In this regard, it has been reported that throughout prenatal development female rats had higher densities of dopaminergic fibers and GABAergic neurons in the striatum (48), and that female rats had greater density of neurons expressing GABA immunoreactivity in the bed nucleus of the stria terminalis (67). Gonadectomy enhanced rotational behavior induced by electrical stimulation in the SNR in female but not in male rats (57). Extensive electrophysiological studies also showed marked sex differences in the SNR (86–88). The basal firing rate of neurons in the SNR is higher in intact male rats compared to orchidectomized male, intact female, and ovarectomized female rats, and neither sex nor castration affected GABA sensitivity and responsiveness of the SNR to BZs (acute IV diazepam attenuated firing rate similarly in intact and castrated male and female rats). Furthermore, intact male rats chronically exposed to diazepam-filled implants (90 mg/10 days) showed decreased responsiveness of the SNR to systemically administered diazepam and the compensatory increase in the spontaneous firing rate upon administration of flumazenil. In contrast, in ovaractomized female rats chronic diazepam treatment failed to modified the ability of additional diazepam (IV bolus) to inhibit the firing rate of the SNR, and the administration of flumazenil did not elevate the firing rate above prediazepam baseline. Taken together, it can be speculated that the higher intensity of behaviorally manifested withdrawal in male than in female diazepam-treated rats after intranigral administration of flumazenil reflects gender difference in the flumazenil-induced compensatory increase in firing rate of the neurons in the SNR.

Convulsive Phenomena

Gender does not seem to play a role in convulsion-evoking abilities of the CBR and PBR antagonists int he SNR. Central application fo flumazenil or PK 11195 into the SNR did not evoke convulsions in either sex (at least in the range of doses employed herein). Further, the total number of twitches and jerks was similar in male and female rats (61 vs. 76 and 123 vs. 101 for flumazenil and PK 11195, respectively). It should be mentioned, however, that convulsive phenomena following peripheral administration of antagonists seemed to be related to sex. Accordingly, IV infusion of 3.9 mg/kg of flumazenil evoked convulsions in 70% of diazepam-treated male rats (93), while 46 mg/kg was required to induce convulsions in female rats (37). The incidence of flumazenil-evoked (IP) convulsions was significantly different in male and female mice chronically implanted with diazepam pellets (54). Furthermore, a bolus IV injection of flumazenil or PK 11195 evoked more tonic–clonic and clonic convulsions and twitches and jerks in male than in female rats chronically exposed to a high dose of diazepam (63,65). Lack of gender differences in convulsive phenomena after focal but not after systemic administration of flumazenil and PK 11195 seems to be in line with data showing that the hormonal milieu affects responses of SNR neurons (inhibition in firing rate) to systemically but not to locally administered BZs (86).

Other Signs

There were marked sex-related differences in the quantitative and qualitative aspect of focally evoked dose-related signs of withdrawal. Thus, both flumazenil-induced turning and PK 11195-induced tachypnea had higher scores in male than in female rats, $F(1, 55) = 16.1$, $p = 0.025$, and $F(1, 69) = 6.0$, $p = 0.05$, two-way RM ANOVA; for turning and respiration, respectively. Further, flumazenil- and PK 11195-induced twitches and jerks were significantly related to dose in male but not in female rats; flumazenil- and PK 11195-induced head bobbing was dose-related in female rats, while flumazenil-induced head and body tremors and backing were dose- related in female and male rats, respectively. These data suggest that the $GABA_A$ receptor subunit expression, the PBR, and/or neuroadaptation elicited by chronic BZ treatment are gender selective. The SNR is enriched in the CBR (Type 1), and is thought to have a homogenous $GABA_A$ receptor population ($\alpha_1\ \beta_2\ \gamma_2$) (47,53). However, the α_1, along with α_2 and α_3 subunits, have

been detected in the SNR (8). It is well established that the pharmacological properties of the $GABA_A$/CBR/ionophore complex are defined by subunit composition, and that the neuroactive steroids are potent and selective modulators of GABA function. Sex-related differences in modulation of the GABA-activated chloride influx (female > male) were found for the glucocorticoid-derivative but not for progesterone-derivative or androgenic steroids (90). Further, reduction of stress-induced brain levels of 3α-5α-THP and corticosterone were not significantly different in male and female rats chronically treated with diazepam (91). Structurally similar neuroactive steroids (allopregnanolone and alphaxalone) were differentially influenced by the αγ and βγ $GABA_A$ subunits, and the α subunit played a role in determining the efficacy while the γ subunit influenced both the efficacy and potency of allosteric coupling between steroids and $GABA_A$, which suggest multiple steroid recognition sites on the $GABA_A$ receptor (32,33). Reduced expression for the α_1 a subunit of $GABA_A$ has been observed in the cortex but not in hippocampus or cerebellum in male rats chronically treated with diazepam (20). It is not known, however, whether similar alteration of the α_1 (and/or other subunits) occurs in female rats, and which brain areas are involved. There is a line of evidence that the PBR are involved in synthesis of neuroactive steroids [see (26) for review]; that chronic BZ treatment enhances the PBR, and that there is lack of correlation between BZ-induced enhancement of the PBR in steroidogenic organs and serum levels of steroids (83,84). It is striking that both the neurosteroids regulation of GABA responses (90) and the modification of response of the PBR to stress by the hormonal milieu (5) are regionally specific. Taken together, in can be speculated that the BZ-induced changes in the $GABA_A$/CBR and PBR in a given brain area (i.e., the SNR) are affected by hormonal state.

EEG

Female and male rats differ in the spectral contents of the EEG in the SNR. In comparison to female rats, male rats have less slow (1–4 Hz)-frequency bands in the TP_{EEG} both prior to (about 75 vs. 55% in female and male rats, respectively) and during chronic exposure to diazepam (about 50 vs. 35% in female and male rats, respectively). In both sexes chronic diazepam treatment results in a significant shift of the spectral content of the EEG toward the fast-frequency band, an effect though to be associated with development of tolerance to the sedative activity of BZs (25,39,41,73). Focal injections of flumazenil and PK 11195 into the SNR decreased power of the 12–30 Hz frequency range (beta activity), and this effect was more pronounced in male than in female rats. It should be mentioned that the flumazenil- and PK 11195-induced reversal of the diazepam-induced increase of the beta activity in the SNR was in the same direction as changes in the EEG that were associated with spontaneous withdrawal phenomena. Taken together, the above comparisons indicate concordance between gender differences in withdrawal syndrome manifested by the PAS (male > female) and decrease of beta activity in the EEG (male > female). Whether gender difference in the power spectrum of the EEG show regional brain heterogeneity needs to be determined. It can be speculated that the sex-related intrinsic difference in the proportion of slow- and high-frequency bands in the TP_{EEG} of the EEG in the SNR reflect different brain sensitivity to BZ in male and female rats.

In summary, it can be concluded that gender effects on the focally precipitated withdrawal syndrome manifested both by the PAS and the EEG effect (male > female) in the SNR are in agreement with the flumazenil-induced compensatory increase in spontaneous firing rate of the neurons in the SNR (male > female) [87,88] but not with plasma levels of BZs (male < female).

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