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Substantia Nigra: The Involvement of Central and Peripheral Benzodiazepine Receptors in Physical Dependence on Diazepam as Evidenced by Behavioral and EEG Effects

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WALA, E. P., J. W. SLOAN AND X. JING. *Substantia nigra: The involvement of central and peripheral benzodiazepine receptors in physical dependence on diazepam as evidenced by behavioral and EEG effects.* PHARMACOL BIOCHEM BEHAV **64**(3) 611–623, 1999.—Male rats chronically exposed to diazepam (DZ) slowly released from subcutaneously implanted silastic capsules along with empty capsule control 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, 25 μg] and PK 11195 [(PK) 3.125, 6.25, 12.5, 25 μg], respectively (weekly intervals; Latin square design). Rats were observed for signs of withdrawal and the EEG was recorded simultaneously from the site of injection (SNR), caudate putamen, thalamus, hippocampus, and frontal cortex. In DZ-dependent rats the Precipitated Abstinence Score (PAS) was significantly related to dose of FLU. The PAS increased with increasing doses of PK (3.125–12.5 μg); however, the highest dose of PK (25 μg) showed less effect. The rapid onset of the PAS was accompanied by a rise in the total power (1–32 Hz) of the EEG (TP_{EEG}) in the SNR and other brain areas. The PAS and TP_{EEG} had similar time courses. Intranigrally injected FLU and PK did not evoke clonic and tonic–clonic convulsions; however, both antagonists induced dose-related twitches and jerks. Additionally, FLU precipitated a dose-related tachypnea and increases in turning and backing. Chronic DZ treatment altered the spectral content of the EEG, as indicated by a decrease and an increase of the slow and fast frequency bands, respectively. FLU and PK rapidly but transiently reversed the EEG. Data suggest that in the SNR the CBR mediate autonomic and motor signs of DZ withdrawal, while both the CBR and PBR are responsible for twitches and jerks and alteration of the EEG. It is possible that PK also acts on the site linked to a $GABA_A$/CBR/ionophore. © 1999 Elsevier Science Inc.

Substantia nigra Central benzodiazepine receptors Peripheral benzodiazepine receptors Focal injection Flumazenil PK 11195 Precipitated withdrawal EEG

THE long-term administration of benzodiazepines (BZs) may be associated with physical dependence, as indicated by a withdrawal syndrome induced by termination of the treatment. The signs of BZ withdrawal are complex, and are manifested in human and laboratory animals by convulsions, changes in autonomic function, sensory and perceptual disturbance, and impaired motor function [for references, see (38)]. The specific antagonist of the central BZ receptors (CBR), flumazenil, inhibits the central effects of BZs, and can unmask all signs of withdrawal that are mediated through the gamma-aminobutyric ($GABA_A$) /CBR/ionophore complex [for review, see (82)]. As is the case for flumazenil, PK 11195, a specific antagonist of the peripheral BZ receptor (PBR), administered intravenously (IV), intramuscularly (IM), or centrally (the CA1 area of hippocampus and spinal subarachnoid space) precipitates a withdrawal syndrome that includes dose-related convulsions and/or twitches and jerks in diazepam-dependent rats (40,66,72,74). Both flumazenil and PK11195 reverse the convulsant action of the PBR agonist, Ro 5-4864 [for references, see (12)]. Further, the PBR have been dem-

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onstrated to play a role in the pathogenesis of epileptic seizures in EL mice [for references, see (50)]. These findings suggest that both the PBR and CBR are involved in the mediation of convulsive activity within the CNS.

The substantia nigra, a brain area that has an intensive GABAergic innervation and is tonically inhibited by GABA input, is thought to be a critical locus in the neuronal control of propagation of convulsions. The following findings suggest that the substantia nigra might constitute a site of anticonvulsant action of BZs: 1) the enhancement of GABAergic transmission within the substantia nigra prevents the motor manifestation of both chemically induced and kindled convulsions [for references, see (75)]; 2) transplantation of fetal GABAergic neurons into the substantia nigra significantly reduces kindled convulsions in the rat (30); 3) BZs (diazepam, clonazepam) inhibit the firing of pars reticulata cells in doses that terminate tonic–clonic status epilepticus and pentylenetetrazol (PTZ) evoked convulsions (75); 4) bilateral injections of BZs (flurazepam, midazolam) into the substantia nigra induce dose-related anti-PTZ convulsive activity, while the intranigral coadministration of midazolam with flumazenil reverses the anticonvulsant effects of midazolam (84); 5) flumazenil reverses an inhibitory effect of acutely administered diazepam on the firing rate in the substantia nigra (58). Although the firing of reticulata neurons is similar in diazepam-treated and control male rats, flumazenil decreases GABA sensitivity and enhances the firing rate in diazepam-exposed but not in control rats (78,80,81).

Pilot data from our laboratory revealed that the unilateral injection of flumazenil into the substantia nigra induced a well-defined withdrawal syndrome (statistically significant signs of precipitated withdrawal such as tremors, turning, tachypnea, blinking, digging), but surprisingly failed to evoke motor manifestation of convulsions in female rats chronically treated with a high dose (540 mg/week) of diazepam (73). Although sex has been reported to have no effect on GABA sensitivity and responsiveness to BZs in the substantia nigra, the basal firing rate of nigral neurons was higher in male than in female rats, and flumazenil induced a compensatory increase in spontaneous nigral firing in male but not in ovariectomized female rats (76,77). Furthermore, there was a trend for more flumazenil- and PK 11195-evoked (IV) clonic and tonic–clonic convulsions in male than in female rats exposed to diazepam (540 mg/week) (62,63). These data suggest that convulsive phenomena evoked by the focal injection of flumazenil or PK 11195 into the substantia nigra in diazepam-dependent male rats are likely to be pronounced.

There is a line of evidence that in the rat acutely administered diazepam affects the cortical electroencephalogram (EEG), as indicated by occasional bursts of 2–4-Hz waves, lengthening of the 7–12-Hz spindles, and the appearance of 15–30-Hz waves. Chronic diazepam treatment markedly reduces spindle bursts and increases the high frequency waves, an EEG effect thought to be associated with development of tolerance to the sedative effect of diazepam (22,29,41,43,51,70). The involvement of both the CBR and PBR in the EEG effect is suggested by results of the following studies: 1) systemically injected flumazenil rapidly reverses the diazepam-altered EEG (27,37,49); 2) the concomitant administration of diazepam and PK11195 prevents the induction of tolerance to the EEG effect of diazepam in the rat (41); 3) the focal application of both flumazenil and PK 11195 into the CA1 field of the hippocampus reduces the total power of fast frequency bands of the EEG in female rats chronically exposed to diazepam implants (90 mg/week) (66).

In the rat, the substantia nigra is enriched predominantly in Type I of the CBR (42,52,83), and has a low population of the PBR (10,54). Thus, the aim of the present study is to assess whether antagonists with specificity for the CBR (Type I and Type II), flumazenil, and the PBR, PK11195, precipitate a withdrawal syndrome and affect the EEG following unilateral injections into the substantia nigra in male rats chronically exposed to diazepam (90 mg/week) slowly released from the silastic implants.

METHOD

Animals

Male Sprague–Dawley rats (approximately 90 days old, weighing about 350 g) were used in this experiment. The rats were housed in accordance with the "Principles of laboratory animal care" (NIH publication No 85-23, revised 1985) in a climate-controlled facility with 12 D/12 L cycles with a light onset at 0600 h. Each rat was kept separately in a transparent cage with a sawdust-covered floor and free access to standard laboratory chow and tap water. The experiment was conducted according to a protocol approved by the University of Kentucky Animal Care and Use Committee. The body weights were recorded weekly (before implantation of the successive capsule). All surgeries were performed under sterile condition and ketamine chloride anesthesia (80 and 40 mg/kg IP for brain surgery and subcutaneous implantation of capsules, respectively). Rats were euthanatized with pentobarbital sodium (120 mg/kg IP).

Drugs and Chemicals

Silastic capsules (7 cm long) were made out of Medical Grade Silastic Tubing (inner diameter 0.147 cm × outer diameter 0.195 cm), and were sealed on both ends with Silicon Type A Silastic Medical Adhesive. Each capsule contained 90 mg of crystallized diazepam [recystalization of diazepam in ethyl alcohol increased its in vitro release from silastic tubing (39)] or was empty (control). Before implantation the capsules were conditioned by soaking for 15 min in ethyl alcohol and bovine albumin (3%), consecutively. Flumazenil and PK 11195 were dissolved in dimethylsulfoxide (DMSO). Solutions were freshly prepared on the day of the experiment and were protected from light. Diazepam and flumazenil were gifts of Hoffmann–LaRoche (Nutley, NJ), PK 11195 was purchased from Research Biochemical International, silastic tubing and adhesive were from Dow Corning (Midland, MI), while DMSO and HPLC standards were from Sigma (St. Louis, MO).

Implantation of Injecting Cannulae and Electroencephalogram (EEG) Electrodes

Anesthetized rats were mounted in a Kopf stereotaxic instrument, and their skulls were exposed. An indwelling guide cannulae-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 substantia nigra pars reticulata [anterior posterior (AP; mm) = 3.7; lateral (L; mm) = 2.4; vertical (V; mm) = 5]. Intracranial (E363/1) stainless steel electrodes (Plastic One, Roanoke, VA) directed toward some areas involved in the nigrostriatal, nigrothalamic, and mesocortical projections (11) such as caudate putamen (AP = 8.7; L = 3.2; V = 5), ventromedial thalamus (AP = 6.7; L = 1.6; V = 3), frontal cortex (AP = 10.2; L = 2; V = 8.2), and hippocampus (AP = 3.7; L = 3; V =

6) were implanted ipsilaterally using interaural stereotaxic coordinates, as previously described in detail (73). 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 guide cannula was kept with the help of close-fitting obturator. After completion of surgery the rats were injected with sterile saline (total volume ~5 ml, injected IM in several places) 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.

Implantation of Silastic Capsules

After recovery from the surgery (about 7 days) the rats were subcutaneously implanted in the back with silastic capsules containing diazepam (90 mg) or empty capsules (control rats) according to the protocol of Gallager (16) with minor modification (39). The rats were initially implanted with two capsules (2 × 90 mg of diazepam or empty) and thereafter an additional capsule (1 × 90 mg of diazepam or empty) was implanted at weekly intervals (Friday) for 7 consecutive weeks. The entire diazepam (or empty capsule) treatment lasted 8 weeks (4 weeks prior to and 4 weeks during precipitation of abstinence). Because capsules were almost empty 1 week after implantation (10% of diazepam remained), it was assumed that the rate of diazepam release from Silastic capsules was approximately equal to 40 mg/kg/day. The implantation procedure was simple and fast (about 5 min). Previously implanted capsules were not removed to avoid complicated surgery (implants were incapsulated in the tissue).

Focal Precipitation of Withdrawal

Intranigral injections were initiated 3 days after the fifth implantation of Silastic capsules (4 weeks of chronic exposure) and were continued for 4 weeks. Flumazenil (6.25, 12.5, or 25 μg); PK 11195 (3.125, 6.25, 12.5, or 25 μg) and DMSO-vehicle were rapidly injected in a volume equal to 1 μl [10% of approximate volume of substantia nigra (11)]. The highest dose of antagonists was selected from pilot studies, which indicated that 25 μg of intranigrally injected flumazenil precipitated a significant withdrawal in female rats dependent on 540 mg/week of diazepam (71). The lowest dose was based on approximately two times lower K_d for PK 11195 [0.64 ± 0.03 nM (2)] than for flumazenil [1.0 nM (47)] in rat cerebral cortex. The doses increased in order 1:2:4 (flumazenil) and 1:2:4:8 (PK 11195). The highest doses of PK 11195 and flumazenil were equal. The assumed initial total (protein bound + free) concentrations of flumazenil and PK 11195 in target tissue were equal to 2.06–8.2 × 10^{-8} M and 0.88–7.1 × 10^{-8} M, respectively. Thus, it is likely that free levels of antagonists were in the order of magnitude of K_d (10^{-9} M). The concentration of injected solutions was limited by flumazenil solubility in DMSO (25 mg/ml). Flumazenil or vehicle were injected on Monday while PK 11195 was injected on Thursday. There was no significant order effect when flumazenil and PK 11195 were systemically administered in 3-day intervals (65). The order of doses was balanced by a replicate block Latin square design (4 × 4). At the end of the study all rats had received all three doses of flumazenil, all four doses of PK 11195, and vehicle. One diazepam-dependent and two control rats were excluded from the study, and thus, data from seven diazepam-exposed and four control rats that completed the experiment are presented herein.

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). The chemotrode (stainless steel tubing, G-28;) was inserted into the guide cannula. 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 preantagonist baseline values. Thereafter, the chemotrode was connected to a Hamilton syringe with PE-20 polyethylene tubing and either flumazenil, PK 11195, or vehicle was rapidly injected, without handling the rat, into the substantia nigra (V = 1.8 mm). The tight fit between the guide cannula 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. The EEG was recorded for 20 min, and simultaneously the rat was observed (in four 5-min epochs) for signs of withdrawal. All rats were observed by the same observer (J.W.S.), who was blind to the treatment, and verified periodically by two other investigators. 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. Each rat served as its own control with respect to a preinjection baseline and the effect induced by DMSO-vehicle.

Precipitated Withdrawal Signs

The scored withdrawal signs included convulsive phenomena such as clonic convulsions (symmetrical clonic spasm of bilateral limbs), tonic–clonic convulsions (tonic spasm, clonic jerking, full extension in some cases), and twitches and jerks (isolated spasms of head, body, or limbs); motor signs manifested by head and body tremors, jumping (violent episodic movement), backing (episode of backward walking), turning (rapid change of direction); motor dyskinesia indicated by writhing, affective signs such as vocalization and severely arched back as well as change in an autonomic sign, 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) as previously described in detail (39). The PAS served as an indication of the overall intensity of the withdrawal syndrome. Several other withdrawal signs such as 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, symptoms of ataxia, head and body scratching, rearing (upright position), episodes of chewing, blinking, and ear twitching, as well as some signs related to activity such as fixed posture (sitting, standing), walking, exploring the cage, or curled position were also counted and recorded in 5-min epochs, but they were not included in the calculation of the PAS. The above listed withdrawal signs were frequently observed after bolus IV injection of flumazenil in rats chronically exposed to diazepam implants (39,74). The data are presented both as the PAS and scores for individual withdrawal signs. To normalize the data for between-subjects differences in baselines values, individually derived mean preinjection scores were subtracted at each observation

period from the postinjection scores for each rat. The areas under the normalized time action curves ($AUC_{0-20min}$) were used to construct dose–response curves.

Brain Electrical Activity

The EEG was recorded from the site of injection (substantia nigra), hippocampus, caudate putamen, ventromedial thalamus, and frontal cortex. Each rat served as its own control with respect to a baseline EEG recorded for 10 min prior to focal injection of flumazenil, PK11195, or DMSO. 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 normalize data for differences in the EEG baselines for each rat the post injection change in total power of the EEG (μV^2) was calculated with respect to an individually derived baseline and presented as the percentage of increment or decrement of (1–32 Hz) total power (TP_{EEG}) over the individual 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 (percentage of TP_{EEG}). The data are presented as: 1) the time action curves of TP_{EEG} ; 2) dose–response curves for the maximum (5 min) relative TP_{EEG} (normalized for an individual derived baseline); and 3) changes in distribution of different frequency bands within the TP_{EEG}.

Plasma Levels

Blood samples (about 0.5 ml) were collected at weekly intervals (Friday; 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 (39).

Histology

After completion of the studies the locations of the guide cannulae and electrodes were histologically identified. Rats were anesthetized with pentobarbital sodium and perfused 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. Rats used in the data presented here had chemotrode tips placed within the substantia nigra. Wrong placement of the cannula resulted in lack of significant PAS. Although in some brain sections there were signs of damage (the tissue lost was not quantitatively evaluated), presumably resulting from the multiple injections, rats did not show neurological impairments, nor did the time factor significantly affect the intensity of withdrawal.

Data Analysis

The data were tested for normal distribution and equal variance prior to the use of parametric statistics (Kolmogorov–Smirnov normality test and Levine median equal variance test). Time courses of the PAS and scores for individual signs (raw data) were analyzed by repeated measures (RM) analysis of variance (ANOVA), with dose (0–25 μg) and time [average preinjection and 5, 10, 15, 20 min postinjection] taken as the factors. The effect induced by DMSO-vehicle (dose 0) was compared with the effect induced by each dose of flumazenil or PK11195 at each post injection epoch with the help of the post hoc all pairwise Student–Newman–Keuls method. Using data normalized for preantagonist baseline, the effects induced by different doses of flumazenil and PK 11195 on the PAS and on the individual signs of precipitated withdrawal as well as effects on the EEG (changes in TP_{EEG}) were analyzed by one-way RM ANOVA, with dose used as a factor. The between-doses differences 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 both flumazenil and PK11195 were analyzed by a two-way ANOVA with treatment (diazepam vs. empty capsules) and dose [flumazenil (6.25–25 μg) or PK 11195 (3.125–25 μg)] taken as the factors. Spectral content of the EEG at preinjection (baseline) and postinjection (5 min) was compared by paired *t*-tests, while differences in the spectral content of the EEG in control and diazepam-dependent rats were evaluated by unpaired *t*-tests. 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; Kruskal–Wallis one-way ANOVA on ranks; Mann–Whitney rank test; Wilcoxon signed rank test; Spearman ranks order correlation). A probability level of 0.05 or less was considered significant. All statistical calculations were made with the help of Sigma Stat statistical software.

RESULTS

There was a marked difference in the body weight gain in male rats exposed for 8 weeks to diazepam-filled and empty capsules. Accordingly, the between-weeks difference in the weight gain was of statistical significance in control, $F(7, 21) = 2.7$, $p < 0.05$; one-way RM ANOVA, but not in diazepam-treated rats, $F(7, 42) = 1.8$. There was a trend toward more weight gain in control than in diazepam-dependent rats both prior to (weeks 1 to 4: 39.25 ± 14.04 g vs. 25.1 ± 3.3 g) and after (weeks 5 to 8: 48.6 ± 19.0 g vs. 20.9 ± 2.3 g) focal injections of flumazenil and PK11195, respectively; however, differences were below the level of statistical significance (unpaired *t*-test). Intranigral injections of flumazenil and PK 11195 did not significantly affect body weight gain either in diazepam-dependent or control rats (Mann–Whitney rank sum test).

In rats chronically exposed to diazepam implants (90 mg/week) the average steady-state plasma levels of diazepam and its metabolites, nordiazepam, oxazepam, temazepam, and 4-hydroxydiazepam, were equal to 0.71 ± 0.05; 0.25 ± 0.07; 0.10 ± 0.06; 0.16 ± 0.11; and 0.13 ± 0.04 μg/ml, respectively, and they did not significantly change across the time of the experiment (one-way RM ANOVA and RM ANOVA on ranks). It should be pointed out that mean plasma levels of diazepam were lower prior to (weeks 1 to 4: 0.61 ± 0.04 μg/ml) than after (weeks 5 to 8: 0.81 ± 0.07 μg/ml) focal injections of antagonists ($p = 0.0515$; Wilcoxon signed rank test). The significance of this observation is ambiguous, however, due to the

lack of an appropriate control (8 weeks of diazepam treatment without focal injections).

In male rats chronically exposed to diazepam focal injections of graded doses of flumazenil and PK 11195 into the substantia nigra induced a PAS that peaked within 5 min and then gradually declined. The withdrawal syndrome was not induced by intranigral injections of flumazenil and PK 11195 in control rats and by DMSO-vehicle in diazepam-treated rats. Between doses, $F(3, 72) = 4.8$, $p < 0.025$, and between time, $F(4, 72) = 32.2$, $p < 0.0001$, differences and dose–time interaction, $F(12, 72) = 2.4$, $p < 0.025$) for the flumazenil-evoked PAS as well as between the time points difference, $F(4, 96) = 10.8$, $p < 0.0001$, for the PK 11195-induced PAS were of statistical significance (RM ANOVA). Post hoc analysis indicated that the PAS induced by 6.25 μg (5 min) and 25 μg (5, 10, 15 min) of flumazenil as well as by 25 μg (5 min) of PK11195 were statistically significantly different from the preinjection baseline ($p < 0.05$; Student–Newman–Keuls method). In empty-capsule control rats the differences between doses, time and dose–time interactions were lacking statistical significance.

Figure 1 shows examples of the time courses of the PAS (Fig. 1A and B) and total power (1–32 Hz) of the EEG (TP_{EEG}) (Fig. 1C and D) following focal injections of flumazenil (25 μg) and PK 11195 (12.5 μg) into the substantia nigra in diazepam-exposed and control rats (doses that induced a maximum PAS). In diazepam-treated rats, a rapid onset of flumazenil- and PK 11195-induced PAS (within 5 min) was accompanied by the simultaneous enhancement of the TP_{EEG}. The rate constants for decrease of behavioral and EEG effects were similar: PAS = 0.0305 ± 0.005 min^{-1}; TP_{EEG} = 0.0318 ± 0.009 min^{-1} (flumazenil), and PAS = 0.0257 ± 0.007 min^{-1}; TP_{EEG} = 0.0324 ± 0.005 min^{-1} (PK 11195) [mean values for pooled doses of flumazenil (6.25–25 μg) and PK 11195 (3.125–25 μg), respectively]. Intranigral injection of DMSO in diazepam-treated rats as well as injections of flumazenil, PK 11195, and DMSO in control rats had a minor effect on the PAS and EEG. In other brain areas such as hippocampus, thalamus, caudate putamen, and frontal cortex the time action curves for flumazenil- and PK 11195-induced changes in TP_{EEG} were similar to these in the substantia nigra (data not shown).

Figure 2 indicates that in diazepam-dependent rats there is a good concordance across doses between flumazenil-induced maximum withdrawal score (PAS_{max}) and relative (% of the baseline) maximum TP_{EEG} (Fig. 2A and C). The flumazenil-induced PAS_{max} showed a significant between-doses difference, $F(2, 12) = 4.9$, $p < 0.05$; one-way RM ANOVA, and a statistically significant dose–response relationship, $F(1, 20) = 4.05$, $p < 0.05$. The flumazenil (12.5 μg)-induced effect was lower than expected (both for the raw and baseline-normalized data). Although the PAS_{max} increased with increasing dose of PK 11195 (3.125–12.5 μg), the highest dose of PK 11195 (25 μg) induced less effect (Fig. 2B). The between-dose differences and dose–response curve for maximum TP_{EEG} were below the level of statistical significance both for flumazenil and PK 11195 (RM ANOVA on ranks). The correlation between maximum PAS and TP_{EEG} was not of statistical significance (Spearman ranks order correlation). There was a statistically significant difference between the PAS_{max} in diazepam-treated and control rats, $F(2, 32) = 32.9$ and $F(3, 43) = 15.0$ for flumazenil and PK 11195, respectively; $p < 0.001$; two-way ANOVA. The effects of both flumazenil and PK 11195 on the TP_{EEG} failed to reach statistical significance in diazepam-dependent and control rats. In diazepam-exposed

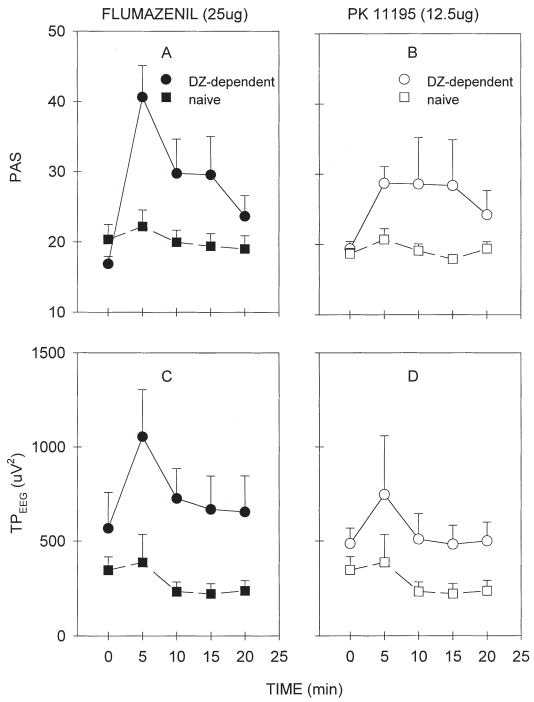

FIG. 1. Time courses of the Precipitated Abstinence Score (PAS) (A and B) and total power (1–32 Hz) of the EEG (TP_{EEG}) (C and D) after focal administration of flumazenil (25 μg) and PK 11195 (12.5 μg) into the substantia nigra in diazepam-dependent (90 mg/week) ($n = 7$) and empty capsule control (naive) ($n = 4$) male rats. Data are mean + SEM of n rats.

rats, there was a trend for higher behaviorally and EEG-manifested withdrawal effects after administration of flumazenil than after PK 11195 (except for the 12.5-μg dose). In control rats, flumazenil and PK 11195 did not induce a significant PAS (Fig. 2A and B); the flumazenil-induced enhancement of the TP_{EEG} was not related to dose (Fig. 2C); PK 11195 did not affect the TP_{EEG} (Fig. 2D).

Figure 3 shows that as in the substantia nigra, in other brain areas such as the hippocampus, caudate putamen, thalamus, and frontal cortex, the increase of the TP_{EEG} was more pronounced after administration of flumazenil than after PK 11195. Although enhancement of the TP_{EEG} seemed to be higher in the caudate putamen than in other brain areas, regional differences were not of statistical significance (Kruskal–Wallis one-way ANOVA on ranks). EEG recording in other brain areas showed a trend for the maximum TP_{EEG} to increase with increasing doses of intranigrally injected flumazenil and PK 11195 (data not shown).

In male diazepam-dependent rats unilateral injection of flumazenil and PK 11195 in the substantia nigra did not evoke behavioral manifestation of convulsions. However, as illustrated in Fig. 4A and B, both antagonists precipitated twitches and jerks (isolated spasms of head, body, or limbs) that emerged rapidly (within 5 min) and then progressively declined. Flumazenil-induced twitches and jerks showed a significant between-doses difference, $F(3, 27) = 5.7$, $p < 0.01$; one-way RM ANOVA, and a significant dose–response relationship, $F(1, 27) = 15.3$, $p < 0.001$ (C). Although PK11195-induced twitches and jerks differed between doses ($\chi^2 = 11.1$, $p < 0.05$; RM ANOVA on ranks), and had a statistically sig-

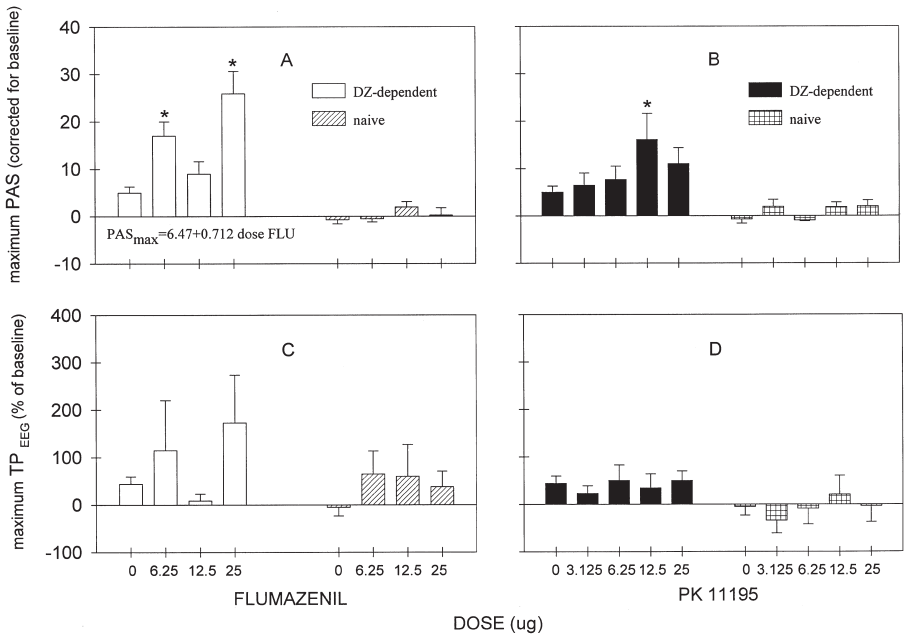

FIG. 2. Dose–response curves for the maximum Precipitated Abstinence Score (PAS_{max}) (A and B) and maximum total power (1–32 Hz) of the EEG (TP_{EEG}) [C and D] induced by focal injections (1 μl) of graded doses of flumazenil and PK 11195 into the substantia nigra in male rats chronically exposed to diazepam (diazepam dependent; $n = 7$) and empty capsules (naive; $n = 4$). The PAS_{max} was corrected for baseline (postinjection − average preinjection); TP_{EEG} is presented as increment over preinjection baseline (% of baseline). Vehicle (DMSO) = 0 dose. Data are mean + SEM of *n*. *Significantly different from vehicle (*t*-paired test).

nificant dose–response relationship, $F(1, 34) = 4.9$, $p < 0.05$, the highest dose of PK 11195 (25 μg) showed a plateau (Fig. 4D). Scores for twitches and jerks induced by the highest dose (25 μg) of flumazenil and PK 11195 were similar (1.18 ± 0.399 and 1.57 ± 0.683, respectively). However, it should be noted that the total number of twitches and jerks (across the 20-min observation period; pooled three doses of each antagonist: 6.25, 12.5, and 25 μg) was higher for PK11195 than for flumazenil (123 vs. 61). Twitches and jerks were not evoked by DMSO-vehicle in diazepam-treated rats nor by flumazenil and PK 11195 in empty-capsule control rats. With few exceptions twitches and jerks were accompanied by spikes and high amplitude low frequency waves on the EEG tracing.

In diazepam-dependent rats flumazenil-induced tachypnea (breath/min), turning (rapid change of direction), and backing (backward walking) showed a significant difference between

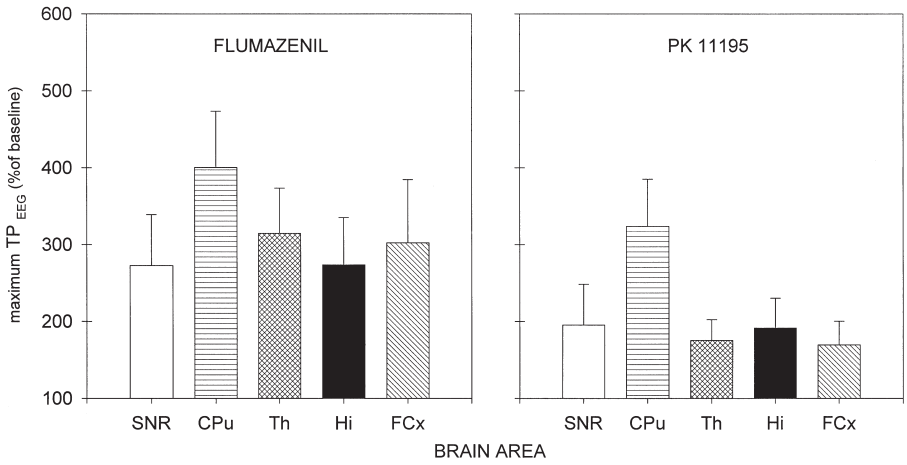

FIG. 3. The maximum Total Power (1-32 Hz) of the EEG (TP_{EEG}) in the substantia nigra (SNR), caudate putamen (CPu), thalamus (TH), hippocampus (HI) and frontal cortex (FCx) recorded within 5 min after focal injections of flumazenil and PK 11195 into the substantia nigra in diazepam-dependent (90 mg/week) male rats (n=7). Data are presented for pooled doses of flumazenil and PK 11195 (6.25 - 25 μg) and are mean + SEM of "n" rats.

doses, $F(2, 27) = 5.8$, 5.6, and 5.3, respectively, $p < 0.01$; one-way RM ANOVA, and had a statistically significant dose–effect relationships, $F(1, 27) = 13.5$, 15.2, and 14.7, respectively, $p < 0.025$. None of the above (or any other) withdrawal sign was related to the dose of PK 11195. In control rats flumazenil and PK 11195 did not precipitate dose-related signs of abstinence.

In diazepam-dependent rats the average PAS ($AUC_{1–20\ min}$), maximum PAS and scores for individual signs of abstinence (twitches and jerks, tachypnea, turning, and backing) did not significantly change across time (4 weeks) of focal intranigral injections of flumazenil and PK 11195 (one-way RM ANOVA or one-way RM ANOVA on ranks).

Figure 5 illustrates the relative band power of the EEG power spectrum (% of TP_{EEG}) in the substantia nigra prior to (preinjection baseline) and immediately after (within 5 min) focal injections of flumazenil and PK 11195 in diazepam-exposed and empty-capsule control rats. Changes in the power spectrum of the EEG (y-axis) in different frequency bands (x-axis) were plotted as a function of the experimental conditions (z-axis). Comparison of the average preinjection baselines in diazepam-treated (Fig. 5A and B) and control (Fig. 5C and D) rats revealed that in the substantia nigra chronic exposure to diazepam significantly reduced slow (1–4 Hz: about 38 vs. 57%), and enhanced fast frequency bands (about 33 vs. 28%; 11 vs. 7.8%; 10 vs. 5% and 8 vs. 3% for 4–12; 12–18; 18–26, and 26–32 Hz, respectively) within the TP_{EEG} ($p < 0.05$; *t*-unpaired test). In diazepam-exposed rats focal injections of flumazenil and PK 1195 into the substantia nigra rapidly (within 5 min) shifted the spectral content of the EEG toward the prediazepam condition, as indicated by trend toward enhancement of the slow and reduction of the fast frequency bands within TP_{EEG} . The power spectrum of the EEG declined across 20 min toward preinjection baselines. Although in diazepam-dependent rats there was a trend for slow waves to increase and for high frequency bands to decrease with increasing doses of flumazenil (Fig. 5A) and PK 11195 (Fig. 5B) the between doses differences were below the level of statistical significance [(1–4 Hz) flumazenil: $F(2, 12) = 3.75$, $p = 0.054$, and PK 11195: $F(3, 18) = 3.11$, $p = 0.052$ (one-way RM ANOVA)]. It is noteworthy, however, that for pooled doses (flumazenil or PK 11195) in comparison to preinjection baselines, the contribution of fast frequency waves in the TP_{EEG} (%) was significantly reduced ($p < 0.05$; *t*-paired test) within 5 min after focal injections of both flumazenil (11.8 ± 0.49 vs. 9.0 ± 0.50% and 10.3 ± 0.79 vs. 8.1 ± 0.69% for 12–18 Hz and 18–26 Hz, re 6spectively), and PK 11195 (10.6 ± 0.69 vs. 8.8 ± 0.74% and 8.3 ± 0.63 vs. 7.3 ± 0.69% for 18–26 Hz and 26–32 Hz, respectively) in diazepam-dependent rats. There was lack of trend for dose-related changes in the EEG in control rats. It is interesting that the highest dose of flumazenil (25 μg) seemed to affect the spectral content of the EEG in an opposite directions in control (shift toward high-frequency bands) and diazepam-treated rats (shift toward low-frequency bands). PK 11195 had a minor and inconsistent effects on the EEG in control rats (Fig. 5D). DMSO administered alone, produced inconsistent and a small effect on the EEG both in diazepam-dependent and control rats. As was the case for the site of injection, in other brain areas there was a trend for the slow- and fast-frequency bands to increase and decrease, respectively, with increasing doses of flumazenil and PK 11195 in diazepam-dependent but not in control rats (data not shown).

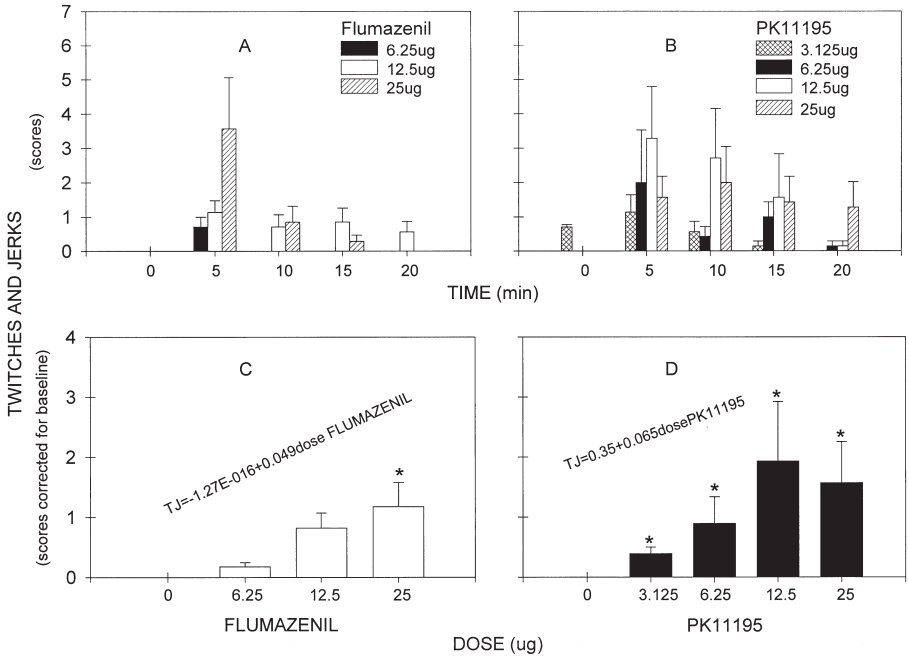

FIG. 4. Time courses for twitches and jerks (score) evoked in the substantia nigra by flumazenil (6.25, 12.5, and 25 μg) (A) and PK 11195 (3.125, 6.25, 12.5, and 25 μg) (B). Dose–response curves for twitches and jerks [TJ; average scores ($AUC_{0–20min}$) corrected for preinjection baseline] induced by focal injections of graded doses of flumazenil (C) and PK 11195 (D) into the substantia nigra. Data are mean + SEM for seven diazepam-dependent (90 mg/week) male rats. Vehicle (DMSO) = 0 dose. *Significantly different from vehicle (post hoc Student–Newman–Keuls method).

DISCUSSION

In diazepam-treated rats, flumazenil (6.25 and 25 μg) and PK 11195 (3.125–12.5 μg) induced a PAS in the substantia nigra that increased with increasing dose. Flumazenil has high affinity for the CBR [mainly localized to the symptosomal fraction of neurons (4,48)] but does not bind to the PBR. PK 11195 has specificity as an antagonist of the PBR [localized on the outer mitochondrial membrane, mainly in the peripheral tissues, but also found within the CNS, on glial cells and neurons (see [60] for references)]. Thus, the present data suggest that in the substantia nigra an abstinence syndrome was mediated by both the CBR and the PBR. The effect induced by the middle dose of flumazenil (12.5 μg) was lower than expected. Because this observation cannot be explained by an unexpectedly high baseline score, the involvement of different subsets of receptors is possible. Although the plateau effect of the highest dose of PK 11195 suggests saturation of the PBR in the substantia nigra, without additional data a biphasic curve cannot be ruled out. Thus, testing of a wider range of doses of flumazenil and PK 11195 is in order. In comparison to flumazenil, PK 11195-evoked withdrawal was characterized by a lower PAS. The same phenomenon was observed when flumazenil and PK 11195 were injected systemically (74) and centrally into the CA1 area of the hippocampus (66) and spinal subarachnoid space (72). This can likely be explained by a difference in population of the CBR and PBR in the CNS. The flumazenil- and PK 11195-induced PAS peaked rapidly and declined with half lives equal to 25 ± 5.18 min and 26.4 ± 7.32 min, respectively [mean values for pooled doses of flumazenil (6.25–25 μg) and PK 11195 (3.125–25 μg)] that approximated disappearance of systemically administered flumazenil ($T_{0.5}$ = 16 min) from brain in the rat (28). Whether resemblance of the PAS time curves for flumazenil and PK 11195 can be explained by similar pharmacokinetics of these antagonists in the brain remains to be determined.

Flumazenil precipitated several dose-related abstinence signs in the substantia nigra such as twitches and jerks, tachypnea, turning, and backing. The one possible explanation of different types of signs is that the withdrawal syndrome is mediated in the substantia nigra through different subtypes of the GABA/CBR/ionophore complex. In the rat, the substantia nigra is predominantly enriched with CBR Type I (42,52,83), and is thought to have a homogeneous $GABA_A$ receptor population in terms of subunit composition. With this regard the abundance of α_1 subunit along with β_2 and γ_1 and γ_2 subunits was detected by in situ hybridization (53,55), while immunofluorescence staining revealed the α_1 $\beta_{2,3}$ γ_2 subunits combination (15). It must be noted, however, that polymerase chain reaction analysis showed α_2 and α_3 subunits in the substantia nigra in rat where these subunits were not detectable with in situ hybridization or were found at very low levels in comparison to the α_1 subunit (9). It is well known that the choice of the α subunit of $GABA_A$ increases the diversity of BZ responses. For example, diazepam (anxiolytic, anticonvulsant, sedative) showed no differences in binding to receptors containing α_1, α_2, or α_3 subunits while affinity of zolpidem (hypnotic) was markedly higher in α_1- than in α_2- and α_3-containing receptors [for references, see (44,46)].

In female rats chronically exposed to diazepam implants (90 mg/week), levels of free (unbound) diazepam in the extraneuronal brain space resembled levels of its metabolites, nordiazepam, and oxazepam, indicating similar availability of these BZs at the receptor site (39). Interestingly, despite the fact that diazepam, nordiazepam, and oxazepam bind with similar affinities to the CBR (4,48), they produced different type of physical dependence, as indicated by qualitatively and quantitatively different flumazenil-induced abstinence syndromes both in the dog and rat models (23,24,38). Thus, it can be speculated that in a given brain area (i.e., substantia nigra) diazepam and its metabolites interact with different subtypes of $GABA_A$ receptors producing different pharmacological effects. This hypothesis seems to be supported by a line of evidence that the substantia nigra responds differently to various BZs. In this regard, in rats chronically treated with flurazepam tolerance developed to dose-related suppression of spontaneous firing of nigral neurons (69) as well as significant downregulation of the CBR (68) and differential regulation of behavioral effects of GABA agonists in the substantia nigra (57) was observed. Furthermore, tolerance developed to the contralateral circling behavior evoked by unilateral nigral microinjections of flurazepam in the rat (67). On the contrary, chronic exposure to diazepam failed to change the sensitivity of reticulata neurons to iontophoretically applied GABA, the continued presence of diazepam potentiated the GABAergic response, while flumazenil reduced GABA sensitivity in the substantia nigra (78,80,81).

It is important to mention, however, that results of some other studies contradict the hypothesis that a given $GABA_A$ receptor subtype is specifically linked to a given behavioral output and rather suggest dynamic regulation of $GABA_A$ receptor structure in response to BZ-induced changes in neuronal function [for review, see (8)]. It must be emphasized that the pharmacokinetcs of flumazenil after focal administration into the brain is not known, and therefore, rapid distribution and reaction from adjacent and/or distant brain areas cannot be ruled out. The types of abstinence signs evoked from different structures can reflect specific function of each brain region. Pilot studies from our laboratory indicated that patterns of flumazenil-induced signs of withdrawal markedly differed between brain areas in diazepam-dependent but not in control rats (64). Additionally, effects due to the reabsorption of antagonist (6.25–25 μg) into blood is unlikely, because IV injection of a higher dose of flumazenil (250 μg) did not precipitate withdrawal in diazepam-dependent rats (72).

In male rats chronically exposed to diazepam (90 mg/week) unilateral injections of flumazenil and PK 11195 into the substantia nigra did not evoke, at least in the range of doses employed herein, behaviorally manifested convulsions. This observation is consistent with the lack of convulsions after unilateral intranigral injection of flumazenil (25 μg) in female rats chronically treated with a high dose (540 mg/week) of diazepam (73), and further implies that convulsions induced in diazepam-dependent rats by systemically administered flumazenil and PK 11195 (16,39,62,63,74,79) were not initiated in the substantia nigra. It is important to mention, however, that bilateral injections of BZs (midazolam, flurazepam) into the substantia nigra showed a dose-related anti-PTZ effect, and that flumazenil reversed the anticonvulsant effect of midazolam when both were infused into the substantia nigra (84).

The present data showed that in diazepam-dependent rats unilateral intranigral injections of both flumazenil and PK 11195 precipitated twitches and jerks (which are classified as convulsive phenomena). Twitches and jerks was a sole abstinence sign significantly related to the dose of PK 11195. Whether or not this is accounted by a homogenous population of the PBR in the substantia nigra cannot be judged herein. Occupation of the PBR by a single BZ ligand was also possible. With regard to the latter, diazepam bound with high

affinity to the CBR and PBR, while its metabolite, nordiazepam, exhibited very low affinity for the PBR (19). Taken together, the present data suggest that in the substantia nigra the CBR and PBR are involved in the generation of twitches and jerks while other withdrawal signs are mediated solely by the CBR. Interestingly, results similar to the present data were obtained in the spinal cord (72) and in the CA1 field of the hippocampus (66), where twitches and jerks were a common withdrawal sign evoked by flumazenil and PK 11195. This occurred despite the fact that the substantia nigra has a high population of CBR Type I, while the CA1 of hippocampus and spinal cord are enriched with CBR Type II (3,52,59) and despite the fact that there are marked differences in the population of the PBR (spinal cord >> CA1 > substantia nigra) (10,54,71) and specific binding of [^{3}H]PK11195 [6.1 ± 1.9 and 12.7 ± 3.3 fmol/mg, for CA1 and substantia nigra, respectively (25)].

Alternatively, it is possible that PK 11195-evoked twitches and jerks are mediated by a site that is functionally linked to a $GABA_A$ /CBR/ionophore complex. Although the PBR are not coupled to GABA receptors, there is a line of evidence suggesting a connection between the PBR and GABA-regulated anion channels: 1) neither GABA nor chloride ion affected the density and affinity of the PBR binding site, however, agonists of $GABA_A$ and $GABA_B$ altered both the CBR and PBR [for references, see (60)]. 2) Both PK 11195 and flumazenil antagonized the convulsive effect of the PBR agonist, Ro 5-4864, in rats [for references, see (60)]. 3) The electrophysiological responses of the PBR agonist, Ro 5-4864, and antagonist, PK 11195, were in opposite directions; however, some intrinsic actions of PK 11195 (proconvulsant, anxiogenic, and elevation of plasma corticosterone) were similar to those of Ro 5-4864. Because PK 11195 possessed intrinsic activity at concentrations that were higher than those that saturate the PBR binding site its action on the CBR binding site was suggested [for references, see (12)]. 4) The PBR agonist, Ro 5-4864, modulated binding of the cage convulsant [^{35}S]TBPS at the $GABA_A$/CBR/chloride ionophore, and this effect was blocked by PK 11195 but not by flumazenil (17,18). 5) Both the $\alpha_2\,\beta_1$ and $\alpha_2\,\beta_1\,\gamma_1$ receptor subtypes reconstituted a site(s) that interacted with Ro 5-4864, which suggests potential heterogeneity of a site functionally coupled to a $GABA_A$ receptor and a [^{35}S] TBPS labeled chloride ionophore (26). 6) The PBR are thought to be involved in the production of steroids, which in turn, alters $GABA_A$ [for review, see (21)]. 7) Similar regulation of the CBR and PBR by GABA agonists and by stress suggests a common endogenous ligand for both types of BZ recognition sites [for references, see (60)]. 8) Concurrent administration of PK 11195 and lorazepam prevented the development of tolerance (motor activity and anti-

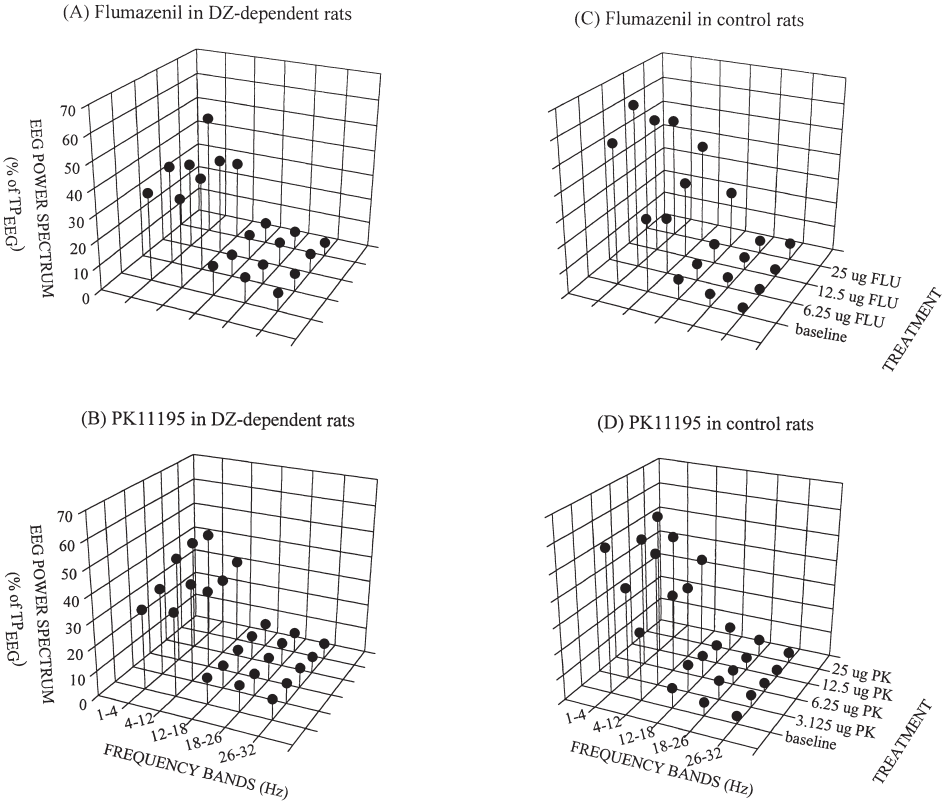

FIG. 5. Changes in the EEG power spectrum [% of total power (1–32 Hz) of the EEG (TP_{EEG})] in the substantia nigra (y-axis) as function of frequency bands (x-axis) and treatment conditions (preinjection baseline and 5 min after administration of graded doses of antagonists) (z-axis) in diazepam-dependent rats (n = 7) focally injected into the substantia nigra with flumazenil (A) and PK 11195 (B) and in empty capsule control rats (n = 4) injected with flumazenil (C) and PK 11195 (D). Data are mean of n rats.

convulsant effect), reduced downregulation of GABA-dependent chloride uptake and attenuated the discontinuation effect of lorazepam on the PTZ-induced seizure threshold in mice (6,45). Furthermore, in rats that were administered diazepam plus PK 11195, behavioral and EEG tolerance to the sedative effect of diazepam did not develop (41). These observations led to the speculation that the effect of PK 11195 in the presence of a BZ was mediated through a direct modulation of the function of a chloride channel rather than by indirect action on the PBR (45).

In diazepam-treated rats the behavioral manifestation of flumazenil- and PK 11195-induced withdrawal was mirrored by concomitant changes in the EEG (increase in the TP_{EEG}). This suggests that activation of both the CBR and PBR affects brain electrical activity. Although flumazenil-induced enhancement in the TP_{EEG} increased with increasing dose, as was the case for the PAS, the middle dose of flumazenil (12.5 μg) had less effect on the EEG than expected. Electrophysiological studies showed that flumazenil neither affected GABA sensitivity nor the firing rate of reticulata neurons in control rats, while it reduced GABA sensitivity and produced a significant overshoot in diazepam-dependent rats (78,80,81). The present data suggest that flumazenil-induced enhancement of firing rate in the substantia nigra is manifested by both the emergence of signs of abstinence and enhancement of the TP_{EEG}. In comparison to flumazenil, the PK 11195-induced withdrawal scores and alteration of the EEG were less pronounced. The effect of PK 11195 on the nigral firing rate has not been determined to the best of our knowledge. Across time, changes in the TP_{EEG} were similar in the site of injection (substantia nigra) and in other brain areas, which pointed to rapid propagation of the nigrally stimulated EEG effect. Taken together, the combination of behavioral observation with the EEG recording proved to be a useful tool for monitoring the withdrawal syndrome.

A power spectral analysis revealed that in comparison to empty capsule controls, chronic diazepam treatment resulted in a reduction of 1–4-Hz frequency band and enhancement of 4–32-Hz frequency bands in the EEG recorded from the substantia nigra. This finding confirms and extends our data in female rats chronically exposed to a high dose of diazepam (72) and is in agreement with reports that the tolerance that develops to the sedative effect of chronically administered diazepam is accompanied by concomitant changes in the EEG manifested by replacement of the low-frequency spindles with high-frequency waves (22,41,43,70). During discontinuation of the chronic BZ treatment, fast frequency waves gradually decrease toward the baseline (20,36,43,56).

Focal injections of both flumazenil and PK 11195 into the substantia nigra instantaneously reverses diazepam-induced changes in the spectral content of the EEG. Thus, it can be concluded that antagonist-induced enhancement of the TP_{EEG} reflect transient shifts of the power spectrum toward high-amplitude, low-frequency waves accompanied by statistically significant reduction of the 12–26 Hz (flumazenil) and 18–32 Hz (PK 11195) frequency bands. Because an increase of the EEG beta activity (12–30 Hz) has been commonly used as a measure of the pharmacological effect of BZs [for references, see (32)] the flumazenil- and PK 11195-induced reversal of beta waves suggests that alteration of the EEG by diazepam is mediated in the substantia nigra by activation of both the CBR and PBR. This speculation seems to be supported by the following data: 1) systemically administered flumazenil rapidly but transiently reversed the BZ-induced enhancement of the EEG beta activity, an effect thought to reflect the competitive nature of flumazenil-BZ interaction at the CBR site (5,14,31,33–35). 2) The EEG tolerance (manifested by a preponderance of the beta-like activity over the spindle burst) developed in rats chronically treated with diazepam but not in rats exposed to BZs that lack selectivities for the PBR, such as clonazepam and CL 218,872 (given in doses 100 and 50 times higher, respectively, than the minimal effective doses for inducing EEG changes) (70). 3) Coadministration of diazepam and PK11195 prevented development of EEG tolerance to the sedative effect of diazepam, while the concomitment administration of clonazepam and the PBR agonist, Ro 5-4864, induced signs of EEG tolerance in rat (41). 4) The behavioral manifestation of Ro 5-4864-induced convulsions was accompanied by dose-dependent changes in the EEG while administration of PK 11195 antagonized the effect of Ro 5-4864 on the EEG (41).

The question remains as to whether the flumazenil- and PK 11195-induced shift in the spectral content of the EEG is related to the common abstinence sign, twitches and jerks. It has been reported that the anticonvulsant activity of diazepam is related to the termination of a spike–wave complex (7), and that flumazenil rapidly but transiently antagonizes diazepam-induced suppression of the spontaneous EEG discharges in rat (37). In general, in the present study, twitches and jerks were preceded or accompanied by a train of sharp spikes and/or slow waves on the EEG tracing; however, in some cases twitches and jerks could not be identified by visual inspection of the EEG records, and occasional spikes and trains of slow waves were not associated with episodes of twitches and jerks.

In control rats, the highest dose of flumazenil (25 μg) had intrinsic activity with regard to the EEG that was like the acute effect of diazepam (shift toward beta activity). The present observation is in line with controversial reports on the effects of acutely administered flumazenil (IV) on the EEG which range from no intrinsic efficacy to an effect similar to that of diazepam (5,13,33,61). The mechanism of this effect is unclear, because in the concentration range of saturation of BZ binding sites, flumazenil did not produce changes in the EEG in the rat (32). Further, chronic treatment with flumazenil was found to reduce the EEG amplitude in cortex and hippocamus in the rats, and this effect was reversed by GABA, pentobarbitone, and picrotoxin, agents know to act at different sites on the GABA/BZ/ionophore complex, but not by BZ agonist, diazepam (1). In control rats, focally injected PK 11195 did not affect the EEG, which is in agreement with a lack of the EEG changes after systemic injection of PK 11195 (0.5–10 mg/kg, IV) in rats (41).

Taken together, the present data show that in the substantia nigra flumazenil and PK 11195 antagonize the effect of diazepam as indicated by dose-related behavioral and EEG effects. This suggests that both the CBR and PBR are involved, or that the action of PK 11195 may be mediated at a site functionally linked to a $GABA_A$ /CBR/ionophore complex.

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