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Pharmacology

Diazepam dependence prevented by glutamate antagonists

(benzodiazepine/tolerance/withdrawal)

KARIN G. STEPPUHN AND LECHOSLAW TURSKI

Research Laboratories of Schering AG, Müllerstrasse 78, D-13342 Berlin, Germany

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ABSTRACT **Long-term treatment leads to tolerance to and dependence on benzodiazepines. Abrupt termination of benzodiazepine administration triggers the expression of signs of dependence. Mice withdrawn from chronic treatment with diazepam showed a time-related evolution of anxiety, muscle rigidity, and seizures between days 4 and 21 after treatment discontinuation. A period between withdrawal days 1 and 3 was symptom-free. Surprisingly, during this "silent phase" the susceptibility of mice to α-amino-3-hydroxy-5-*tert*-butyl-4-isoxazolepropionate (ATPA) and kainate seizures and the magnitude of monosynaptic reflexes mediated by non-*N*-methyl-D-aspartate (NMDA) mechanisms were enhanced. In apparent contrast, the "active phase", between withdrawal days 4 and 21, was characterized by increased susceptibility to NMDA seizures and enhanced magnitude of polysynaptic reflexes, which are NMDA dependent. Treatment of mice with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) antagonists 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466) or 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline but not with the NMDA antagonist 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate (CPP) during the silent phase prevented signs of dependence. In contrast, treatment with CPP but not with GYKI 52466 during the active phase prevented the symptoms. The development of tolerance to and dependence on diazepam was prevented by concurrent treatment of mice with CPP but was not prevented by GYKI 52466. These data indicate that NMDA-dependent mechanisms contribute to the development of tolerance to diazepam and to the expression of signs of dependence in mice after termination of long-term treatment with diazepam. Nevertheless, the non-NMDA-mediated silent phase is essential for triggering the symptoms. Therefore, AMPA antagonists may offer a therapeutic approach for preventing dependence on benzodiazepines that is an alternative to NMDA antagonism.**

The benzodiazepines (BDZs) are the most commonly prescribed psychoactive drugs for therapy of disorders such as anxiety and sleep disturbances (1). The most important disadvantage of the treatment with BDZs lies not in their side-effect profiles but in the development of dependence in many patients (2). Clinical experience documents that anxiety, muscle spasms, and seizures belong to the major signs of dependence seen after the discontinuation of treatment with BDZs or other sedative drugs in humans (2). Several studies have examined which mechanisms underlie dependence (3, 4) and how best to terminate medication with sedative drugs (2). Nonetheless, there are no generally accepted guidelines for the treatment or prevention of dependence on sedative drugs.

Several reasons may be responsible for this status. (*i*) Dependence on BDZs seen after chronic treatment, although described as early as 1961 (5), was neglected by clinicians for at least 10–15 yr after the introduction of BDZs into psychiatric therapy (2, 6). (*ii*) Most attempts to understand BDZ dependence were directed to the function of the γ-aminobutyrate/BDZ/Cl^--ionophore receptor–effector complexes (7). (*ii*) Dependence was broadly understood as a time-related but homogeneous clinical and pathophysiological entity dependent on the length of the chronic treatment and pharmacokinetic properties of abused drugs (3, 4). (*iv*) The scientific armamentarium lacked sufficiently sensitive methods necessary for the objective description of the signs of dependence in experimental animals (8).

For these reasons we have developed methods for electroencephalographic (EEG) monitoring of seizures, for electromyographic (EMG) monitoring of muscle tone, and for detecting anxiety-like behavioral changes after withdrawal following long-term treatment with sedative drugs in mice. Using these experimental approaches, we show the dynamics and heterogeneity of diazepam dependence. We also show involvement of excitatory amino acid-mediated processes in the development of tolerance to diazepam and in the expression of the signs of dependence after withdrawal, and finally we propose a therapeutic strategy for preventing dependence.

MATERIALS AND METHODS

Electroencephalography. Male NMRI mice (Schering AG), 20–24 g in weight, were injected s.c. once a day for 12 days with diazepam at 15 mg/kg (Hoffmann–La Roche) or vehicle (sesame oil). Diazepam was dissolved in sesame oil and administered in a volume of 0.04 ml per 10 g of body weight. For long-term EEG monitoring, mice were stereotaxically implanted with bipolar twisted electrodes positioned in the dorsal hippocampus (anterior-posterior 2.5; lateral 2.0; ventral 3.5) (9) under sodium pentobarbital (50 mg/kg i.p.) anesthesia. Surface recordings were led from screws positioned bilaterally over the occipital cortex. EEG monitoring started on the day after the last administration of diazepam or vehicle [withdrawal day (WD) 1] at 9:00 a.m., was continued for up to 21 days, and was discontinued for periods of 10–15 min/day (between 8:30 and 9:00 a.m.) because of the daily care. Video- and EEG signals were stored on computer discs and magnetic tape. Seizure recognition was done on-line using a detection program (Monitor 5.0; Stellate, Westmount, Quebec) (10). Computer-identified seizure patterns were reanalyzed off-line by an observer unaware of drug treatment, and artifacts were discarded.

Electromyography. The spontaneous activity in the EMG was recorded from the gastrocnemius muscle of the mice,

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Abbreviations: ATPA, α-amino-3-hydroxy-5-*tert*-butyl-4-isoxazolepropionate; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine; BDZ, benzodiazepine; EEG, electroencephalogram/electroencephalographic; EMG, electromyogram/electromyographic; WD, withdrawal day(s); β-CCE, ethyl β-carboline-3-carboxylate; KA, kainate; CPP, 3-[(±)-2-carboxypiperazin-4-yl]-propyl-1-phosphonate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*f*)quinoxaline.

using pairs of Teflon-insulated stainless-steel wire electrodes inserted percutaneously into the muscle (11). The electrical signals were amplified, band-pass filtered (5–10 kHz), full-wave rectified, and stored on discs and paper. The EMG was recorded continuously, and the average integrated activity was determined over 1 hr. Recordings were made between 9:00 and 11:00 a.m.

Locomotor Activity and Movement Tracking. A computerized Digiscan-16 animal activity monitoring system (Omnitech, Columbus, OH) was used to quantify and track locomotor activity in mice. The total number of interruptions of the horizontal sensors was taken as a measure of horizontal activity (11). Furthermore, time spent by mice moving in a close proximity to walls (<1 cm) or in the box center (>1 cm) was recorded. The locomotor activity monitoring and movement tracking was done in independent groups of nonhabituated mice for 10 min between 9:00 and 11:00 a.m. on each experimental day. Mice showing a pattern of activity limited to movements along cage walls or in one cage compartment (i.e., avoiding the center of the cage) were termed "anxious." Therefore, the center time/margin time ratio was used as a measure of anxiety. A similar pattern of locomotor activity was induced in mice by drugs such as picrotoxin, pentylenetetrazol, and ethyl β-carboline-3-carboxylate (β-CCE) (12), reported to produce anxiety-like effects in other models.

Threshold for Clonic Seizures Induced by Excitatory Amino Acids. Diazepam withdrawn or vehicle-treated mice received intracerebroventricular infusions of α-amino-3-hydroxy-5-*tert*-butyl-4-isoxazolepropionate [ATPA–α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) agonist; 1 nmol per 5 μl], kainate (KA; 1 nmol per 5 μl) or *N*-methyl-D-aspartate (NMDA; 1 nmol per 5 μl) at 5 μl/min by means of Harvard pumps until clonic seizures were triggered. Freely moving mice were individually observed in Perspex bell-shaped cages (diameter, 80 cm) until convulsion was triggered or for 180 s. The time in s between beginning the infusion and induction of a clonic seizure was used as an endpoint for determining the susceptibility of mice to convulsions (13); the threshold was calculated in nmol.

Spinal Reflexes. The spinal reflexes of male NMRI mice, 35-40 g in weight, were recorded under α-chloralose (Merck; 80 mg/kg i.p.)/urethane (Sigma; 400 mg/kg i.p.) anesthesia. To record muscle- (M-) wave and Hoffmann- (H-) reflex the tibial nerve was stimulated by single square wave shocks of 0.2-ms duration until the respective maximal response (M_{max} or H_{max}) was reached. EMG was recorded with a pair of skin-clip surface electrodes from the plantar foot muscle. For every measurement 20 consecutive EMG responses were averaged; the magnitude of EMG responses was evaluated by measuring the peak-to-peak amplitude (11). For recording flexor reflexes and determining the reflex stimulation–response relationship the tibial nerve was stimulated electrically (5 square-wave shocks at 500 Hz, 0.2-ms duration) at 1.5, 1.8, 2.0, 2.5, and 3.0 times the nerve threshold (T_n). EMG was recorded with a pair of wire electrodes inserted percutaneously into the ipsilateral tibial muscle. For every stimulation level 20 consecutive EMG responses were averaged; the magnitude of flexor reflexes was evaluated by measuring the area bounded by the averaged response and the baseline (11).

Glutamate Antagonists and Tolerance to Diazepam. For concurrent administration of glutamate antagonists and diazepam, mice were implanted i.p. with osmotic minipumps (Alza type 2002, one pump per mouse) 2 days before the first administration of diazepam s.c. This procedure minimized the possible effects of anesthesia and surgery on the development of tolerance to diazepam and allowed constant plasma concentrations of the glutamate antagonists to be reached. 3-[(±)-2-Carboxypiperazin-4-yl]-propyl-1-phosphonate (CPP; Tocris, Buckhurst Hill, U.K.) and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (GYKI 52466; Drug Research Institute, Budapest) were infused at 1 mg/kg per hr for 14 days. The exploratory activity was monitored daily for 10 min 2 hr after each administration of diazepam at 15 mg/kg or vehicle s.c. using the Digiscan-16 animal activity monitoring system. The total number of interruptions of the horizontal sensors was used as a measure of exploratory activity.

Glutamate Antagonists and Dependence on Diazepam. Mice were implanted i.p. with osmotic minipumps type 1003D (four pumps per mouse) on the first WD or with osmotic pumps type 2002 (one pump per mouse) on the third WD under light ether anesthesia. CPP and GYKI 52466 were infused at 1 mg/kg per hr for 72 hr beginning on WD 1, or for 14 days beginning on WD 3. Furthermore, a competitive AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX; Novo Nordisk, Copenhagen) was infused at 1 mg/kg per hr for 72 hr beginning on WD 1. NBQX has not been used for long-term administration by means of osmotic minipumps because of insufficient solubility. Monitoring of EEG, EMG, and locomotor activity started on WD 2, 24 hr after the beginning of drug or vehicle infusion in the experiment with minipumps type 1003D and was discontinued during WD 3 in the experiment with minipumps type 2002.

Treatment Regimen with Glutamate Antagonists. The treatment regimen with the glutamate antagonists GYKI 52466, NBQX, and CPP was chosen to give an approximately equivalent effect on the threshold for seizures induced by the respective agonists (Table 1). Furthermore, the selected doses of the glutamate antagonists were devoid of sedation (Fig. 1) and did not disturb performance of mice on the rota-rod (Table 1). To ensure that the antagonists were present in relevant concentrations in the brain to interact with respective receptors and to determine whether tolerance to the glutamate antagonists develops on long-term treatment,

Table 1. Threshold for clonic seizures induced by excitatory amino acids and performance on the rota-rod in mice continuously administered i.p. with glutamate antagonists or vehicle by means of osmotic minipumps

Treatment	Day	Seizure threshold, mean nmol ± SEM (% control; *n*) ATPA	KA	NMDA	*F/R*
Vehicle	3	0.71 ± 0.04 (100; 5)	0.72 ± 0.04 (100; 6)	0.68 ± 0.03 (100; 6)	0/17
GYKI 52466	3	1.62 ± 0.12** (228; 6)	0.93 ± 0.07 (129; 5)	0.73 ± 0.04 (107; 6)	0/17
NBQX	3	1.48 ± 0.14* (205; 6)	1.28 ± 0.13* (180; 6)	0.75 ± 0.02 (110; 6)	0/18
CPP	3	0.73 ± 0.04 (103; 6)	0.75 ± 0.02 (104; 6)	1.41 ± 0.11** (207; 6)	0/18
Vehicle	14	0.73 ± 0.03 (100; 6)	0.72 ± 0.03 (100; 6)	0.70 ± 0.04 (100; 6)	0/18
GYKI 52466	14	1.51 ± 0.09** (207; 5)	0.96 ± 0.09 (133; 6)	0.67 ± 0.05 (96; 6)	0/17
CPP	14	0.81 ± 0.02 (111; 6)	0.93 ± 0.05 (129; 5)	1.32 ± 0.07** (189; 6)	0/17

CPP and GYKI 52466 were administered at 1 mg/kg per hr for 3 or 14 days, whereas NBQX was administered at 1 mg/kg per hr for 3 days. ATPA, KA, and NMDA were infused into the brain lateral ventricle at 1 nmol per 5 μl with a rate of 5 μl/min. The threshold for seizures was determined in nmol ± SEM. n, Number of animals. *F/R*, Number of animals falling/remaining from/on the rota-rod. *, $P < 0.05$; **, $P < 0.01$ vs. respective vehicle-treated controls (Student's *t* test).

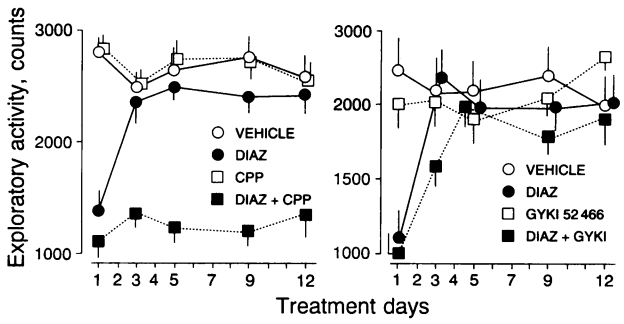

FIG. 1. Time course of changes in exploratory activity of mice during chronic treatment with diazepam and effect of concurrent treatment with glutamate antagonists CPP and GYKI 52466 on development of tolerance to the sedative action of diazepam (DIAZ). Circles represent mean ± SEM number of counts recorded in groups of eight mice treated with vehicle (○) or diazepam at 15 mg/kg (●). Squares represent mean ± SEM number of counts recorded in groups of eight mice treated with vehicle (□) or diazepam at 15 mg/kg (■) and concurrently treated with continuous i.p. infusion of CPP at 1 mg/kg per hr (*Left*) or GYKI 52466, 1 mg/kg per hr (*Right*) by means of osmotic minipumps. Exploratory activity was monitored in nonhabituated mice 2 hr after s.c. administration of vehicle or diazepam for 10 min. ANOVA showed that the effect of treatment was significant [$F(1,70) = 9.95$, $P < 0.05$], revealing that CPP prevented evolution of tolerance to diazepam. GYKI 52466 had no effect on the development of tolerance to diazepam [$F(1,70) = 1.03$, $P > 0.05$].

the threshold for clonic seizures induced by excitatory amino acids given into the lateral brain ventricle was determined in mice administered with glutamate antagonists or vehicle by osmotic minipumps i.p. and sesame oil s.c. 2 hr after the last administration of sesame oil on the third and 14th day of treatment. Furthermore, to ensure that the glutamate antagonists did not produce neurotoxicity on long-term treatment, the motor coordination was tested immediately before convulsive tests by placing mice for 2 min on a horizontal rod (diameter, 3 cm) rotating at a rate of six rotations per min, 17 cm above the bench. Those mice that fell three times during a 2-min period were considered ataxic.

Statistics. The experimental data were analyzed statistically by means of either analysis of variance (ANOVA), analysis of covariance, Mann–Whitney *U* test or Student's *t* test.

RESULTS

Tolerance to Diazepam and Effects of Glutamate Antagonists. Long-term treatment with diazepam led to a rapid loss of its depressant action on exploratory activity in nonhabituated mice measured as the number of interruptions of horizontal sensors (Fig. 1). Concurrent treatment of mice with diazepam and the NMDA-antagonist CPP, but not with the AMPA-antagonist GYKI 52466, prevented the development of tolerance (Fig. 1). Long-term treatment with CCP or GYKI 52466 at 1 mg/kg per hr did not affect the exploratory activity of mice (Fig. 1) or their performance on the rota-rod (Table 1). GYKI 52466 elevated the threshold for ATPA seizures by ≈100% and had apparently little effect on the threshold for KA and NMDA seizures (Table 1). CPP elevated the threshold for NMDA by ≈100% and did not affect thresholds for ATPA and KA seizures (Table 1). After long-term treatment with either GYKI 52466 or CPP, no tolerance to the anticonvulsant effects or dependence was seen (Table 1, Fig. 2).

Dependence on Diazepam. Mice withdrawn from diazepam showed electrographic seizures, increase in EMG activity, and a pattern of exploratory activity reminiscent of anxiety (Fig. 2). No seizures were detected in EEG, no changes in

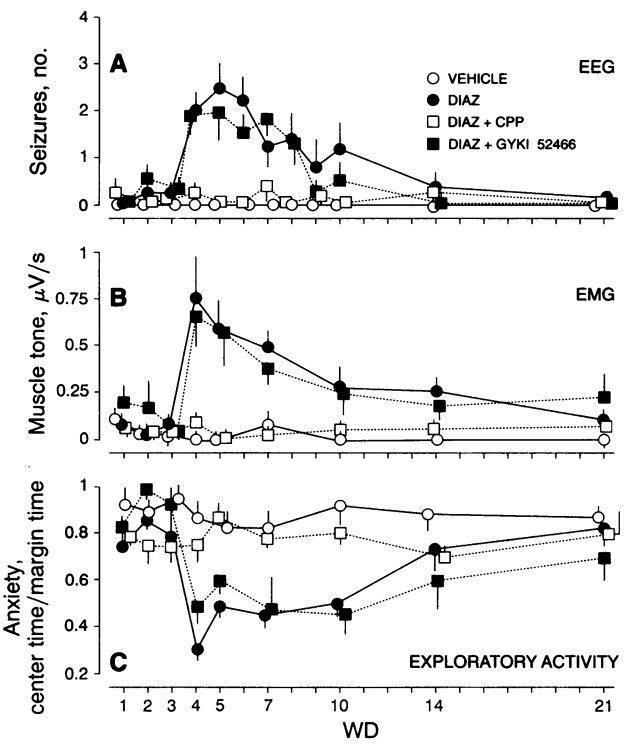

FIG. 2. Time course of seizures (*A*), changes in muscle tone (*B*), and anxiety (*C*) in mice subjected to chronic treatment with diazepam (DIAZ), diazepam and CPP, CPP, diazepam and GYKI 52466, GYKI 52466, or vehicle on withdrawal. Circles and squares represent mean ± SEM number of seizures per animal recorded in groups of eight mice (*A*), mean ± SEM magnitude of EMG activity in μV/s recorded in groups of five mice (*B*), mean ± SEM magnitude of anxiety expressed as center time/margin time ratio (*C*) from groups of eight mice withdrawn from vehicle (○), diazepam (●), diazepam and CPP (□), or diazepam and GYKI 52466 (■). Mice withdrawn from chronic treatment with either CPP or GYKI 52466 did not differ from those withdrawn from vehicle (data not shown). ANOVA on EEG, EMG, and exploratory activity for diazepam and CPP showed that the effects of treatment were significant [$F_{EEG}(1,84) = 28.55$, $P < 0.005$; $F_{EMG}(1,48) = 12.62$, $P < 0.005$; $F_{EXP}(1,84) = 13.71$, $P < 0.005$], revealing that chronic treatment with diazepam resulted in seizures, muscle rigidity, and anxiety on withdrawal, whereas concurrent treatment with diazepam and CPP did not cause signs of dependence in mice. ANOVA on EEG, EMG, and exploratory activity for diazepam and GYKI 52466 showed that the effects of treatment were not significant [$F_{EEG}(1,84) = 1.74$, $P > 0.05$; $F_{EMG}(1,48) = 0.34$, $P > 0.05$; $F_{EXP}(1,84) = 1.91$, $P > 0.05$], revealing that concurrent treatment with diazepam and GYKI 52466 did not affect evolution of seizures, muscle rigidity, and anxiety on withdrawal from diazepam.

muscle tone were registered in EMG, and no change in the pattern of exploratory activity, measured as a center time/margin time ratio, was observed in animals withdrawn from long-term treatment with vehicle (Fig. 2).

Seizures. The electrographic seizures were most frequent between WD 4 and 10 (Fig. 2*A*). No or few seizures were detected during the first 3 and after 21 WD. The duration of seizures rarely exceeded 60 s. Most seizures were first seen in the hippocampus and then spread rapidly to cortical recordings. In terms of behavior, the seizures were characterized by initial akinesia, automatisms, forelimb clonus, rearing, and falling. No seizures were detected during the entire observation period (up to 21 days) after cessation of the treatment of mice with vehicle (Fig. 2*A*).

EMG Activity. EMG monitoring showed increased activity in the gastrocnemius muscle in mice withdrawn from diazepam (Fig. 2*B*). EMG activity was maximal between WD 4 and 14. Little or no muscle rigidity was registered on WD 1–3 and 21. Abrupt termination of the treatment of mice with vehicle

did not cause changes in the muscle tone during the entire monitoring period (Fig. 2*B*).

Anxiety. In mice withdrawn from diazepam, anxiety was maximal between WD 4 and 10 (Fig. 2*C*). Little anxiety was recorded during WD 1–3 and on WD 14 and 21 (Fig. 2*C*). A decrease in the center/margin time ratio, indicating that the animals spent more time moving at the cage walls, reflected the changes in locomotor activity patterns in mice withdrawn from diazepam (Fig. 2*C*). No difference between diazepam- and vehicle-withdrawn mice was detected over the entire observation period in exploratory activity measured as horizontal activity (counts) or total distance (cm). No change in the center/margin time ratio was detected in mice withdrawn from vehicle (Fig. 2*C*).

Seizure Threshold. The threshold for seizures induced by ATPA and KA in diazepam-withdrawn mice was lower at days 2 and 3 but did not differ from vehicle-treated animals between WD 4 and 21 (Fig. 3 *A* and *B*). At times of increased susceptibility of mice to ATPA and KA the threshold for NMDA seizures was unchanged. However, at WD 4–7 the threshold for NMDA seizures decreased and then slowly normalized up to day 21 (Fig. 3*C*). Abrupt cessation of the treatment with vehicle did not cause changes in susceptibility to seizures induced by excitatory amino acids during the entire period (Fig. 3 *A–C*).

Spinal Reflexes in Diazepam-Dependent Mice. Recording of spinal reflexes showed that H-reflex was increased during the first 3 WD, being maximal at WD 3 (Fig. 4*A*). Magnitude of the flexor reflexes was higher between WD 4 and 10 (Fig. 4*B*). No difference between flexor reflexes in vehicle- and diazepam-withdrawn mice was detected on WD 1–3 and 21 (Fig. 4*B*).

Effect of Glutamate Antagonists on Diazepam Dependence. Treatment with GYKI 52466 or NBQX during the first 3 days after termination of diazepam administration (silent phase) almost totally prevented electrographic seizures and increase in the EMG activity and reduced anxiety (Fig. 5*A*). Similarly to GYKI 52466, on long-term treatment, NBQX elevated the threshold for ATPA seizures by ≈100%, had no effect on the

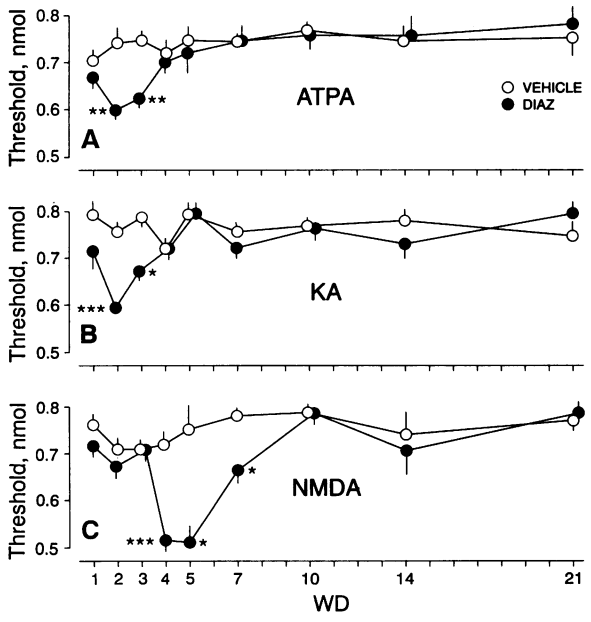

FIG. 3. Evolution of changes in the threshold for seizures induced by agonists activating different subtypes of glutamate receptors in diazepam and vehicle-withdrawn mice. Threshold for clonic seizures was calculated in nmol ± SEM. Experimental groups in convulsive experiments consisted of 7–16 mice. ANOVA showed that the effects of treatment were significant for ATPA and KA during the first 3 days of withdrawal [$F_{ATPA}(1,90) = 4.57$, $P < 0.05$; $F_{KA}(1,90) = 4.18$, $P < 0.05$], revealing that chronic treatment with diazepam lowered the threshold for seizures induced by non-NMDA agonists on withdrawal. The threshold for seizures induced by NMDA was lower between WD 4 and 21 [$F_{NMDA}(1,180) = 3.98$, $P < 0.05$]. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle-treated mice, ANOVA.

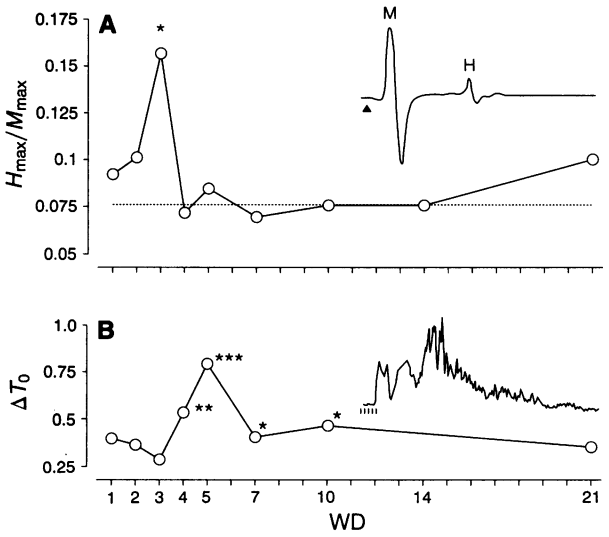

FIG. 4. Changes in Hoffmann- (H-) (*A Inset*) and flexor (*B Inset*) reflexes in diazepam- or vehicle-withdrawn mice. (*A*) Magnitude of H-reflexes is expressed as a ratio between H_{max} and M_{max} (median values). *, $P < 0.05$ vs. vehicle-treated mice (Mann–Whitney U test). (*B*) Differences between flexor reflexes are shown as a shift of the reflex stimulation–response curve established on different WD and expressed as nerve-threshold difference (ΔT_0) between vehicle-treated and diazepam-withdrawn mice. Withdrawal produced a parallel shift of the reflex stimulation–response curve to the left [$F(8,176) = 3.36$, $P < 0.005$]. The reflex magnitude in diazepam-withdrawn mice was significantly increased vs. vehicle on days 4 [$F(1,8) = 7.65$, $P < 0.01$], 5 [$F(1,8) = 13.46$, $P < 0.0005$], 7 [$F(1,8) = 4.83$, $P < 0.05$], and 10 [$F(1,8) = 5.89$, $P < 0.05$]. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle-treated mice, analysis of covariance.

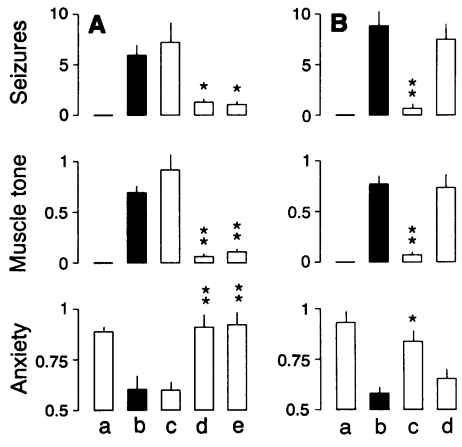

FIG. 5. Effect of CPP, GYKI 52466, and NBQX on seizures, changes in the muscle tone, and anxiety in diazepam-withdrawn mice during the silent (*A*) and active (*B*) phase. Bars represent mean ± SEM number of seizures per animal, mean ± SEM EMG activity in μV/s, and mean ± SEM anxiety measured as a center time/margin time ratio in exploratory activity experiments. Groups of eight mice were implanted i.p. with osmotic minipumps type 1003D (*A*) on the first WD or type 2002 (*B*) on the third WD. CPP, GYKI 52466, and NBQX were infused at 1 mg/kg per hr for 72 hr in *A*, whereas CPP and GYKI 52466 were infused for 14 days in *B*. Bars: a, vehicle; b, diazepam; c, diazepam + CPP; d, diazepam + GYKI; e, diazepam + NBQX. *, $P < 0.05$; **, $P < 0.01$ vs. diazepam-treated mice, ANOVA.

threshold for NMDA seizures, and did not affect the performance of mice on the rota-rod. In contrast to GYKI 52466, NBQX also elevated the threshold for KA seizures by 80% (Table 1). In contrast, 3-day infusion of CPP did not affect diazepam dependence (Fig. 5*A*). When treatment with GYKI 52466 was initiated on the third WD (active phase) and was continued for the next 14 days, dependence on diazepam was unaffected (Fig. 5*B*). Interestingly, CPP attenuated expression of the signs of dependence when treatment was initiated on WD 3 (Fig. 5*B*). Treatment of mice with vehicle during the first 3 days after discontinuation of diazepam administration or between WD 3 and 17 did not affect intensity of dependence signs, as monitored in EEG and EMG and seen in locomotor activity patterns (Fig. 5).

DISCUSSION

Long-term electrophysiological monitoring shows that termination of chronic treatment of mice with diazepam leads to signs of dependence—such as electrographic seizures, changes in muscle tone, and anxiety. Two major phases can be recognized in the occurrence of signs of dependence during diazepam withdrawal in mice. The initial phase lasts for ≈3 days and is symptom-poor (silent phase); the second phase is symptom-rich (active phase) and is characterized by distinct dynamics. The onset of the signs of dependence is rapid, the symptoms being most pronounced during the next 3–7 days and abating slowly up to WD 21. Seizure threshold experiments with glutamate agonists and spinal reflex pharmacology suggest that the initial phase after diazepam withdrawal is mediated by non-NMDA mechanisms. This phase is symptom-poor but is critically important for triggering signs of dependence. Blockade of AMPA-mediated excitation by the antagonists GYKI 52466 (14) and NBQX (11) during the initial silent phase is sufficient for the prevention or reduction of the signs of dependence. The NMDA-antagonist CPP is inactive. These results suggest that the activation of non-NMDA receptors is essential for induction of a withdrawal syndrome. The second phase after diazepam withdrawal is most probably mediated by glutamate acting via NMDA receptors. Blockade of NMDA receptors with CPP during the active phase eliminates signs of dependence. GYKI 52466 is inactive against dependence signs when administered during this phase. Interestingly, the development of tolerance to diazepam in mice can be prevented by concurrent administration of the NMDA-antagonist CPP but not of the AMPA-antagonist GYKI 52466. Furthermore, electrophysiological monitoring shows that chronic treatment of mice with diazepam and CPP (but not with GYKI 52466) does not lead to signs of dependence on withdrawal. The silent phase after diazepam withdrawal might be attributed to the slow elimination kinetics of the compound. There is reason to believe, however, that differentiation of diazepam dependence into silent and active phases is not dependent on the time course of the disappearance of diazepam or its metabolites from the brain after discontinuation of the chronic treatment. Analysis of the plasma concentrations of diazepam, *N*-methyldiazepam, and 3-hydroxydiazepam in mice withdrawn from chronic treatment with 15 mg/kg for 12 days showed that diazepam and its metabolites are hardly detectable as soon as after 24 hr (WD 1) and are not detectable at 48 hr (WD 2) (12). Furthermore, little oxazepam can be detected in plasma after 24 hr (12). Perhaps long-term treatment with diazepam leads to a compensatory enhancement of glutamate activity that counteracts the action of the BDZ on γ-aminobutyrate-produced inhibition, thus resulting in a new balance between excitatory and inhibitory neurotransmission. If this mechanism underlies tolerance to BDZs (and our own experiments suggest that NMDA-mediated mechanisms may be involved), then it may well be that rapid discontinuation of diazepam treatment leads to relative overactivity of excitatory neurotransmission (mediated by glutamate) versus inhibitory neurotransmission (mediated by γ-aminobutyrate). One consequence of such rapid changes in the balance between inhibitory and excitatory neurotransmission might be activation of glutamate receptors, leading to such consequences as seizures, increases in muscle tone, and anxiety. Although at the present time, very little evidence can be offered in support of such a hypothesis, there is evidence that glutamate may be involved in mediating dependence on sedative drugs. The NMDA antagonists MK-801 and ketamine have been reported to block naloxone-precipitated withdrawal signs in morphine-dependent rodents (15). The persistence of undefined adaptive processes initiated to counter the effects of abused drugs has also been suggested to be involved in dependence on opiates, psychostimulants, and ethanol (16, 17). Such considerations suggest that the signs of diazepam dependence seen during the active phase after withdrawal are mediated by NMDA.

The relevance of our studies lies in the detection of two different phases in the occurrence of signs of dependence on withdrawal from diazepam. Whatever the ultimate explanation, our experiments document clear differences between these two phases with regard to their pathophysiology and pharmacology. If such differences can be confirmed in clinical practice, pharmacological manipulation during the early period after withdrawal may be critical for the following therapeutic result. Our data suggest that the AMPA antagonists are of value for preventing the signs of dependence during the initial phase after withdrawal. NMDA antagonists may be indicated in the course of chronic treatment with BDZs or during the active phase after withdrawal. These principles may lead to additional therapeutic approaches to the preventive treatment of BDZ tolerance and dependence. Should our observations also be valid for other withdrawal syndromes seen after abrupt discontinuation of treatment with sedative drugs such as barbiturates, opiates, and ethanol, which are clinically very similar (1, 2), then a therapeutic strategy, based on AMPA antagonism, for the preventive therapy of dependence could be offered.

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1. Roy-Byrne, P. P. & Hommer, D. (1988) *Am. J. Med.* **84,** 1041–1052.
2. Duncan, J. (1988) *Human Psychopharmacol.* **3,** 171–180.
3. File, S. E. (1990) *Neurosci. Biobehav. Rev.* **14,** 135–146.
4. Woods, J. H., Katz, J. L. & Winger, G. (1987) *Pharmacol. Rev.* **39,** 251–413.
5. Hollister, L. E., Motzenbecker, F. P. & Degan, R. O. (1961) *Psychopharmacology* **2,** 63–68.
6. Sellers, E. M. (1988) *Can. Med. Assoc. J.* **139,** 113–120.
7. Lukas, S. E. & Griffiths, R. R. (1982) *Science* **217,** 1161–1163.
8. Brady, J. V. (1991) *Neurosci. Biobehav. Rev.* **15,** 35–43.
9. Montemurro, D. G. & Dukelow, R. H. (1972) *A Stereotaxic Atlas of the Diencephalon and Related Structures of the Mouse* (Futura, Mount Kisco, NY).
10. Gotman, J. (1985) in *Long-Term Monitoring in Epilepsy*, eds. Gotman, J., Ives, J. R. & Gloor, P. (Elsevier, Amsterdam), pp. 133–145.
11. Turski, L., Jacobsen, P., Honoré, T. & Stephens, D. N. (1992) *J. Pharmacol. Exp. Ther.* **260,** 742–747.
12. Steppuhn, K. G. & Turski, L. (1991) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **344,** R68.
13. Steppuhn, K. G. & Turski, L. (1993) *J. Pharmacol. Exp. Ther.* **265,** 1063–1070.
14. Donevan, S. D. & Rogawski, M. A. (1993) *Neuron* **10,** 51–59.
15. Trujillo, K. A. & Akil, H. (1991) *Science* **251,** 85–87.
16. Collier, H. O. J. (1980) *Nature (London)* **283,** 625–629.
17. Koob, G. F. & Bloom, F. E. (1988) *Science* **242,** 715–723.