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Different kinetics of tolerance to behavioral and electroencephalographic effects of chlordiazepoxide in the rat

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Abstract

The daily oral administration of chlordiazepoxide (40 mg/kg) over 9 weeks in rats elicited full tolerance to muscle relaxant effects within 7 weeks, as revealed by twice weekly evaluations of abdominal tone myorelaxation and decreased grip strength. No full tolerance was achieved, however, during the 9 weeks of treatment in terms of ataxia. Electroencephalographic (EEG) studies showed that this tolerance to the behavioural effects was accompanied by a progressive decrease in mean power spectra, associated with a progressive decrease in the β band, but in this case, full tolerance was reached within 4 weeks. Once weekly evaluations of the ability of chlordiazepoxide to protect the animals against pentylenetetrazole seizures revealed a similar pattern. Treatment with flumazenil (50 mg/kg p.o.) 24 h after the last chlordiazepoxide administration induced a clear withdrawal syndrome associated with EEG changes which consisted of an increase in total power spectra associated with an increase in the δ band (in comparison with chlordiazepoxide-dependent rats not given the antagonist). These findings suggest that the different kinetics of the tolerance to anticonvulsant and EEG effects in comparison to myorelaxant effects can be attributed to a different involvement of benzodiazepine receptor subtypes.

Keywords: Chronic chlordiazepoxide; Behavior; EEG (electroencephalographic); Tolerance; Dependence; (Rat)

1. Introduction

The depressant effects of acutely administered benzodiazepines on the central nervous system (CNS) are well documented (Greenblatt and Shader, 1974), and there is evidence that tolerance to their sedative, anticonvulsant and anxiolytic effects may develop rapidly (File, 1985, 1990).

Tolerance to the sedative effects of chlordiazepoxide in terms of a decreased reduction in lever-pressing activity has been reported after 3–5 days of treatment in rats (Cook and Sepinwall, 1975). Decreased reduction in spontaneous locomotor activity after 14 days in mice and rats (File and Hyde, 1978; File, 1982a), motor activity, walking and righting reflexes after 9–10 days in rats (Ryan and Boisse, 1983), and a reduced barbiturate-sleeping time after 3–14 days of chlordiazepoxide treatment (Orme et al., 1972) have also been reported.

Tolerance to the anticonvulsant effects of benzodiazepine against pentylenetetrazole-induced seizures has been demonstrated after 4–20 days in mice (File, 1983; Gent et al., 1985; Scherkl et al., 1988), rats (Gonsalves and Gallager, 1986) and dogs (Frey et al., 1984). Tolerance to their anxiolytic effects has been shown within a few days, using the rat neophobia test (Cooper et al., 1981), the Vogel conflict test (Söderpalm, 1987), the mouse punished-crossing test (Stephens and Schneider, 1985), the defensive burying test (Treit, 1985), the Geller conflict test (Rock and Barret, 1987), the social interaction and elevated plus-maze test (Fritz et al., 1986; Baldwin et al., 1987; File and Baldwin, 1989), as well as in a drug discrimination test (Emmett-Oglesby et al., 1983). However, no tolerance was shown over 7 weeks when a conflict test was used (McMillan and Leander, 1978).

EEG studies have revealed that benzodiazepines elicit characteristic electrocortical patterns in laboratory animals, which consist of repeated typical 7–12 Hz spindle bursts, sometimes interrupted by short high-frequency periods (15–30 Hz) (Mele et al., 1984; Vale-

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rio and Massotti, 1988; Massotti et al., 1990). These different EEG signs are respectively associated with signs of behavioural sedation (a crouched stance, open eyes and myorelaxation) and stimulation (gnawing, running, ear twitching, and sometimes wet-dog shakes). The increase of spindle burst activity and the increase in high frequency waves can be reduced within 3 days using repeated administration of very large doses of diazepam, flunitrazepam and clonazepam; these effects are associated with a decrease in the sedative action of the drugs (Valerio and Massotti, 1988).

A hyperexcitation syndrome (twitches, tremors, tail erection and weight loss) has been reported in rats after the abrupt withdrawal of chlordiazepoxide after 5 weeks of treatment (McNicholas and Martin, 1982; Ryan and Boisse, 1983; Kunchandy and Kulkarni, 1986). Other behavioural changes indicating increased anxiety during spontaneous withdrawal have been observed in a drug discrimination test (Emmett-Oglesby et al., 1983), in the social interaction test (Baldwin et al., 1987) and in the elevated plus-maze test (File and Baldwin, 1989). After prolonged exposure to diazepam, flumazenil-precipitated withdrawal, consisting of convulsions, was shown in rats (Falk and Tang, 1987; Gallaher et al., 1988).

Surprisingly, no reports are available concerning rat EEG modifications during spontaneous or benzodiazepine-antagonist-precipitated withdrawal syndrome after prolonged benzodiazepine administration. Only Steppuhn and Turski (1993a) and Steppuhn et al. (1993b) have reported that mice previously subjected to 12 days of chronic treatment with diazepam show electrographic seizures.

Since tolerance to the various effects mentioned above develops at very different rates, the first aim of the present study was to investigate EEG modifications and the kinetics of tolerance to the myorelaxant and anticonvulsant effects of chlordiazepoxide in rats on a 9-week schedule of oral daily administration. A second aim was to investigate the physical dependence precipitated by a selective benzodiazepine receptor antagonist (flumazenil) as manifested by the behavioural withdrawal syndrome and EEG activity, in rats.

2. Materials and methods

2.1. Animals

The animals used were male Wistar albino rats (Charles River, Calco, Como, Italy), weighing 250–300 g at the beginning of treatment. They were housed singly in an air-conditioned room (22 ± 2°C) with a 12-h light/12-h dark illumination cycle and free access to food and water.

The rats were allowed to acclimatize to the environment for a period of one week prior to the surgical implantation of EEG electrodes.

Animal care was in accordance with the State regulations governing the care and treatment of laboratory animals.

2.2. Apparatus and procedure

2.2.1. EEG

2.2.1.1. Surgery.

The animals were anesthetized using tribromoethanol 200 mg/kg i.p. (Aldrich) (0.5 ml/hg), and four silver-silver chloride ball electrodes were fixed epidurally by means of acrylic dental cement (Palaferm, Kulzer & Co., Wehrheim/Ts, Germany) on the right and left of the parieto-occipital cortex. The target positions for the 4 electrodes were based on coordinates taken from the Atlas of Paxinos and Watson (1982): 2 mm anterior, 2 mm lateral and 3 mm posterior from the bregma. The four electrodes, as well as a micro-screw inserted into the nasal bones for grounding, were connected to a Cannon ITT micro-connector attached to the head of the animal by means of the same acrylic dental cement.

2.2.1.2. EEG recording.

For the EEG recordings, each rat was placed in a Plexiglas cage situated in a special sound-attenuated Faraday chamber which was lit by 40 W fluorescent bulbs located 2 m from the cage on the opposite side of the chamber.

The micro-connector on the head of the animal was then connected to a rotating connector (Air Precision, France) attached to the cage in such a way as to allow the recording of electrophysiological parameters without hampering movement. The rotating connector was connected to a B8P polygraph (Battaglia-Rangoni, Casalecchio di Reno, Italy) and an IBM PS/2 computer by means of an A/D converter (Cambridge Electronic Design). The signals were filtered using a band-pass filter set at cut-off frequencies of 0.2 and 50 Hz. The filtered signals were not only recorded on paper (low speed: 1.5 mm/s; high speed: 30.0 mm/s), but also stored in a computer in order to allow them to be digitized and processed for fast Fourier transform spectral analysis (sampling frequency of 100 Hz, ten 5-s epochs). The power spectra between 0 and 25 Hz were evaluated using a resolution of 0.2 Hz. Quantitative EEG analyses were expressed as the mean power spectra (μV^2) or as the percentage of selected bands over the total power spectra: δ (0.2–4.0 Hz); Θ (4.2–8.0 Hz); α (8.2–13.0 Hz) and β (13.2–25.0 Hz).

Seven days after surgery, the rats were allowed to acclimatize to their new environment for a period of 3

days. Before the start of treatment, they were randomly divided into two groups of seven rats each. Every Wednesday, after a 30-min baseline recording, EEG activity was recorded every 10 min for 2 h between 09.00 and 11.00 a.m. Throughout the recording sessions, the waking state of the animals was assured by means of one long, loud ring immediately before the start of each recording, and their general behaviour was continuously monitored by means of a TV camera (Coger Cctv System, Milan, Italy) aimed from above the cage.

2.2.2. Behavioural testing

2.2.2.1. Muscle relaxant evaluation.

In addition to daily body weight measurements, the degree of chronic intoxication and the time-course of chlordiazepoxide-induced tolerance was assessed by evaluating the sedative effects observed in each animal. Abdominal tone, grip strength according to Irwin (1968), and ataxia according to Majchrowicz (1975), were evaluated, 60 min after treatment, every Tuesday and Friday between 10.00 and 11.00 a.m.

In order to measure abdominal tone, the animal was restrained in a supine position and the abdomen was gently palpated with the index finger; the score was based on the relative presence of muscle resilience (resistance to compression) or flaccidity (softness with continuing cavity deformation after compression). Grip strength (grip-grid resistance) was measured by placing the animal on the grid and applying horizontal pull to its tail in order to draw it backwards (approximately 1–2 s); the score was based on the capacity of the animal to resist the pull.

For both of these parameters, a 0–4 scale was used with the scores of 0, 2 and 4 respectively representing marked, slight and normal behaviour.

The following definitions were used to describe the degree of ataxia: *neutrality* was defined as the presence of transient normal body and muscle tones, and normal reflexes; *sedation* as the presence of reduced muscle tone, general sedation, a dulled and relaxed appearance, and slow locomotor activity; *ataxia 1 (moderate)* as the lowest degree of gait impairment in addition to heavy sedation, a pronounced lack of muscle coordination and sluggish movements, although with a markedly sustained elevation of the abdomen and pelvis during forward movement despite a staggering gait; *ataxia 2 (marked)* as an accentuated staggering gait with considerable elevation of the abdomen and pelvis; and *ataxia 3 (extreme)* as a major impairment of motor coordination indicated by a broad, staggering gait, the absence of pelvic and abdominal elevation, heavily depressed reflexes, flaccid muscles, heavy general sedation and a lethargic appearance.

Once again, a 0–4 scale was used (0, 1, 2, 3 and 4 respectively representing neutrality, sedation, and moderate, marked and extreme ataxia).

2.2.2.2. Anticonvulsant activity.

The anticonvulsant activity of chronic chlordiazepoxide against acute pentylenetetrazole (50 mg/kg i.p.)-induced convulsions was assessed in another group of animals, once a week over 9 weeks, for 10 min in the morning. The animals were injected 60 min after treatment (saline or chlordiazepoxide) with pentylenetetrazole and immediately placed in 30×30 square cm Plexiglas chambers. The saline group received the same dose of pentylenetetrazole according to the same schedule but was replaced weekly by naive rats, because of pentylenetetrazole toxicity. The number of animals (%) exhibiting chronic convulsions, as well as the latency (s) to the first convulsion were scored.

2.2.3. Physical dependence

Behavioural symptoms were observed for the 60 min following the 09.00 a.m. administration of flumazenil. The following characteristic symptoms of withdrawal syndrome were evaluated according to Ryan and Boisse (1983) with some simplification: ear twitches, tremors, tail erection, vertical posture, irritability, head shakes, piloerection, myoclonic jerks, grooming and tooth chatter. A score of 1 was given for the presence of each symptom, and the total of all of the symptoms observed in each rat was recorded. No fluid or food was allowed before the measurements were completed and, after the hour of behavioural observation, EEG recordings were made every 10 min for 30 min.

2.2.4. Drugs

The first group (divided into 2 subgroups of 7 animals each) received daily chlordiazepoxide HCl 40 mg/kg p.o. (Sigma), dissolved in saline, due to its relative solubility in water, in a volume of 0.5 ml/hg, for 9 weeks: the first subgroup was tested for EEG and muscle relaxant effects; the second, not submitted to surgery, for anticonvulsant activity. The second (control) group (divided in 2 subgroups of 7 animals each) received the same volume of saline alone for the same period and was tested as described for the chlordiazepoxide subgroups. Pentylenetetrazole 50 mg/kg (Sigma), dissolved in saline was administered once a week 60 min after chlordiazepoxide. Flumazenil (Roche), dissolved in water to which two drops of Tween 80 had been added, was orally administered 24 h after the last chlordiazepoxide treatment at a dose of 50 mg/kg. All the drug solutions were freshly prepared prior to use.

2.2.5. Statistical analysis

The results for all the normally distributed data were expressed as means ± S.E.M. and analyzed by

means of one-factor analysis of variance (ANOVA) for repeated measurements, followed by Tukey's test where appropriate. Behavioural and withdrawal data were analyzed using the non-parametric analysis of variance (Kruskal-Wallis test) using an χ^2 parameter, followed by post-hoc comparisons where appropriate. For abdominal tone and grip strength, the mean scores of the different groups were compared using periods of 3 weeks each. For anticonvulsant responses, the significance of the differences in percentages between the treated and control group was analyzed every week using the χ^2 test.

Although seven animals were initially included in each group, some lost cortical EEG electrodes or presented with some artifacts during the course of the study; these animals were excluded from the statistical evaluation.

3. Results

3.1. Behavioural testing

Throughout the chronic treatment period, all the animals showed a consistent weight gain with no signif-

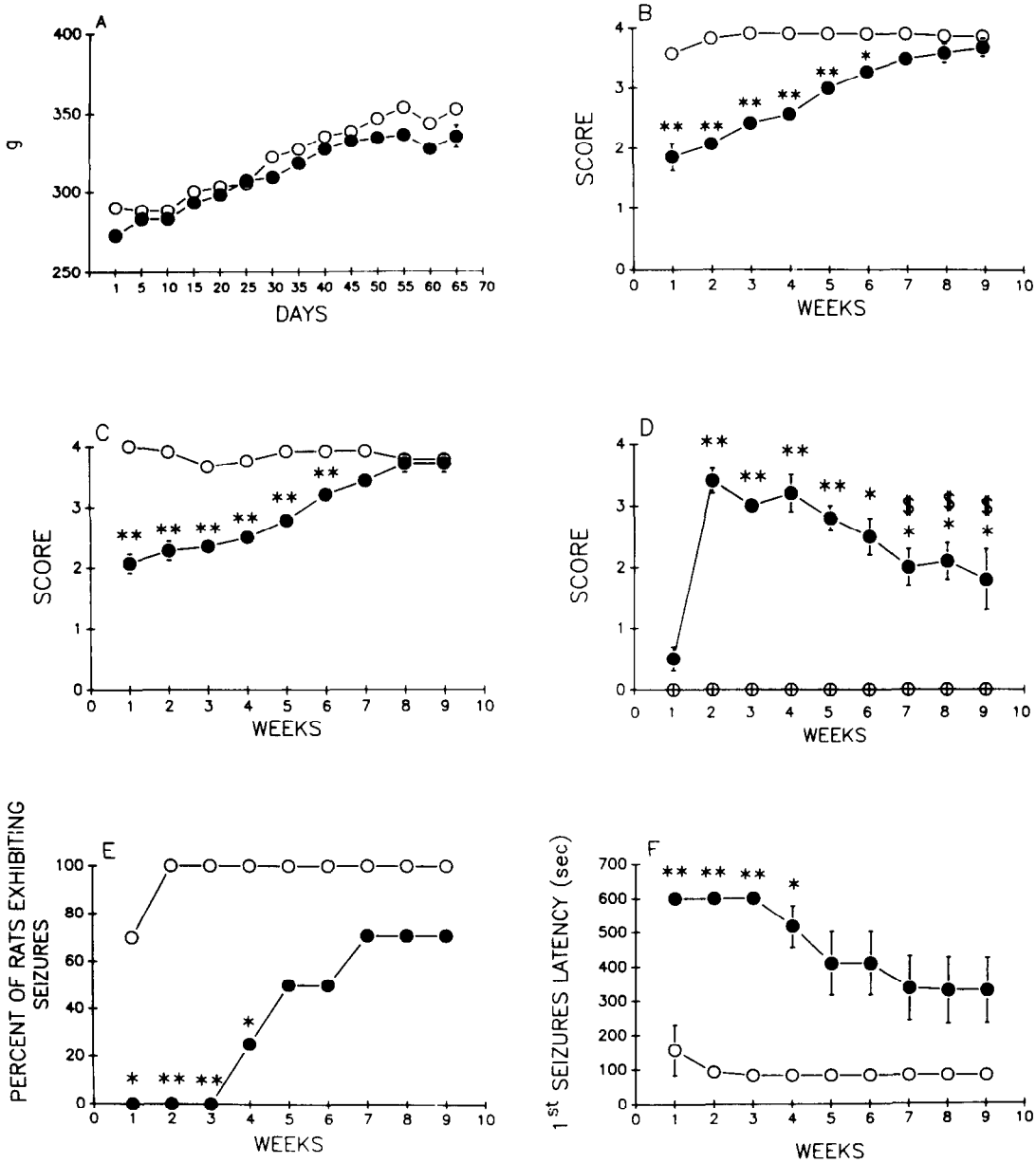

Fig. 1. Effect of daily chronic administration of chlordiazepoxide (40 mg/kg p.o.) (●) or saline (○) over 9 weeks on: (A) body weight (measured daily and reported here as mean ± S.E.M. for 5 days); (B) abdominal tone; (C) grip strength; (D) ataxia (all evaluated twice weekly and here reported as weekly means ± S.E.M.); (E) percentage of CDZ-dependent rats exhibiting seizures, and (F) latency to the first seizure (mean ± S.E.M.) after once-weekly administration of pentylenetetrazole 50 mg/kg i.p. 60 min after chlordiazepoxide treatment. * $P < 0.05$, ** $P < 0.01$ vs. saline group on the same day; $ $P < 0.01$ vs. chlordiazepoxide group at the 2nd week. $n = 7$ for each group.

icant difference being observed between the chlordiazepoxide and saline group (Fig. 1A). The twice-weekly behavioural evaluations revealed a certain degree of motor impairment in the chlordiazepoxide-treated rats, which was characterized by a slight but significant decrease in grip strength ($\chi^2 = 51.2$, $P < 0.0001$) and abdominal tone ($\chi^2 = 53.8$, $P < 0.0001$) for the first 3 weeks (Fig. 1B and C) which was maintained over the second 3-week period ($\chi^2 = 42.6$ and 45.2; $P < 0.0001$ respectively). Tukey's test showed that all the mean values for the first 6 weeks were different between the two groups ($P < 0.01$). It was considered that full tolerance was achieved during the last 3 weeks since no significant differences in the values for the treated and control animals were recorded. Marked ataxia was observed in the chlordiazepoxide-treated group during the 2nd and 3rd week ($\chi^2 = 19.4$, $P < 0.0001$ in comparison with the saline group) (Fig. 1D); this pathological symptom persisted during the two subsequent 3-week periods, although it became less evident ($\chi^2 = 31.4$ and 30.5 respectively, $P < 0.0001$). The differences between the treated and the saline group were always significant (Tukey's test), but partial tolerance developed between the second and the last 3 weeks ($\chi^2 = 22.6$, $P < 0.0001$).

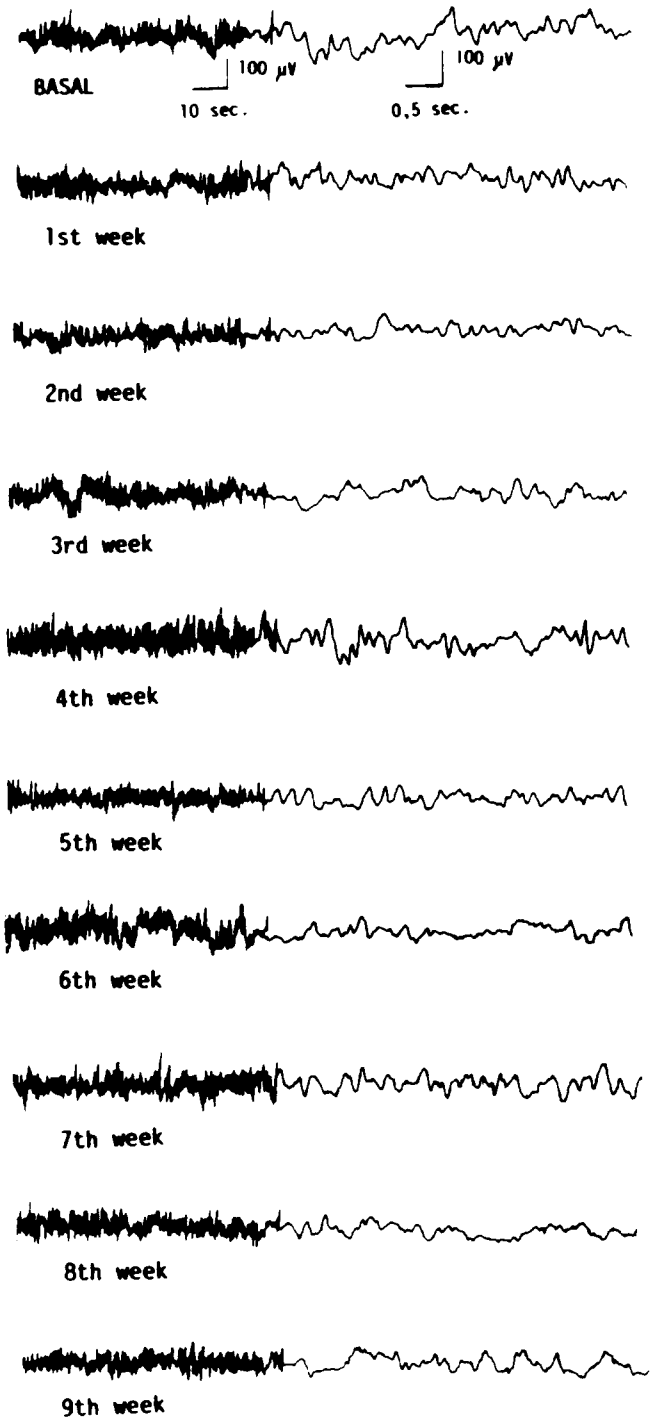

Fig. 2. Qualitative examples of morphological changes in cortical electrical activity recorded for 1 min in one freely moving awake rat once a week, 30 min before and 80 min after the daily administration of chlordiazepoxide 40 mg/kg p.o. for 9 weeks.

Over the first 4 weeks, daily chlordiazepoxide treatment provided complete protection against the tonic-clonic convulsions induced by once-weekly pentylenetetrazole injections, as revealed by the fact that none of the animals showed any signs of seizures within a cut-off period of 600 s ($\chi^2 = 6.45$, 10.21, 7.92, 5.11 respectively) (Fig. 1E). After the 5th week, no difference was observed between the two groups.

The results in terms of latency to the first seizure were similar ($F(17,85) = 8.91$, $P < 0.0001$, ANOVA test), post-hoc comparisons revealing a significant difference between the treated and the control group only for the first 4 weeks (Tukey test, $P < 0.05$, $P < 0.01$) (Fig. 1F), with no difference between the groups being observed from the 5th week.

3.2. EEG

The chronic administration of chlordiazepoxide was found to produce significant EEG changes which were different from those produced by the administration of the vehicle alone. Fig. 2 shows representative examples of the EEG segments recorded once a week before and 70/90 min after drug administration. During the first 3 weeks, chlordiazepoxide induced a marked decrease in the voltage and an increase in the frequency of EEG activity; from the 4th week, there was a slight decrease in high-frequency activity, which returned to baseline values for the remaining 5 weeks.

A quantitative analysis of these patterns is given in Fig. 3, in which the power spectra are expressed as the means of the recordings evaluated at times −30/0, 30/40, 70/90 and 120 min from treatment. The administration of chlordiazepoxide 40 mg/kg p.o. induced a significant increase in the mean total power from the first week ($F(7,48) = 19.1$, $P < 0.0001$). Tukey's test showed that the values at 30/40, 70/90 and 120 min

post treatment were significantly higher ($P < 0.01$) in comparison with both pre-drug levels and the saline group. During the second week, there was also a significant difference between the treated and the control group at the different times ($F(7,46) = 7.54$, $P < 0.0001$) and a shift of the peak. Tukey's test showed that the mean total power detected at 30/40 and 70/90 ($P < 0.05$) and 120 ($P < 0.01$) min was significantly different from the pre-drug and saline group values. Chlordiazepoxide administration also induced a significant increase in mean total power during the third week ($F(7,46) = 4.41$, $P < 0.001$), post-hoc comparisons once again showing that these increases at 30/40, 70/90 and 120 min were significantly different

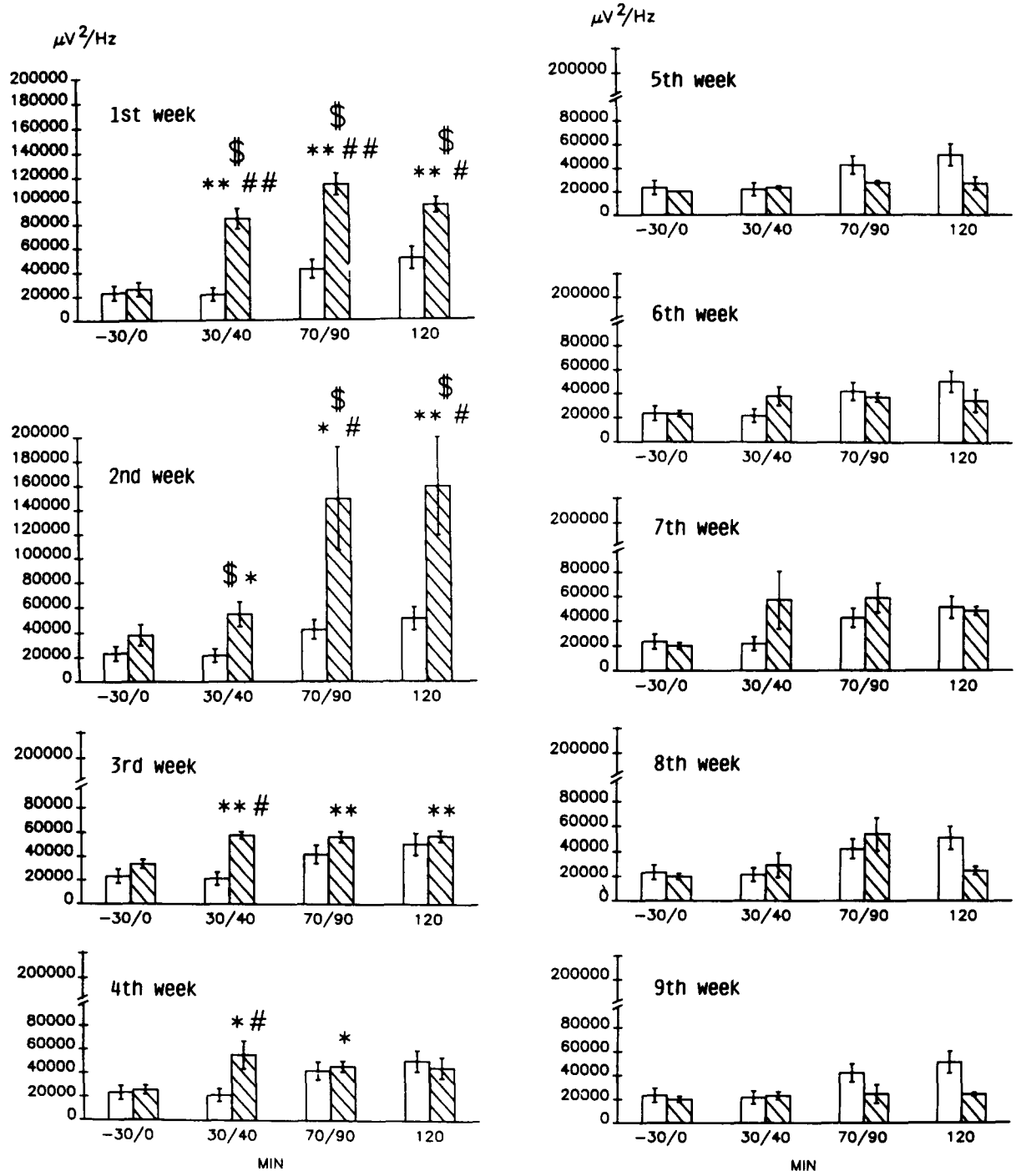

Fig. 3. Cortically derived time-dependent EEG changes in mean (±S.E.M.) total power density of freely moving awake rats evaluated once weekly 30 min before and 120 min after the daily administration of chlordiazepoxide 40 mg/kg p.o. (hatched columns) or saline (open columns) for 9 weeks. * $P < 0.05$; ** $P < 0.01$ vs. its pre drug (−30/0) value; $ $P < 0.01$ vs. pre-drug (−30/0) saline value; # $P < 0.01$ vs. saline group at the same time. $n = 7$ for each group.

from pre-drug and saline group values ($P < 0.01$). From the fourth week, there was a slight but significant difference in total power density between the treated and the control group ($F(7,46) = 3.0$, $P < 0.01$). Tukey's test showed that the values obtained 30/40 and 70/90 min after treatment were higher than the pre-drug value ($P < 0.05$), although there was no difference between the saline and the chlordiazepoxide group 120 min after treatment. ANOVA showed significant differences at 70/90 min during the first 4 weeks of

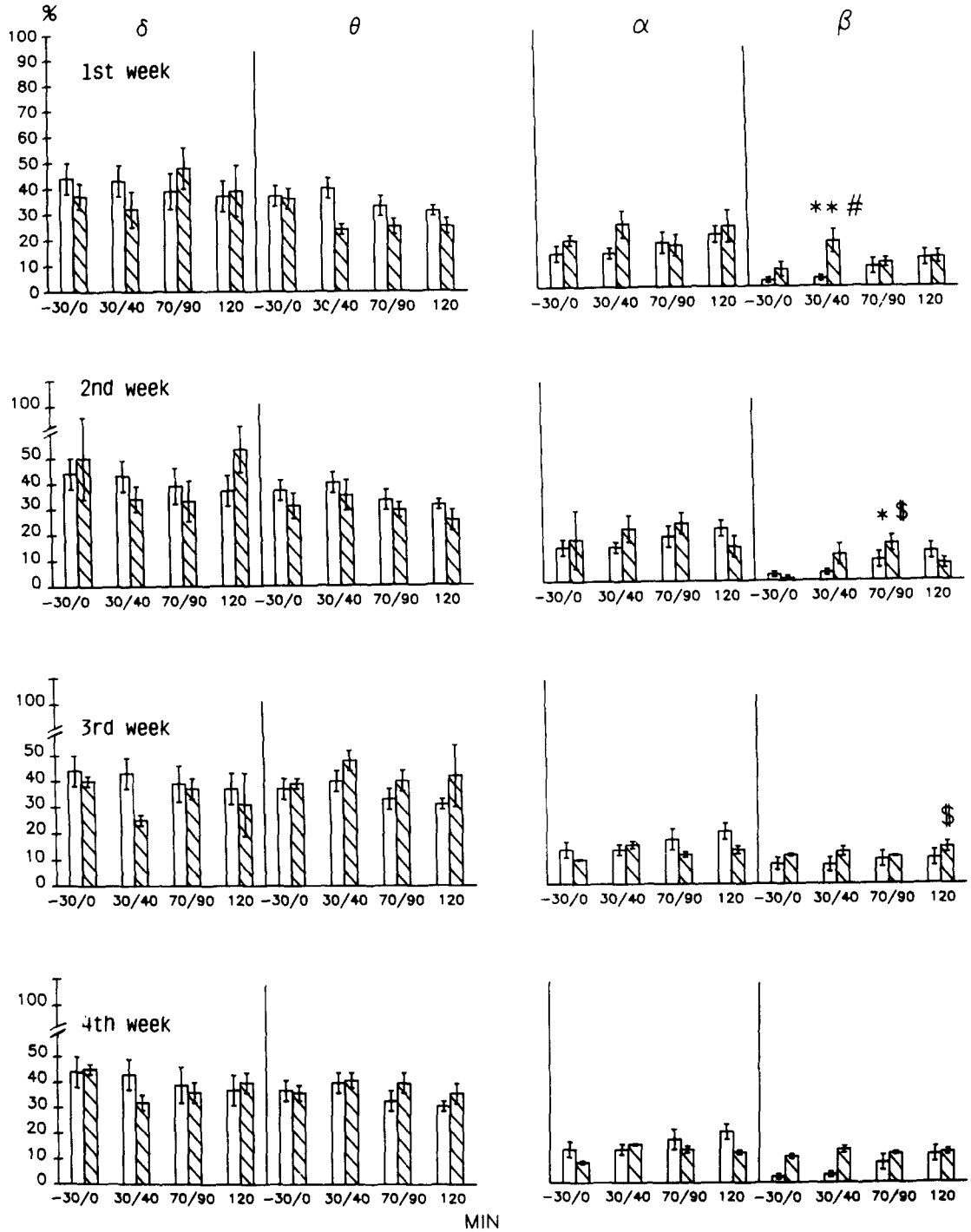

Fig. 4. Time-dependent EEG changes (mean ± S.E.M.) in the δ (0.2–4.0 Hz), θ (4.2–8.0 Hz), α (8.2–13.0 Hz) and β (13.2–25.0 Hz) band frequency distributions of mean spectral power. Once-weekly evaluations were made from cortical leads 30 min before and 120 min after the chronic administration of chlordiazepoxide (40 mg/kg p.o.) (hatched columns) or saline (open columns) during the first 4 weeks to freely moving awake rats. * $P < 0.05$; ** $P < 0.01$ vs. its pre drug (−30/0) value; $^{\#}$ $P < 0.01$ vs. saline group at the same time; $^{\$}$ $P < 0.05$ vs. saline pre-drug (−30/0) value. $n = 7$ for each group.

treatment ($F(3,23) = 16,80$ $P < 0.0001$). Tukey's test showed that the mean total power was higher during the second than during the third and fourth weeks ($P < 0.01$).

From the fifth week, chlordiazepoxide administration did not lead to any significant change in total power, thus suggesting full tolerance. The changes in mean total power were mainly due to the higher number of β waves detected in the chronically treated rats only at the first ($F(7,48) = 3.34$, $P < 0.005$), second ($F(7,47) = 2.47$, $P < 0.03$) and third weeks ($F(7,28) = 2.34$, $P < 0.05$) (Fig. 4). In the treated group, post-hoc comparisons showed a significant increase at 30/40 min during the first week ($P < 0.01$) in comparison with both pre-drug values, and the value at 30/40 min in the saline group; there was also a significant increase at 70/90 min during the second week ($P < 0.05$) in comparison with pre-drug values. During the third week, a significant increase ($P < 0.05$) in comparison with the saline group and pre-drug values was still found 120 min from treatment.

Starting from the fourth week, the percentage distribution of the mean power spectra did not show any significant modifications in comparison with pre-drug or saline group observations.

3.3. Physical dependence

During the period of abstinence, there was a difference in the number of symptoms ($\chi^2(2) = 8.47$, $P < 0.01$) in the chlordiazepoxide + flumazenil, saline + H_2O and saline + flumazenil groups. When the treated animals were given flumazenil p.o. 24 h after the last chlordiazepoxide administration, comparison with the saline + saline and saline + flumazenil groups showed a significant increase in the number of vertical posture, groomings, episodes of diggings and irritability and tooth chattering ($P < 0.05$, Tukey test) (Fig. 5A). At

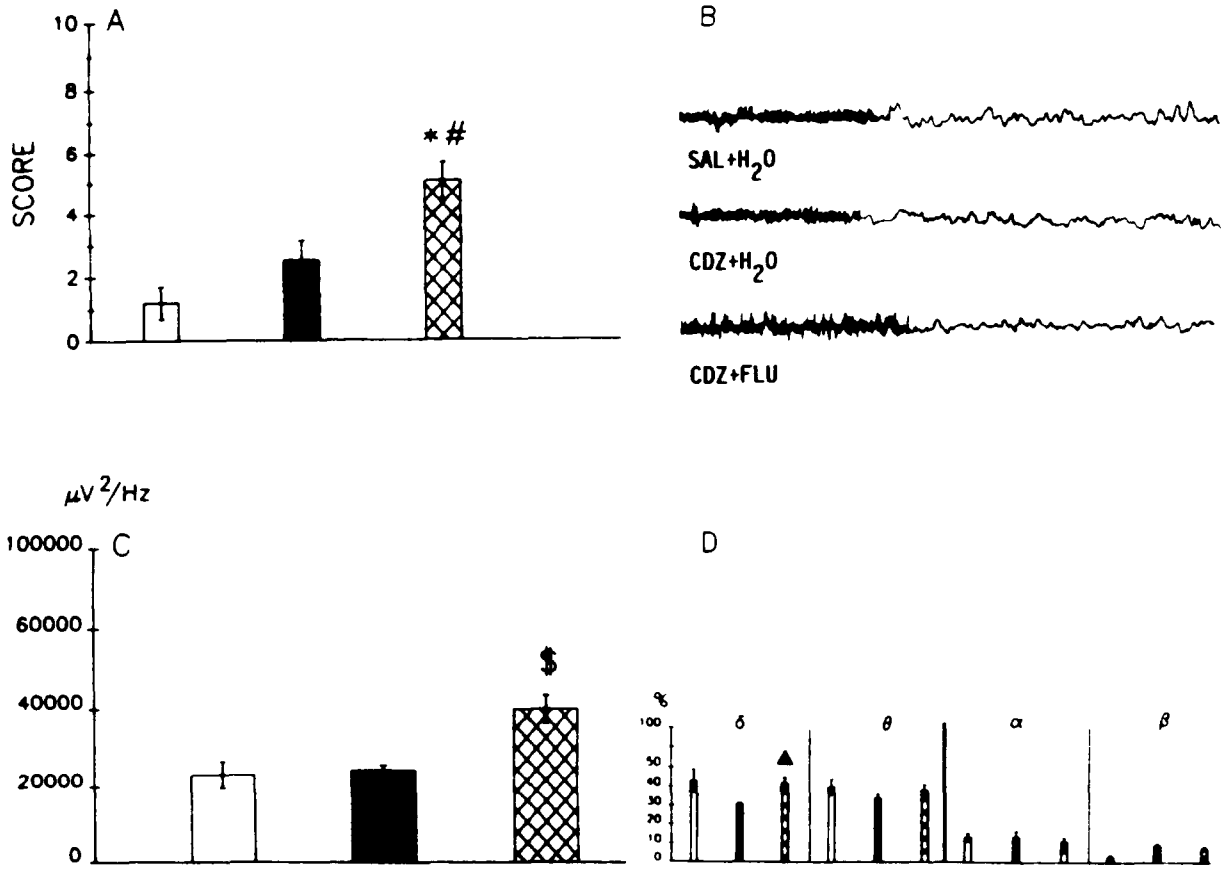

Fig. 5. (A) Score of symptoms observed during the 60 min immediately following the administration of flumazenil (FLU) 50 mg/kg p.o., 24 h after the last chlordiazepoxide (CDZ) administration. Open column: SAL + H_2O, black column: saline (SAL) + FLU, cross-hatched column: CDZ + FLU. * $P < 0.05$ vs. SAL group; # $P < 0.05$ vs. FLU alone. $n = 7$ for each group. (B) Qualitative examples of morphological changes from cortical leads recorded for 1 min after 9 weeks of CDZ or SAL treatment in a control rat after H_2O treatment, a CDZ-dependent rat after H_2O treatment and a CDZ-dependent rat after FLU administration. All recordings were made 80 min after treatment in freely moving awake rats. (C) EEG changes in mean (± S.E.M.) total power density and (D) frequency distribution (mean ± S.E.M.) evaluated for 30 min, 60 min after treatment of (open column) SAL + H_2O, (black column) CDZ + H_2O, (cross-hatched column) CDZ + FLU. $ $P < 0.05$ vs. CDZ + H_2O group; ▲ $P < 0.05$ vs. CDZ + H_2O. $n = 7$ for each group.

70/90 min after flumazenil administration, there was a slight increase in the voltage, and a decrease in the frequency of EEG activity in the chlordiazepoxide + flumazenil group in comparison with both the saline + H_2O and chlordiazepoxide + H_2O groups (Fig. 5B). Analysis of the total spectral power of these patterns evaluated at the same time revealed a significant difference ($F(2,16) = 4.66$, $P < 0.02$) between the groups. Post-hoc evaluation showed a significant increase in total power in the chlordiazepoxide + flumazenil group ($P < 0.05$) (Fig. 5C).

ANOVA revealed a difference in frequency distribution between the period of abstinence and the last week of chlordiazepoxide administration ($F(2,16) = 4.26$, $P < 0.02$) (Fig. 5D). Post-hoc comparisons showed a significant increase ($P < 0.05$) in the δ band of the same group.

4. Discussion

The development of tolerance to the effects of benzodiazepines as revealed by a variety of tests has been widely reported, and all the previously published studies describe the rapid development of tolerance to sedative, anticonvulsant and anxiolytic effects within a few days.

However, we now adopted the oral model to induce tolerance to the sedative effects of chlordiazepoxide and this led to different results. The behavioural consequences of the myorelaxant properties showed that tolerance developed only from the seventh week of treatment for grip strength and abdominal tone, while no complete tolerance was shown in terms of ataxia even after 9 weeks.

Tolerance to the anticonvulsant effects of chlordiazepoxide against pentylenetetrazole-evoked seizures was found after 4 weeks. This last finding disagrees with the report by File (1983), according to which tolerance to the anticonvulsant effect of diazepam developed after 5 days of treatment; however, this study was done in mice and used a higher pentylenetetrazole dose (120 mg/kg). What is interesting is that tolerance to anticonvulsant activity developed more rapidly than that to myorelaxant activity. For the last effect, in addition to different sensitivity to the test chosen, a possible different involvement of spinal receptors or a combination of spinal/cerebral receptors cannot be excluded.

The kinetics of tolerance to the anticonvulsant effect follow the same rate as those of EEG activity. The chlordiazepoxide-induced increase in power spectra gradually decreased until the fourth week, after which full tolerance developed; and the increase in the β power spectrum observed in the first week gradually decreased over the second and third week and there was a progressive shift of peak effect. It can be argued that the existence of a similar rate of tolerance development is probably due to an interaction of the same central receptors subtype or to the same sensitivity of the measures used.

Previous studies have shown that, after repeated benzodiazepine administration, the initial (within 30 min) EEG synchronization of 12 Hz spindle bursts decreases, while β-like activity increases as tolerance to the myorelaxant effects develops (Mele et al., 1984; Valerio and Massotti, 1988). Prolonged administration of diazepam for 10 days induces the progressive appearance of β-like activity associated with behavioural activation (gnawing, eating, and slight motor activity), and the simultaneous disappearance of the 12 Hz spindle bursts associated with behavioural sedation (closed eyes and myorelaxation).

In our study, the myorelaxant effects of chlordiazepoxide were always accompanied by the high (13.2–25.0 Hz) frequency (at least for the first 3 weeks), but the behavioural effects continued even after the level of this β-like activity had returned to baseline values. It remains unclear why this EEG activation occurs at the same time as the drug-induced myorelaxant effects. A possible reason for the enhanced EEG activation at the time of myorelaxation may relate to the animal experiencing loss of control of its muscles as frightening, leading to enhanced arousal. This is the reason cited for the paradoxical effects of benzodiazepine in increasing arousal in cats.

Treatment with flumazenil after 9 weeks of chlordiazepoxide administration induced the appearance of clear signs of behavioural withdrawal syndrome characterized by CNS hyperexcitation, as previously reported in humans (Winokur et al., 1980), primates (Gallager et al., 1986) and rodents (McNicholas and Martin, 1986) after chronic diazepam treatment. Surprisingly, no quantitative data concerning EEG alterations during the withdrawal state are available, except in some studies which show that flumazenil completely reverses the EEG effects of a variety of acutely administered benzodiazepines (Polc et al., 1981; Klotz et al., 1985; Mandema et al., 1992). In the present study, the administration of flumazenil to chlordiazepoxide-dependent rats produced a significant increase in the mean total power spectra in comparison with that observed in the chlordiazepoxide-dependent rats treated with H_2O. This increase was mainly due to an increase in the δ band, which cannot be attributed to the intrinsic properties of flumazenil: two pharmaco-EEG profiles of flumazenil have shown that, unlike benzodiazepine agonists, it has central stimulating activity (Schopf et al., 1984; Santucci et al., 1989). One study has shown that flumazenil does not produce any significant EEG effects (Breimer et al., 1991), and another evidenced partial agonist activity (Higgit et al., 1986) with an

increase in β activity. It can be argued that the observed increase in the δ band may have been due to the complete restoration of EEG activity, making it similar to that of the control group.

In conclusion, the present findings indicate the validity of the animal model used for studying the tolerance to, and physical dependence on, the EEGraphic, anticonvulsant and myorelaxant effects of benzodiazepine after prolonged administration in rats. The results seem to favour the hypothesis that the kinetics of the onset of chlordiazepoxide-induced EEG changes and anticonvulsant effects are different from the kinetics of the onset of chlordiazepoxide-induced myorelaxant effects, suggesting that different benzodiazepine receptor subtypes develop tolerance at different rates. The use of specific antagonists of the different benzodiazepines receptor subtypes will better clarify the mechanism involved in the above effects.

Finally, it cannot be excluded that neurotransmitters other than GABA could mediate some of the behavioural effects of benzodiazepines. File (1982b) reported that chlordiazepoxide induced-ataxia was significantly antagonized by naloxone, suggesting an interaction with the opioid system.

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