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Anticonvulsant Activity of Flumazenil in Rats During Ontogenetic Development

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RATHOUSKÁ, J., H. KUBOVÁ, P. MAREŠ AND J. VORLÍČEK. *Anticonvulsant activity of flumazenil in rats during ontogenetic development.* PHARMACOL BIOCHEM BEHAV 44(3) 581–586, 1993.—The influence of flumazenil on seizures induced by pentylenetetrazol (PTZ) was studied in rats aged 7, 12, 18, 25, and 90 days. Flumazenil in doses of 25, 37.5, and 50 mg/kg IP injected 10 min before PTZ exhibited a dose-dependent anticonvulsant action in all age groups studied. It was more effective against generalized tonic-clonic than against minimal clonic seizures at all developmental stages studied. In the two youngest groups, minimal seizures were elicited only rarely under control conditions. Pretreatment with the two lower doses of flumazenil resulted in an increased incidence of this type of seizure for these two groups. The anticonvulsant activity found in all age groups is in agreement with data from other benzodiazepines and speaks against a pure benzodiazepine-antagonistic action of flumazenil.

Flumazenil Pentylenetetrazol Seizures Ontogenetic development

A marked anticonvulsant effect of benzodiazepines (BDZs) has been described repeatedly [for review, see (11,17)]. The anticonvulsant potency of individual BDZs correlates well with their binding to specific high-affinity BDZ receptors (28), which form part of the supramolecular complex of $GABA_A$ receptor-BDZ receptor-chloride ionophore (10,25). Thus, the anticonvulsant activity of BDZs apparently is exerted by means of their action on this supramolecular complex. This argument also is supported by data on the BDZ receptor antagonist flumazenil (Ro 15-1788, Anexate®, Roche, Brampton, Ontario, Canada), capable of abolishing both the anticonvulsant and other pharmacological effects of BDZs (3,9,13). In addition, for rats continuously exposed to diazepam flumazenil reportedly elicited withdrawal seizures (32). As a receptor antagonist, flumazenil should be devoid of intrinsic activity. This was confirmed by Hunkeler et al. (13) and Bonetti et al. (2), who did not find an effect of flumazenil on pentylenetetrazol(PTZ)-induced seizures. However, Nutt et al. (26), Grecksch et al. (9), and Vellucci and Webster (31) described a suppression of PTZ-induced seizures by flumazenil. Because of these contradictory results, we studied the effect of flumazenil on motor seizures in rats. Lack of data about the action of flumazenil in immature animals and results indicating the possible convulsant action of benzodiazepines during early development (1,27) led us to perform this ontogenetic study in rats.

METHOD

Experiments were performed in random-bred, albino rats of the Wistar strain of both sexes. Five age groups were studied: 7-, 12-, 18-, 25-, and 90-day-old rats, with the day of birth taken as zero. Rat pups were weaned at the age of 28 days. Flumazenil was dissolved in physiological saline with Tween-80 added (1 drop/10 ml). The freshly prepared solution was administered IP. Three doses were used in all age groups: 25, 37.5, and 50 mg/kg. Ten minutes after flumazenil, metrazol (i.e., PTZ) was injected SC in a dose of 100 mg/kg in all age groups except the 18-day-old group; in the latter case, a 90-mg/kg dose was used instead. The 90-mg/kg dose in the 18-day-old group was used because our previous work demonstrated a higher sensitivity of 18-day-old rats to PTZ (23). Each flumazenil-pretreated group consisted of seven to nine animals, whereas all control groups, given PTZ only, consisted of 12–16 rats. The number of males and females used was practically always equal (mostly four and four); the maximum difference was one animal (in age and dose groups where an unpaired number of rats was taken).

Animals were observed in isolation for 30 min after PTZ injection. Body temperature of rats aged 18 days or less was maintained by means of an electrically heated pad. Incidence of two types of epileptic seizures was recorded: 1) minimal (predominantly clonic, involving facial and forelimb muscles,

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i.e., mMS) and 2) major (generalized tonic-clonic, with a loss of righting reflexes, i.e., MMS). Other motor phenomena (e.g., isolated myoclonic jerks) also were registered, and latency to the beginning of seizures always was measured. The severity of epileptic phenomena was quantified according to the following scale based upon previous work (29):

0 – normal behavior;
0.5 – abnormal behavior (e.g., orienting reaction);
1 – isolated myoclonic jerks;
2 – atypical, minimal seizures, that is, only some elements of minimal seizures were present, for example, unilateral forepaw clonus, shuffling of the forelimbs;
3 – typical, minimal seizures, that is, bilateral clonus of the forepaws and head while the hindlimbs were widely abducted;
4 – major seizures without a tonic phase, that is, a sequence of wild running and clonic phase with a loss of righting ability at the beginning of the latter phase;
5 – full-blown major seizures formed by wild running, tonic and clonic seizures of all four limbs; righting reflexes were lost at the beginning of the tonic phase.

Each animal was assigned a value based upon the most severe phenomenon it demonstrated, and an average score was calculated for each group.

Statistical analysis of the data was performed using BMDP programs (7). The incidence of seizures was analyzed using a log-linear hierarchical model of a four-way contingency table with three controlled factors: dose (with four levels—controls and three dose groups), age (five levels), and sex (two levels). An alternative method of stepwise logistic regression was used to compare results.

The latencies to seizures were evaluated by means of a three-factor analysis of variance (ANOVA) with unequal number of replicates in cells and with factors dose, age, and sex. Logarithmic transformation of the latency was used to stabilize variance in cells (4). Simple effects in this model were

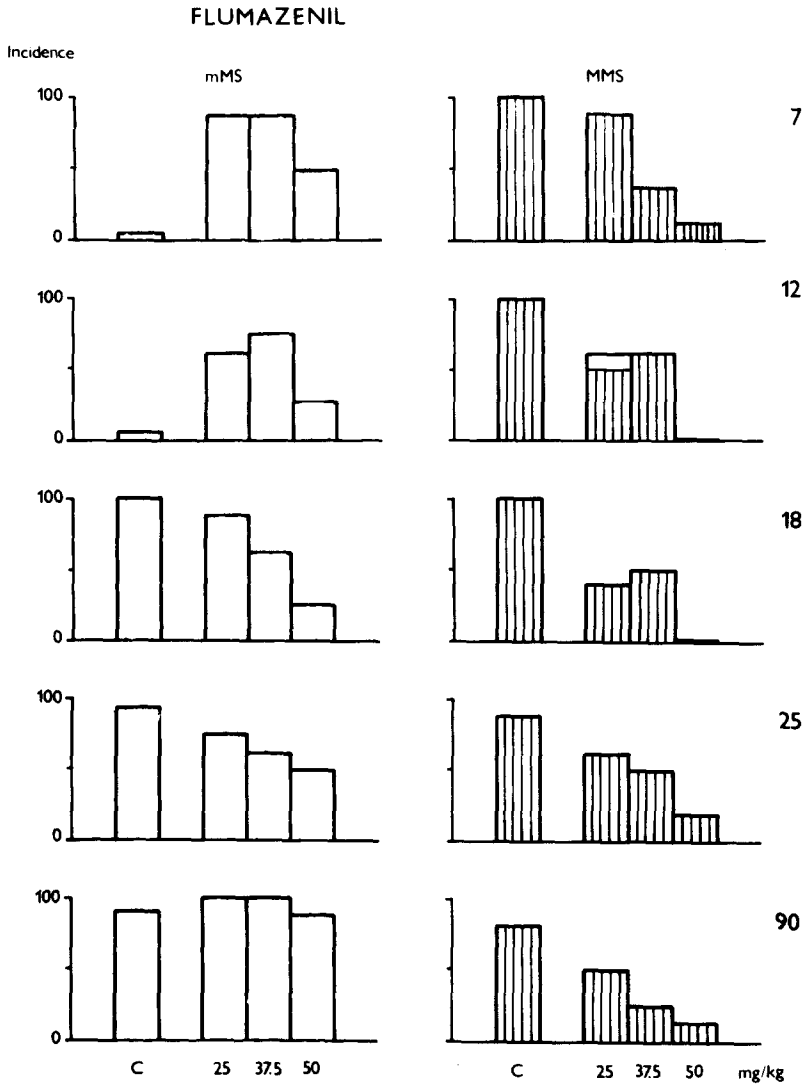

FIG. 1. Incidence of minimal (mMS, left) and major (MMS, right) metrazol seizures in rats 7, 12, 18, 25, and 90 days old (from top to bottom). In each graph, abscissa represents controls (C, no flumazenil pretreatment) and the three doses of flumazenil (25, 37.5, and 50 mg/kg); ordinate: percentage of rats exhibiting seizures.

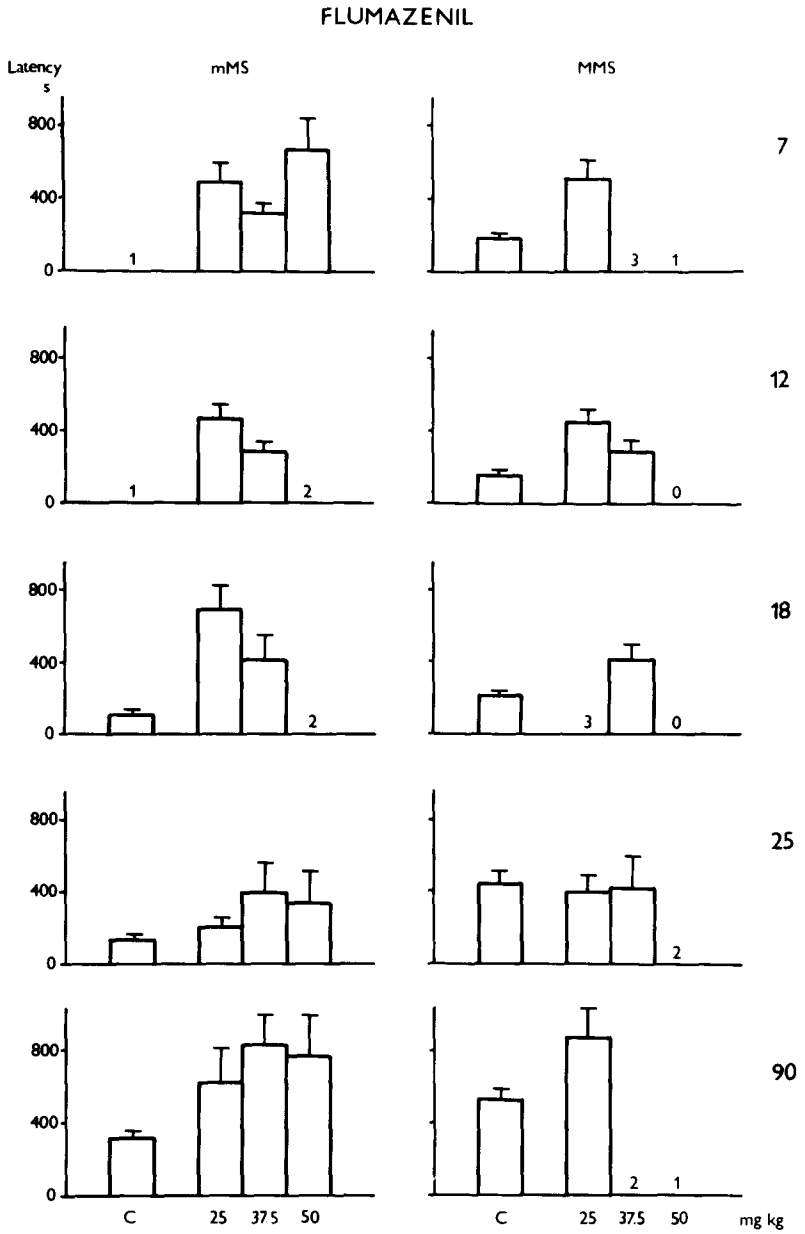

FIG. 2. Latencies (mean ± SEM) of minimal and major pentylenetetrazol (PTZ)-induced seizures. Details as in Fig. 1, only the ordinate here represents latencies in hundreds of seconds. The numbers replacing some columns represent the number of animals exhibiting seizures in the age and dose groups where this number was not sufficient for counting of the mean and variance.

obtained from two-way analysis under the condition of fixed level of the third factor (33). The level of significance of tests of main effect contrasts were adjusted according to Holm's multiple-test procedure (12).

To compare seizure severity scores among dose levels in each age group, a nonparametric three-way ANOVA model was applied after random reduction of the number of observations in controls to obtain an equal number of observations in each cell (30). The level of significance of tests of main effect pairwise contrasts were adjusted according to Holm's multiple-test procedure (12).

Unless stated otherwise, the level of significance was $p < 0.05$.

RESULTS

Incidence of Seizures

Figure 1 presents results on the incidence of seizures.

There was a significant effect of dose. The highest dose of flumazenil used completely abolished MMS in 12- and 18-day-old rats. On the other hand, the incidence of MMS was not

significantly age dependent nor was there a significant influence of sex.

Minimal seizures (mMS) were more resistant to the effect of flumazenil than major seizures. There was no difference between sexes in the incidence of mMS, but there was a strong dependence upon age and dose and upon combinations of levels of these factors. The latter dependence was caused by the incidence in controls; 7- and 12-day-old rat pups exhibited mMS only rarely, in contrast to older animals with almost complete incidence. The two lower doses of flumazenil in combination with PTZ led in the 7- and 12-day-old groups to a markedly increased incidence of mMS; these seizures were often incomplete (e.g., only head jerking, shuffling) and were thus quantified as severity score 2. The 50-mg/kg dose did not change the low incidence of mMS in 7- and 12-day-old animals.

Latencies of Seizures

Figure 2 presents the latencies of seizures.

Dose and age main and interaction effects were significant. In all age groups except 25- and 90-day-old rats, latency of MMS was prolonged in both the 25- and 37.5-mg/kg dose groups in comparison with controls. Age dependence was found only among controls; 90- and 25-day-old groups exhibited longer latencies than other age groups. In the three-way ANOVA, there were no significant (at 10% level) differences between sexes and no interaction of factor sex with the factors of dose and/or age. The 50-mg/kg dose level was excluded from the analysis of MMS latency because of the rare incidence of MMS.

A complete three-factor model of mMS was required for the 25- and 37.5-mg/kg dose levels because of insufficient data. No dose difference or interactions were found. There were longer latencies in the male groups (the only difference between sexes found in our study). Higher values in 90- and 18-day-old age groups than in the 25-day-old group were the only significant age differences. All dose groups of 18- and 90-day-old animals exhibited longer latencies than controls, separately analyzed in a one-way model. The 25-day-old group reached only the 10% significance level.

Severity of Seizures

Figure 3 presents results on the severity of seizures.

The seizure severity score exhibited great differences among dose levels. The score of the control group was higher than that of the 25- and 37.5-mg/kg dose groups, and these groups exhibited higher scores than the 50-mg/kg dose group. Dose dependence of the anticonvulsant effect of flumazenil was demonstrated in all age groups. No other differences in age and sex factors, including interaction effects, were found.

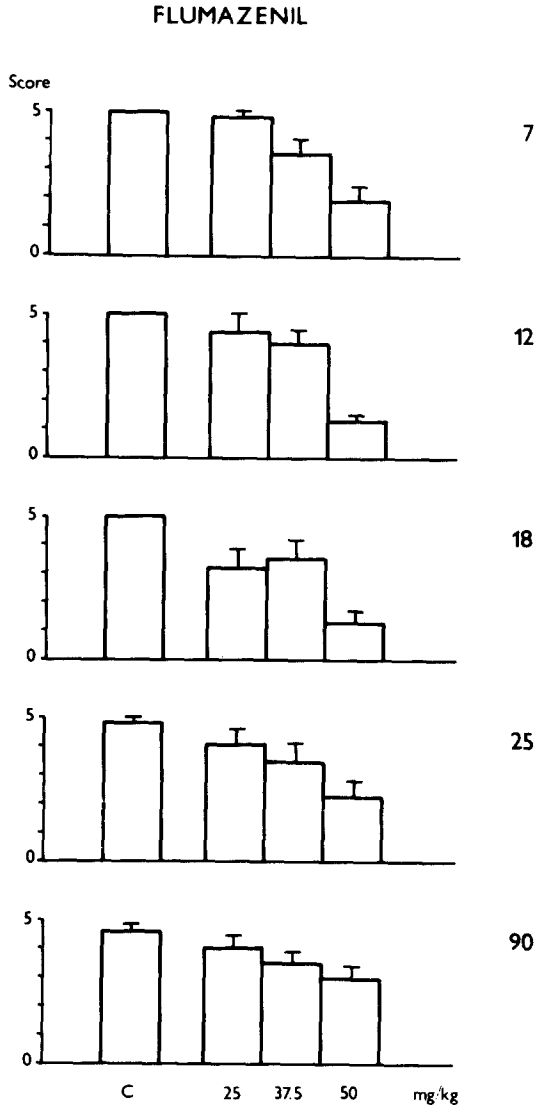

FIG. 3. Severity of seizures (mean ± SEM) in the five age groups. Details as in Fig. 1, only the ordinate here represents score according to Pohl and Mareš (29).

DISCUSSION

Our data are in agreement with authors demonstrating an anticonvulsant action of flumazenil (9,14,31) and may be taken as further evidence that flumazenil does possess an intrinsic activity and cannot be taken as a pure BDZ antagonist. We must conclude that flumazenil is a partial agonist of BDZs. The contradiction with the results of Hunkeler et al. (13) and Bonetti et al. (2) might be explained on the basis of the dose of PTZ used. In the cited studies, a high dose of PTZ was administered in mice (120 mg/kg, IP), whereas our 100-mg/kg dose was injected SC. A direct comparison of these doses administered by these two routes was published previously (23). The anticonvulsant action of flumazenil is probably only moderate; therefore, it might be demonstrated only when the doses of the convulsant drug is not extremely high. Relatively low doses of PTZ were used in our, as well as in other, experiments (9,14,31).

Marescaux et al. (24) described a proconvulsant effect of flumazenil in a model of human absences: spontaneously appearing spike-and-wave electroencephalographic activity in a special inbred strain of rats. Absence seizures are completely different from generalized tonic-clonic seizures with regard to the underlying pathophysiological mechanisms. The inhibition is not suppressed in absence seizures; on the other hand, the suppression of inhibition plays a dominant role in generalized tonic-clonic seizures, where excitation overcomes the inhibitory mechanisms (8).

A dose-dependent, anticonvulsant action of flumazenil against generalized tonic-clonic seizures was found in all age

groups studied. A similar effect, which did not undergo qualitative changes during postnatal development, was demonstrated with other BDZs: nitrazepam (21), clonazepam (15), and midazolam (16). All BDZs studied also exhibited higher efficacy against major, than against minimal, metrazol seizures (in those age groups that displayed both types of seizures). Ontogenetic data on BDZ receptors give evidence for the presence of these receptors in rats at early stages of development, with an increase in their numbers during the first 3 weeks after birth (5,6). As concerns the two subtypes of BDZ receptors (BDZ_1 and BDZ_2), the BDZ_1 may be connected with anticonvulsant effects (19). This subtype is also present after birth and its amount increases steeply during the rat's second postnatal week (18). Thus, BDZ receptors apparently are present in a substantial amount at the age of our youngest group (7 days) and should be capable of mediating the observed effects.

The occurrence of mMS in the two youngest groups only after pretreatment with flumazenil (i.e., a rare phenomenon in control animals) might be taken as a proconvulsant action of lower doses of flumazenil. In any case, mMS was blocked by the highest dose of flumazenil used (50 mg/kg), and a similar increase was not seen after clonazepam or nitrazepam, when relatively high doses of the BDZs were administered (15,21). A similar phenomenon (the triggering of mMS in rats aged 7 and 12 days) has been also observed after pretreatment with both phenytoin (20) and ethosuximide (23). Such findings speak against the possibility that minimal seizures are a specific age-bound phenomenon and have been confirmed in experiments using the convulsant drugs bicuculline (34) and isonicotinehydrazide (22). The latter drugs reliably elicited minimal seizures in the two age groups mentioned above. These findings suggest that there is a triggering of minimal seizures by metrazol, which is age specific, and also that such triggering could be modified by pretreatment with various drugs. The mechanisms of such developmental changes remain to be analyzed.

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