Pharmacology Biochemistry & Behavior, Vol. 29, pp. 791–795. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

Electroencephalographic Changes After Short-Term Exposure to Agonists of Benzodiazepine Receptors in the Rat

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VALERIO, A. AND M. MASSOTTI. *Electroencephalographic changes after short-term exposure to agonists of benzodiazepine receptors in the rat.* PHARMACOL BIOCHEM BEHAV **29**(4) 791–795, 1988.—Naive rats receiving IV diazepam (10 mg/kg), flunitrazepam (2.5 mg/kg) and clonazepam (2 mg/kg) show electroencephalographic (EEG) changes consisting of lengthening of the spindle bursts (7–12 Hz; 200–300 μV) and appearance of 15–30 Hz waves (β-like activity). These EEG manifestations are associated with signs of behavioral sedation (crouched, eyes open and myorelaxation) and stimulation (gnawing, running, ear twitches and sometimes wet-dog shakes), respectively. Bursts of 2–4 Hz waves can be occasionally observed associated with either marked sedation (lying down, eyes closed and presence of righting reflex) or sleep (stretched in the side with absence of righting reflex). Measurements of the periods spent by the animals in the two EEG patterns within the first hour after intravenous injection show the large preponderance of the spindle bursts over the β-like activity. After the triazolopyridazine Cl 218,872 (10 mg/kg) the β-like activity is almost absent, and in no case loss of the righting reflex can be observed. These agonists of BDZ receptors have been injected at the above reported doses for 5 days, once-a-day. At the 5th day, animals receiving diazepam exhibit a preponderace of the EEG and behavioral activation within the first hour after injection. Rats receiving flunitrazepam show a significant increase of the periods of stimulation and a slight decrease of the periods of sedation. These phenomena of "habituation" are absent in animals treated with clonazepam and Cl 218,872.

Diazepam Flunitrazapam Clonazepam Cl 218,872 Repeated administration EEG Rats

BENZODIAZEPINE (BDZ) derivatives, in particular diazepam, are considered the drugs of choice for treatment of anxiety, seizure disorders and insomnia [4,5]. Their superiority over barbiturates appeared evident because of the relative absence of serious side effects [10]. Clinical and experimental data have been accumulated indicating that tolerance can occur to the sedative and anti-convulsant effects of the BDZ, but not to their anxiolytic-anticonflict activity (see [3]). Biochemical studies showed that the appearance of such a tolerance seems to be associated with a decrease of the functional interaction between BDZ binding sites and γ-aminobutyric acid (GABA) receptor (see [9]). On the contrary, only scattered reports concerned with the changes of the brain electrical activity after repeated administration of diazepam in laboratory animals [7, 9, 11].

Electroencephalographic (EEG) studies pointed out that diazepam elicits in laboratory animals characteristic electrocortical patterns which are not normally present in the basal tracing. They consist of repeated typical 7–12 Hz spindle bursts sometimes interrupted by short-lasting periods of high frequency (15–30 Hz) waves. Flunitrazepam and clonazepam also elicit similar electrocortical manifestations. In contrast, the triazolopyridazine derivative Cl 218,872 induces the appearance of the typical spindle bursts, whereas the high frequency waves occur rarely (Longo *et al.*, this volume).

Previous studies from our laboratory have shown that repeated administration of very large doses of diazepam can elicit marked reduction of the spindle bursts and increase of the high frequency waves. Such an EEG effect is associated with a decrease of the sedative action of the drug. The doses of 10 and 20 mg/kg IV, once-a-day, were sufficient to induce such changes already within 5 days of treatment in rats [9] and 10 days in rabbits [11], respectively.

The aim of the present paper is to assess whether similar modifications of the EEG effects also occur after short-term exposure to flunitrazepam, clonazepam and Cl 218,872 in the rat.

METHOD

Male Sprague-Dawley rats, weighing 250–300 g at the be-

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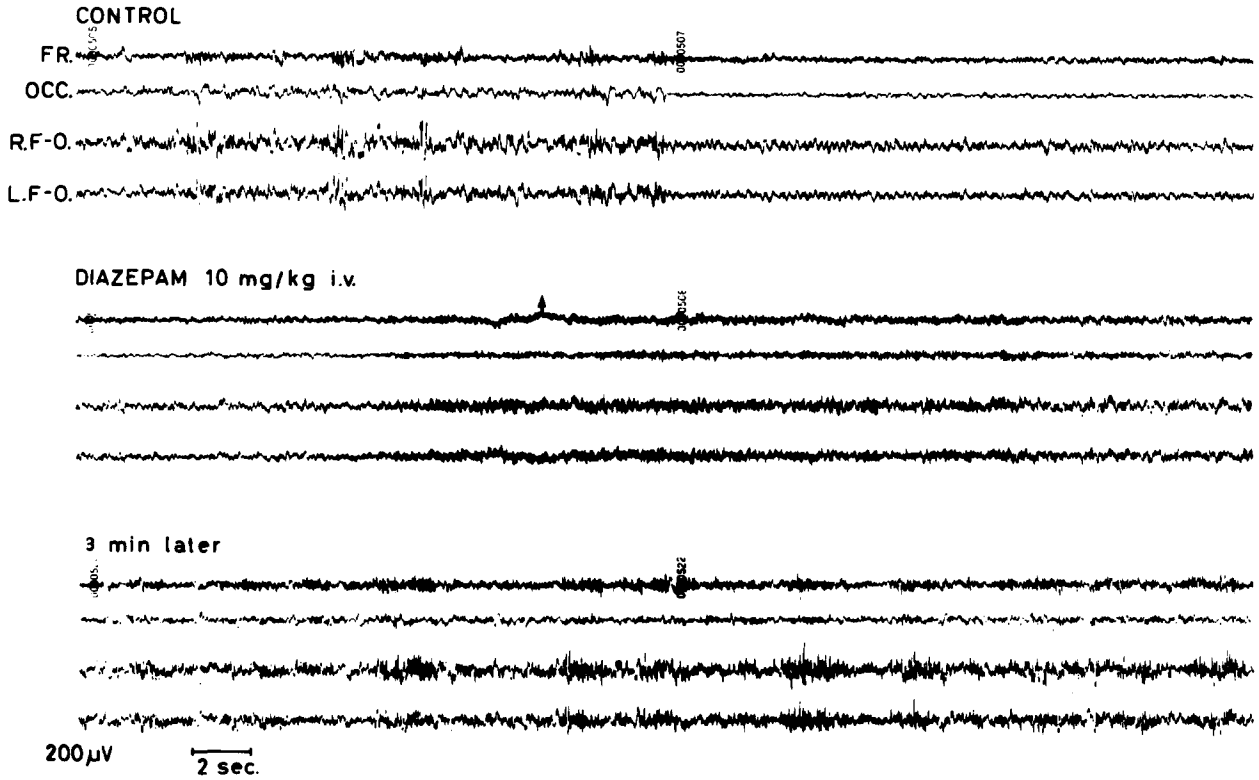

FIG. 1. EEG effects of diazepam in naive rats. This figure depicts the changes of the electrocortical activity induced by 10 mg/kg IV of diazepam. Two EEG patterns can be observed in basal conditions (upper record). In the left part is reported a period of synchronization (intermediate-high voltage, low frequency waves) with spindles (lasting 0.5–1 sec). In the right part is reported a desynchronized record (20–50 μV, high frequency waves). The injection of diazepam (middle record) induces already before the end of injection (arrow) the appearance of 15–30 Hz, 100–150 μV waves associated with signs of behavioral excitation. Later on (lower record) repeated spindle bursts (7–12 Hz, 200–300 μV), each lasting 2–6 sec, occur associated with signs of behavioral sedation. For further details see text. Leads: FR=right-left sensorimotor cortices; OCC=right-left optic cortex; R.F-O=right sensorimotor-optic cortices; L.F-O=left sensorimotor-optic cortices.

ginning of the treatment were used. Surgical procedures were performed under Na pentobarbital (40 mg/kg IP) anesthesia. A chronic indwelling cannula was inserted into the external jugular vein. Cortical electrodes were then implanted at the level of sensorimotor and associative cortices of both hemispheres.

After three days of recovery period, the animals received the first doses of the drugs. Then they were injected once-a-day for 5 days. Brain electrical activity was recorded at the 1st and 5th day of treatment. Each session commenced with a one hour recording period before the injection. Then the EEG was continuously recorded up to 90 min after the injection. The EEG tracing was evaluated by visual inspection. The duration of the various periods of spindle bursts and of the periods of high frequency waves was measured during the first hour following the injections of the drugs.

Diazepam, flunitrazepam and clonazepam were dissolved with two drops of 12 N HCl. The volume was then adjusted with saline. Cl 218,872 was dissolved with propylene glycol 300. Preliminary experiments showed that this solvent (1 and 2 ml/kg) does not influence the brain electrical activity.

All drugs were injected through a catheter connected with the cannula inserted into the jugular vein at slow speed rate (0.1 ml/min) with a volume of 1 ml/kg. This allowed us to inject the drugs without handling of animal.

RESULTS

Diazepam was injected at the dose of 10 mg/kg IV to 10 and 4 animals in acute and chronic studies, respectively. As shown in Fig. 1, in naive rats 50–100 μV, 15–30 Hz waves (thereafter defined β-like activity) emerge already before the end of injection of the drug. This pattern is associated with signs of behavioral activation (gnawing, running, ear twitches and occasionally wet-dog shakes). Within 1–2 min, this record is replaced by repeated 7–12 Hz spindle bursts (200–300 Hz) accompanied by behavioral sedation (crouching, eyes open and myorelaxation and presence of the righting reflex). Each spindle burst lasts 2–5 sec. In some instances, animals show either marked sedation (lying down, eyes closed with the presence of the righting reflex) or sleep (lying on the side, no response to external stimuli and absence of the righting reflex). In both cases, bursts of 2–4 Hz waves are noticed in the record. These EEG patterns last 3–4 hours and can be interrupted by short-lasting (1–3 min) periods of EEG activation, very often associated with signs of behavioral stimulation. The dose of diazepam presently used is the same tested in the previous study [9] and is 100 times higher than the minimal effective one.

As shown in Fig. 2, in naive rats a preponderance of the spindle bursts (48±4 min) over the β-like activity (21±6 min)

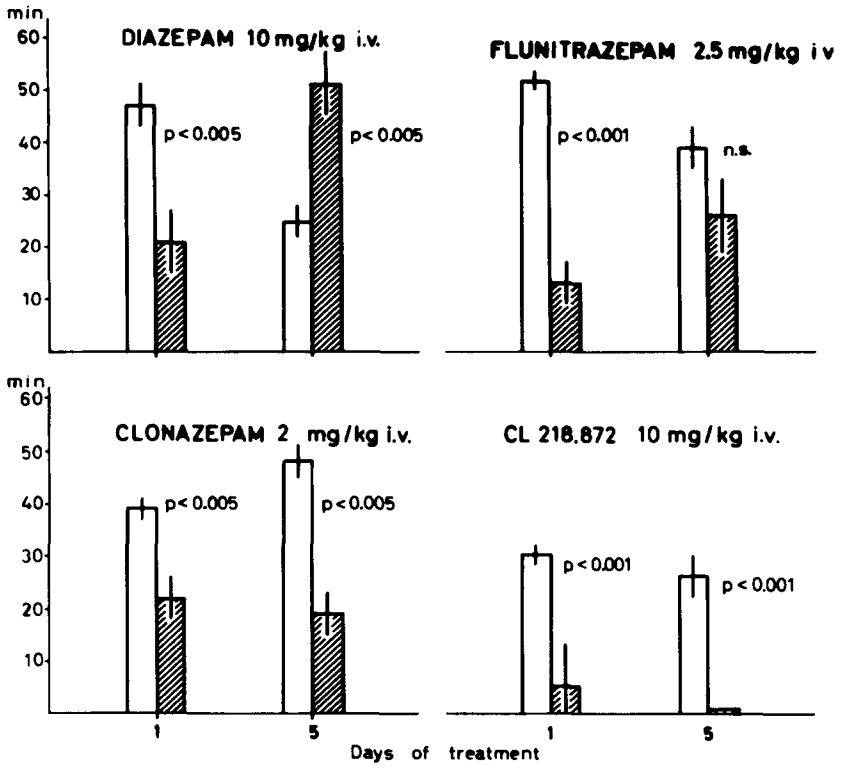

FIG. 2. Relationship between the occurrence of the spindle bursts and β-like activity in the EEG recording of rats after short-term exposure to several agonists of BDZ receptor. The histograms show the duration of the periods of spindle bursts and of the periods of β-like activity observed in naive rats and in animals receiving diazepam, flunitrazepam, clonazepam and Cl 218,872 for 5 days. The drugs were injected every 24 hours. In the abscissa are reported the day of treatment. In the ordinate are reported the total time (min) spent in the two EEG patterns (*namely*, spindle bursts and β-like activity) within the first hour after the injection. Statistical significances of the differences between the two pattern at the testing day for each drug are calculated according to the paired *t*-test. Open columns: spindles; hatched columns: β-like activity.

is noticed within the first hour after the injection. On the contrary, after 5 days of a once-a-day dosing regimen, the presence of the β-like activity throughout the first hour is largely preponderant (51±6 min) over the spindle bursts (24±3 min). Interestingly, administration of 20 mg/kg IV of diazepam to 3 rats, previously exposed to 10 mg/kg of the drug for 4 days, restores the preponderance of the spindle bursts over the β-like activity (data not shown).

Flunitrazepam was injected at the dose of 2.5 mg/kg IV to 10 and 6 rats in acute and chronic experiments, respectively. This dose is 50 times higher than the minimal effective one in this paradigm. In naive animals, similarly to diazepam, this drug elicits before the end of injection a short-lasting period of EEG and behavioral activation. This is followed by long-lasting periods of EEG and behavioral sedation alternated with short-lasting periods of activation. At the first day, within the first hour the periods of spindle bursts attain 52±2 min, and the periods of β-like activity attain 12±3 min. At the 5th day of treatment, no statistically significant difference between the time spent by the animals in the two EEG patterns is noticed. The spindle bursts indeed decrease to 38±4 min, whereas the β-like activity increases to 27±7 min (Fig. 2). Also in this case, the injection of 5 mg/kg IV of flunitrazepam to 3 rats, previously exposed to 2.5 mg/kg of the drug, induces EEG effects similar to those observed in naive animals (data not shown).

Clonazepam was tested at the dose of 2 mg/kg IV to 10 and 6 rats in acute and chronic studies, respectively. This dose is 100 times higher than the minimal effective one in this paradigm. Also in this case, acute administration of the drug produces EEG and behavioral changes similar to those observed after the other two BDZ. In naive rats, within the first hour after injection the total periods of the spindle bursts (39±3 min) are significantly higher than those of the β-like activity (22±5 min). Unlike diazepam and flunitrazepam, at the 5th day of treatment the preponderance of the periods of EEG and behavioral sedation over the periods of activation is still maintained (Fig. 2).

The triazolopyridazine derivative Cl 218,872 was injected at the dose of 10 mg/kg IV to 12 and 9 animals in acute and chronic studies, respectively. This dose is 50 times higher than the minimal effective one in inducing EEG changes. Unlike the above reported BDZ, single injection of the drug induces within 0.5–1 min the appearance of the 7–12 Hz spindle bursts associated with weak behavioral sedation. In no case stretching and/or loss of the right reflex is observed at this dose. The EEG record is interrupted by periods of desynchronization, and only few short-lasting periods of

β-like activity can be observed. In no case, however, are signs of behavioral activation noticed. As shown in Fig. 2, within the first hour after single injection, the total period of EEG spindle bursts is lower than that recorded after the BDZ derivatives (30±2 min; $p<0.01$ in respect to flunitrazepam and $p<0.05$ in respect to diazepam and clonazepam). Very low is the occurrence of the β-like activity (5±5 min). A similar pattern is recorded after 5 days of a once-a-day regimen of treatment with the same dose of Cl 218,872.

CONCLUSIONS

Present findings confirm and extend previous data [9,11], indicating that development of tolerance to the sedative action of diazepam is associated with reduction in the EEG of the typical spindle bursts induced by the BDZ. The diminished efficacy occurs after short-term (3–5 days) exposure to very larger doses of diazepam and, although in a lesser degree, flunitrazepam. This can be clearly evidenced during the first hour after injection of both drugs. Under these experimental conditions, the administration of larger doses (two-fold the ones used during repeated treatment) can restore the EEG and behavioral sedative effects of the two BDZ derivatives. This suggests that the suppression of the spindle bursts can be reasonably ascribed to the development of a state of EEG tolerance.

An intriguing finding is the increase of the β-like activity that occurs with development of tolerance to the sedative effect. The association of this EEG pattern with a behavioral stimulatory syndrome indicates an increase of the excitatory component due to BDZ under repeated administration. Such an EEG pattern can be clearly observed in the early phase as well as in several short-lasting episodes in naive rats after injection of diazepam and flunitrazepam, very often associated with signs of behavioral stimulation. An extensive review on the occurrence of tolerance to the sedative effect of BDZ derivatives has been published by File [3]. The author quotes studies indicating that a rapid tolerance (within 3–5 days) to the sedative effects of moderate–high doses of BDZ derivatives occurs in both rats and mice. In contrast, the stimulatory activity of moderate–low doses does not undergo to tolerance within a 20-day period of exposure to BDZ in mice and up to 14 days in rats. Evidence from our laboratory indicate that only through studies of the brain electrical activity is it possible to evidence an increase of the stimulatory effect of BDZ in rats ([9, 11], present data), rabbits [11] and mice (unpublished observation) after short-term exposure to large doses of diazepam. Based on such observations, one can suggest that when tolerance to the sedative action of BDZ is established, their excitatory effects emerge more clearly. Alternatively, the increase of β-like activity might be generated by central adaptive modifications responsible for the reduced sensitivity to the drug. Time-course studies show that the progressive decrease of the spindle bursts associated with the growing of the β-like activity become evident at the 3rd day of treatment. At this time the stimulatory activity can be also observed in the electrocortical pattern up to 24 hours following the last injection [9].

It is interesting to point out that in animals rendered tolerant to the effects of flunitrazepam, no statistically different values for the duration of the spindle bursts and β-like activity are noticed. An equal degree of EEG tolerance has been found at the 3rd day of repeated exposure to diazepam using the same schedule of treatment [9]. Therefore, one can conclude that such an EEG "habituation" develops more slowly with flunitrazepam than with diazepam.

This reduced effect occurs after a once-a-day regimen of administration of the drugs at doses 50 (flunitrazepam) and 100 (diazepam) times higher than the minimal effective ones in this paradigm. It is interesting to point out that the occurrence of such a tolerance to the sedative action of these drugs is irrespective of their duration of action (*namely*, flunitrazepam and diazepam possessing a short and long half-life, respectively [4,5]), of their relative pharmacological profile (*namely*, more efficacious as hypnotic and anxiolytic, respectively [4,5]) and of the relationship between their potency for enhancing GABA receptor activity and their binding affinity, as shown by Farber *et al.* [2] in chicken embryos (flunitrazepam being more potent than diazepam).

Unlike the above reported BDZ derivatives, signs of EEG tolerance do not seem to occur after clonazepam and Cl 218,872 over a so short-lasting period of exposure at doses 100 and 50 times higher than the minimal effective ones in this paradigm, respectively.

Flunitrazepam and diazepam bind at all three types of BDZ receptors (*namely*, BDZ_1, BDZ_2 and the co-called peripheral receptor). Clonazepam selectively binds to BDZ_1 and BDZ_2 receptor types, and Cl 218,872 selectively binds to the BDZ_1 type (see [13]). This leads to speculation that the so called peripheral BDZ receptor types present in the brain might be relevant in establishing tolerance to the EEG and behavioral effects of agonists of BDZ receptors. However, this possibility is not confirmed by biochemical studies carried out after repeated exposure to several BDZ derivatives. They show that tolerance to the sedative effects is accompanied by reduction of the functional interaction between BDZ and GABA receptors. No consistent data are instead reported in the literature on possible modifications of the BDZ receptor density. It is interesting to mention that two studies report statistically significant changes of ^{3}H-diazepam binding affinity in rats after short-term administration of diazepam (increase [9]) and clonazepam (decrease [12]). These data, however, have not been confirmed by others.

According to Haefely [6], the findings of Chang and Farber [1] would suggest that diazepam and flunitrazepam are full agonists, whereas Cl 218,872 is a partial agonist with low efficacy and clonazepam is a partial agonist with intermediate efficacy. Based on this, present data indicate that the ability of a given BDZ agonist to induce signs of EEG tolerance within a short-term exposure is a function of its pharmacological efficacy. Therefore, the study of the electrocortical activity after short-term exposure to doses that largely exceed the minimal effective ones can be a good model to evaluate the efficacy of agonists of BDZ receptor.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. V. G. Longo for his criticism and advice in the course of this investigation. A.V. is recipient of a Ente Universitario Lombardia Centrale fellowship. This work was supported in part by Contract 86.02039.56 of the National Research Council (Consiglio Nasionale delle Ricerche) of Italy.

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