Naunyn-Schmiedeberg's Arch Pharmacol (1994) 350:393–397

Naunyn-Schmiedeberg's
Archives of
Pharmacology

Convulsant action of a benzodiazepine receptor agonist/inverse agonist Ro 19-4603 in developing rats

H. Kubová[1,2], **P. Mareš**[1,3]

[1] Institute of Physiology, Academy of Sciences of the Czech Republic
[2] Department of Pharmacology and [3] Department of Pathophysiology, 3rd Medical Faculty, Charles University, Prague, Czech Republic

Received: 7 September 1993/Accepted: 5 May 1994

Abstract. An inverse benzodiazepine receptor agonist Ro 19-4603, administered intraperitoneally, was found to induce two types of motor seizures, i.e. minimal, predominantly clonic and major, generalized tonic-clonic, in rats at all developmental stages studied (7, 12, 18 and 25 days old). The developmental profile of the two types of seizure was different. Minimal seizures could be induced easily in the two youngest groups, whereas there were no marked differences in the induction of major seizures between the age groups. A lethal outcome was more common in 18- and 25-day-old rats than in younger animals. The convulsant action of the benzodiazepine agonist/inverse agonist Ro 19-4603 shows only quantitative changes during post-natal development in the rat.

Key words: Seizures – Development – Rat – Benzodiazepine – Inverse agonist

Introduction

Disinhibition is one of the ways to induce epileptic activity in vivo and in vitro (Woodbury 1980; Engel 1989), Heinemann and Jones 1990). Interference with GABA-ergic inhibitory systems results in seizures in adult (review: Meldrum 1975) as well as in immature animals (review: Mareš 1991). We have demonstrated the convulsant action of inhibitors of glutamate decarboxylase, $GABA_A$ receptor antagonists and chloride channel blockers during development in rats (review: Mareš 1991). Because of slight differences between the effects of these drugs it was of interest to study the effect of benzodiazepine receptor agonists/inverse agonists. There is only one abstract in the literature in which a convulsant action of methyl-beta-carboline-3-carboxylate (beta-CCM) in immature rats is described (Cavalheiro et al. 1987). These authors found that this benzodiazepine receptor agonist/inverse agonist was first able to induce clearly discernible seizures (i.e. the adult pattern) in rats at the age of 35 days. Such a late appearance of the convulsant effect is in sharp contrast with the marked anticonvulsant action of benzodiazepine agonists which can already be demonstrated one week postnatally (Kubová and Mareš 1989, 1992). Therefore, we wished to re-examine the action of benzodiazepine receptor agonists/inverse agonists during development. By courtesy of F. Hoffman La Roche AG it was possible to do this with the benzodiazepine receptor agonist/inverse agonist Ro 19-4603 (Pieri 1988).

Abbreviations: DPPC, Diplamitoylphosphat

Correspondence to: P. Mareš, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220, Prague 4, Czech Republic

Methods

Motor seizures. Experiments were done with specific pathogen free, male, Wistar albino rats ($n = 112$). Four age groups were used: 7, 12, 18 and 25 days old. Ro 19-4603, a imidazothieno-diazepinone (tert-butyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]thieno[2,3-f] [1,4]diazepine-3-carboxylate; Hoffman La Roche, was freshly dissolved in dimethylsulfoxide (100 mg/ml) and administered intraperitoneally in doses of 25, 50, 75 or 100 mg/kg. Each experimental group consisted of eight animals.

Rats were observed individually for 30 min after the injection. The body temperature was maintained by means of a heating pad. The incidence of the two types of motor seizures, i.e. minimal, predominantly clonic seizures, and major, generalized tonic-clonic seizures, as well as their latencies, were recorded. Other epileptic phenomena (e.g. isolated myoclonic jerks), and behavioral abnormalities, and lethality were also recorded. The severity of epileptic phenomena was scored by means of the following scale (Pohl and Mareš, 1987):

0 – no change
0.5 – abnormal behavior (e.g. scratching, tremor, orienting reaction in a familiar cage)
1 – isolated myoclonic jerks
2 – atypical minimal seizures, i.e. only some elements of minimal seizures
3 – minimal seizures, predominantly clonic, involving head and forelimb muscles, with preservation of righting reflexes
4 – major seizures without a tonic phase
5 – complete major seizures, i.e. generalized tonic-clonic seizures with loss of righting reflexes.

Each animal was scored according to the most severe phenomenon observed; then, for each group, an average and a standard error of the mean were calculated.

Incidences of seizures were evaluated by means of Fisher's exact test, latencies were statistically evaluated by analysis of variance (BMDP) with subsequent sequential comparison according to Holm (1979). CD_{50}s for major seizures were calculated by means of Finney's probit analysis (BMDP). Seizure severity scores were statistically compared by means of the Kruskal-Wallis nonparametric test. The level of statistical significance was set at five percent.

Electrocorticographic recordings. These experiments were made on 24 male Wistar rats, which had been bred at the same time, when they were 7, 12, 18 and 25 days old. The rats were implanted with electrodes under ether anesthesia according to the method described previously (Schickerová et al. 1984). Flat silver electrodes were placed, epidurally, over the sensorimotor as well as over the occipital cortex of both hemispheres and an indifferent electrode was placed on the nasal bone. All electrodes were fixed with fast-curing, dental acrylic cement. One hour after preparation, the animals were neurologically examined and, if normal, were used for EEG recording. Both reference and bipolar connections were used. Ro 19-4603 was dissolved as described above and injected i.p. at a dose of 100 mg/kg in all age groups. The EEG was recorded for 30 min after the administration of the drug.

At the end of the experiments, the animals were killed with an overdose of ether.

Results

Motor seizures. The first behavioral change observed in all age groups was restlessness accompanied by hyperventilation. This was followed by isolated myoclonic jerks. In rats aged 18 days or more, hyperactivity alternated with periods of behavioral freezing.

Ro 19-4603 induced both basic types of seizures, i.e. minimal, clonic and major, generalized tonic-clonic, in rats of all age groups studied.

Minimal seizures. This type of seizure was elicited, in rats of all age groups, in a dose-dependent manner (Fig. 1); all animals exhibited clonic seizures which involved muscles of the head and the forelimbs. In 18- and 25-day-old rats, the hindlimbs were widely abducted and the tail was erected in a 'Straub-like' fashion. An incomplete pattern of minimal seizures often occurred in younger rats. After lower doses of Ro 19-4603, repetitive minimal seizures were observed in 7- and 12-day-old animals. Atypical minimal seizures, continuing for several minutes (status of minimal seizures), occurred only exceptionally in 7-, 12- and 18-day-old animals. A significantly higher incidence of minimal seizures after the 50-mg/kg dose was observed in 7- and 12-day-old rat pups in comparison with the 25-day-old group and the appearance of these seizures after the 25-mg/kg dose in the two youngest groups demonstrated that minimal seizures can be induced at an early stage of development.

Neither age- or dose-dependence of the latencies of minimal seizures could be found (Table 1).

Major seizures. This type of motor seizure was also elicited in a dose-dependent manner in all age groups (Fig. 1). Generalized tonic-clonic seizures were observed at all developmental stages studied, although the incidence of the complete tonic phase in fore- and hindlimbs was higher

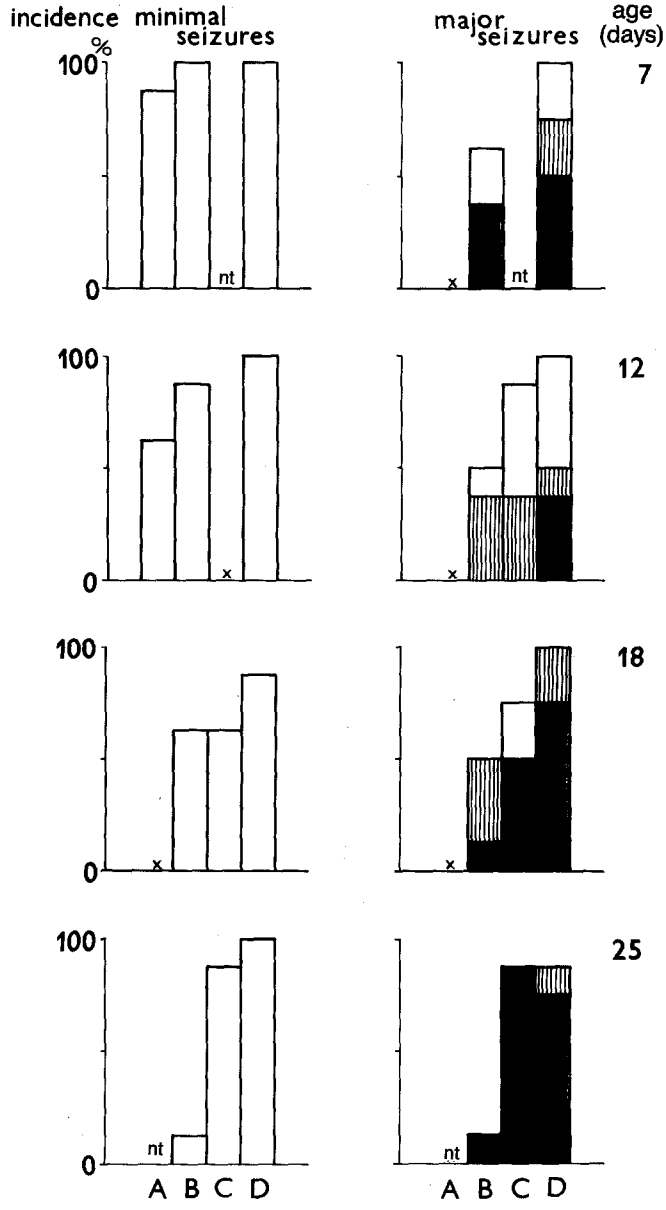

Fig. 1. Incidence of minimal seizures (*left*) and major seizures (*right*) in rat pups aged 7, 12, 18, and 25 days ($n = 8$ for each group). *Abscissae*: doses (mg/kg, i.p.) of Ro 19-4603: A = 25, B = 50, C = 75, and D = 100. *Ordinates*: percentage of animals exhibiting seizures. x: seizures not elicited, nt: dose not tested. Right row of columns: *black areas* represent complete tonic-clonic seizures, i.e. the tonic phase involved all four limbs; *shaded areas* represent seizures in which the tonic phase was restricted to the forelimbs; white areas represent purely clonic seizures

in 18- and 25-day-old animals than in younger rats. A dose of 100 mg/kg elicited complete tonic-clonic seizures in 50% and 37.5% of 7- and 12-day-old animals, respectively, whereas complete tonic-clonic seizures occurred in 75% of the 18- and 25-day-old rats. The major seizures started with a short phase of wild running (in the youngest animals this was replaced by swimming – like, poorly coordinated movements) which was followed by a loss of righting reflexes at the beginning of the tonic phase. The tonic phase was usually made up of tonic extension of the forelimbs. Involvement of the hindlimbs was less com-

Table 1. Latencies (seconds) of seizures induced in rats by Ro 19-4603

Age (days)	Dose (mg/kg i.p.)			
	25	50	75	100
Minimal seizures				
7	331 ± 62	475 ± 184	nt	131 ± 15
12	431 ± 154	408 ± 86	–	541 ± 446
18	–	553 ± 151	86 ± 8	84 ± 5[a]
25	nt	[1]	89 ± 6	252 ± 104
Generalized tonic-clonic seizures				
7	–	330 ± 62	nt	193 ± 17
12	–	622 ± 191	200 ± 69	134 ± 16[a]
18	–	188 ± 18[a] [3]	189 ± 56	115 ± 21[a,b]
25	nt	[1]	305 ± 37	254 ± 71

Values are means ± SEM, $n = 8$. nt, not tested; –, seizures did not appear during the 30 min observation period; [1], only one rat exhibited seizures; [3], values from three rats only

[a] Significantly different from the corresponding value in 7-day-old rat pups

[b] Significantly different from the value in 25-day-old rats

mon in the two younger groups (Fig. 1), and, in these groups, the tonic extension of the hindlimbs was observed only exceptionally. The CD_{50} for major seizures was nearly the same throughout development (56.9 ± 6.0; 51.3 ± 5.0; 52.5 ± 5.6; and 64.0 ± 6.5 mg/kg for the four age groups studied).

The latencies of generalized tonic-clonic seizures showed a tendency to shorten, in a dose dependent manner, in all age groups (Table 1). The latencies of major seizures induced by the 100 mg/kg dose were shortest in 18-day-old rats.

Severity of seizures (Table 2). The severity of seizures increased in a dose-dependent manner in all age groups studied. There were significant differences between the two youngest groups and the 18-day-old rats (at a dose of 25 mg/kg) and between the three younger groups and the 25-day-old rats (at a dose of 50 mg/kg). This demonstrates a decreasing sensitivity to the convulsant effects of Ro 19-4603 during maturation.

Lethality. Some of the rat pups, at all ages, died after the highest dose of Ro 19-4603 whereas the 75-mg/kg dose was lethal only for 18- and 25-day-old animals (Table 3). This demonstrates an increasing sensitivity with age.

Table 2. Severity of seizures induced by Ro 19-4603

Age (days)	Dose (mg/kg i.p.)			
	25	50	75	100
7	2.38 ± 0.26	3.63 ± 0.50	nt	4.75 ± 0.16
12	2.00 ± 0.33	3.25 ± 0.59	4.00 ± 0.46	4.50 ± 0.19
18	0.54 ± 0.06[a]	3.38 ± 0.65	3.88 ± 0.55	5.00
25	nt	1.31 ± 0.53[a]	4.75 ± 0.25	4.75 ± 0.25

Values are scores (see Methods) and are means ± SEM; $n = 8$; nt = not tested

[a] Significantly different from values for all younger groups

Table 3. Lethality of Ro-19-4603

Age (days)	Dose (mg/kg, i.p.)			
	25	50	75	100
7	0/8	0/8	nt	1/8
12	0/8	0/8	0/8	4/8
18	0/8	0/8	2/8	4/8
25	nt	0/8	1/8	6/8

nt: not tested

EEG recordings. Episodes of spike-and-wave rhythm (with a frequency from 4 to 6 Hz) were the first sign of the action of Ro 19-4603 in 25- and 18-day-old rats. This activity was invariably accompanied by an arrest of locomotion and, often, by minute movements of facial muscles at the frequency of the EEG spike-and-wave complexes. Younger animals did not exhibit similar rhythmic activity; isolated sharp elements or EEG seizures (described below) represented the first changes.

Seizures were recorded in all age groups studied. Both patterns of motor seizures, i.e. minimal and major, were observed. Exceptionally, the 100-mg/kg dose was too high to elicit both types of seizures sequentially and, as a result, some animals exhibited generalized tonic-clonic seizures as the first seizure activity. Minimal seizures were usually accompanied by spike-and-wave rhythm [with a somewhat higher frequency (up to 8 Hz) than in the case of rhythmic activity in 25- and 18-day-old animals]. Twelve-day-old rats exhibited rhythmic complexes of sharp and slow waves. Such complexes were sometimes recorded also in 7-day-old rat pups, but, more often, minimal seizures were not accompanied by a specific epileptic EEG activity.

Major seizures were characterized by a wide variety of EEG graphoelements. At the start of such seizures (running and tonic phases) spikes were observed in 25- and 18-day-old rats, sharp waves in 12-day-old rats, and slow waves in 7-day-old pups. The two oldest groups then exhibited spike-and-wave rhythm, polyspike-and-wave rhythm and, from time to time, short periods of suppressed EEG activity. These changes in the EEG pattern took place during the clonic phase when the motor pattern of seizures remained practically unchanged. Similar EEG recordings were obtained in 12-day-old animals, however, the frequency of individual graphoelements was somewhat lower. Seven-day-old rats exhibited large delta waves, which were sometimes followed by fast spikes with low amplitude, during the whole course of the major seizures. The electroclinical correlation was poor in all age groups, but was especially so in the youngest groups.

Discussion

Minimal and major seizures represent two basic types of motor seizure in rodents. They can be elicited in adult an-

imals by many drugs having different mechanisms of action (for review Swinyard 1973; Woodbury 1980; Löscher and Schmidt 1988). Ontogenetic development of these two types of seizure shows substantial differences: generalized tonic-clonic seizures, the generator of which was, hypothetically, localized in the brainstem structures (Browning 1985; Browning and Nelson 1985), could be induced at all developmental stages by every drug studied (for review Mareš 1991). The exact pattern of the seizures is determined by the level of maturation of the motor system. In contrast, minimal seizures could not be elicited by some convulsant drugs (strychnine: Engelhardt and Esbérard 1968; Kubová and Mareš, unpublished; NMDA: Mareš and Velíšek 1992; homocysteic acid; Kubová et al., unpublished. Convulsants interfering with the GABAergic inhibitory system elicited minimal seizures at different developmental stages. Isonicotinylhydrazine and 3-mercapto-propionic acid, inhibitors of glutamate decarboxylase, and picrotoxin and Ro 5-3663, drugs which bind to the chloride channel, induce minimal seizures during the first postnatal week. Bicuculline causes such seizures from the age of 12 days and pentylenetetrazol does so from the third postnatal week (review: Mareš 1991). The generator of these seizures, localized the basal forebrain (Browning and Nelson 1985, 1986), is able to generate minimal seizures even at very early stages of maturation, but the ability of this generator to be triggered by different drugs (probably in different ways) matures unevenly.

Our data, demonstrating a convulsant action of the benzodiazepine receptor agonist/inverse agonist Ro 19-4603, even in the youngest rat pups studied, correspond with the postnatal development of benzodiazepine receptors (Braestrup and Nielsen 1978; Candy and Martin 1979; Lippa et al. 1981) as well as with the development of marked anticonvulsant action of benzodiazepine agonists (Kubová and Mareš 1989, 1992). The contradiction of the results of Cavalheiro's group cannot be explained at present. The dose of beta-CCM that was used by these investigators (20 mg/kg might be insufficient for young animals, but the absence of marked developmental changes in the CD_{50} of Ro 19-4603 for generalized tonic-clonic seizures speaks against this possibility. Beta-CCM is rapidly metabolized in adult rats (Schweri et al. 1983) and it seems improbable that its metabolism might be even faster in immature animals. Another factor which must be taken into account is the heterogeneity of benzodiazepine receptors (Klepner et al. 1979; Sieghart 1983). Molecular biological studies have demonstrated that different alpha subunits of the GABA/benzodiazepine receptor complex may play a role in this heterogeneity (Pritchett et al. 1989). In addition, Ro 19-4603 was found to have a high affinity for diazepam-insensitive benzodiazepine receptors (Wong and Skolnick 1992). There are not yet sufficient developmental data to decide which (if any) of the above mentioned factors plays a role in the differences between the action of Ro 19-4603 and beta-CCM.

Differences in the development of sensitivity to the various effects of Ro 19-4603, i.e. decreasing sensitivity of the generator of minimal seizures with age, almost the same incidence of generalized tonic-clonic seizures in all age groups and the higher lethality in 18- and 25-day-old rats, reflect quantitative changes in the action of the benzodiazepine during maturation. Brain regional differences in these changes (with a probable role for heterogeneity of benzodiazepine receptors) might explain the uneven development of the phenomena observed. This possibility remains to be analyzed in future experiments.

Acknowledgements. We wish to thank Hoffmann La Roche Co, Basel for a gift of Ro 19-4603. This study was supported by grant No. 71157 from the Academy of Sciences of the Czech Republic.

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