

Action of Two Neuroactive Steroids Against Motor Seizures Induced by Pentetrazol in Rats During Ontogeny

P. MAREŠ, R. HAUGVICOVÁ, A. KASAL¹

Institute of Physiology and ¹Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Summary

The anticonvulsant action of two neuroactive steroids, 3 α -hydroxy-5 β -pregnan-20-one (pregnanolone) and triethylammonium 3 α -hydroxy-20-oxo-5 α -pregnan-21-yl hydrogensuccinate (THDOC-conjugate), was tested against motor seizures induced by pentetrazol in immature rats. Five age groups (7, 12, 18 and 25 days old and adult rats) were pretreated with the steroids in doses from 2.5 to 40 mg/kg i.p. Twenty minutes later pentetrazol (100 mg/kg s.c.) was administered. Minimal seizures (clonic seizures of head and forelimb muscles with preserved righting ability) could be induced in the three older age groups. They were suppressed by pregnanolone in all these tested groups (this effect was best expressed in 18-day-old rats and decreased with age), whereas significant changes in THDOC-conjugate-pretreated animals appeared only in 18-day-old rats. Generalized tonic-clonic seizures were suppressed by both neuroactive steroids in all age groups, this effect being more marked with pregnanolone and again decreased with age. The 7- and 12-day-old rats exhibited higher sensitivity of the tonic phase so that generalized clonic seizures were observed. Duration of the effect was studied in 12- and 25-day-old animals; it was substantially shorter in the older rats than in 12-day-old animals. Both drugs exhibited an anticonvulsant action in developing rats but, unfortunately, their effect was only shortlasting.

Key words

Convulsions • Rat • Development • Pregnanolone • New neuroactive steroid • Anticonvulsant action • Time course of action

Introduction

Behavioral properties of neuroactive steroids include sedative, general anesthetic, analgesic, anxiolytic and anticonvulsant action (Lambert *et al.* 1995, Gasior *et al.* 1999, Rupprecht and Holsboer 1999). All these actions can be due to their positive allosteric modulation of the GABA_A receptor (Lambert *et al.* 2003). The main effect is a prolongation of the opening time of the

chloride channel (Akk *et al.* 2004). Anticonvulsant action of progesterone metabolites was described a long time ago (Selye 1942) and extensive literature on this action in different chemically induced models of epileptic seizures in adult laboratory animals has appeared since that time (Belelli *et al.* 1989, Kokate *et al.* 1994, Wieland *et al.* 1995, Carter *et al.* 1997, Gasior *et al.* 1997). Various neurosteroids were found to suppress clonic seizures induced by pentetrazol (Belelli *et al.* 1989, Kokate *et al.*

1994, Wieland *et al.* 1995, Carter *et al.* 1997, Gasior *et al.* 1997), seizures elicited by bicuculline, picrotoxin, kainic acid, cocaine (Belelli *et al.* 1989, Kokate *et al.* 1996, Carter *et al.* 1997, Gasior *et al.* 1997), partial seizures induced by 6-Hz corneal stimulation (Kaminski *et al.* 2004). Their efficacy against pilocarpine-induced *status epilepticus* was also described (Kokate *et al.* 1996). In contrast they do not suppress strychnine- (Belelli *et al.* 1989, Carter *et al.* 1997) and N-methyl-D-aspartate-induced seizures (Gasior *et al.* 1997) and allopregnanolone was found to be ineffective against maximal electroshock seizures (Belelli *et al.* 1989, Kokate *et al.* 1994, Carter *et al.* 1997). The weak aspect of most progesterone derivatives is their fast catabolism in the body and thus a short half-life. A successful attempt was made to prevent catabolism by introduction of beta-methyl group in position 3 to the molecule of allopregnanolone, a metabolite of progesterone. The drug, ganaxolone, is under clinical trials (Perucca and Kupferberg 2002).

Data for developing animals are scarce in spite of the fact that majority of human epilepsies start in infants and children (Hauser 1998). The pioneer work of Liptáková *et al.* (2000) has demonstrated an effect of ganaxolone against flurothyl-induced seizures in four age groups of rats. We started developmental studies with the aim to elucidate possible developmental changes in the action of neurosteroids. The first results with allopregnanolone are in press (Mareš *et al.*). Because of the accessibility of new compounds synthesized in the Institute of Organic Chemistry and Biochemistry CAS in Prague we extended our study to some of them. One of these compounds was promising – triethylammonium 3 α -hydroxy-20-oxo-5 α -pregnan-21-yl hydrogensuccinate (THDOC-conjugate), hence we performed complete screening in developing rats. Pregnanolone as an established drug and a substance present in the brain (i.e. real neurosteroid – Rupperecht and Holsboer 1999) was taken for comparison. For the first testing of anticonvulsant action in immature rats we are using motor seizures elicited by pentetrazol. Two types of seizures can be induced by an appropriate dose of pentetrazol: clonic seizures involving mainly muscles of head and forelimbs with preserved righting reflexes (minimal metrazol seizures), and after a longer latency, generalized tonic-clonic seizures with a loss of righting ability. The tonic and clonic phases of these generalized seizures exhibit different sensitivity to antiepileptic drugs – the tonic phase can be selectively suppressed (e.g. by

carbamazepine – Kubová and Mareš 1993). This model is routinely used in our laboratory and its developmental profile was described in detail (Velíšek *et al.* 1992). As far as the pharmacology of this model is concerned, we have at disposal data on other drugs augmenting GABAergic inhibition (Kubová and Mareš 1991, 1992, Kubová *et al.* 1993, Haugvicová *et al.* 2000), antagonizing excitatory amino acid receptors (Mareš and Mikulecká 2004, Mareš *et al.* 2004) or with another mechanism of action (e.g. Kubová and Mareš 1993). Five age groups are always used to cover different stages of postnatal development in rats: 7-day-old rat pups with a level of brain maturation corresponding to premature babies, 12-day-old ones corresponding to human early postnatal period (Dobbing 1970), 18-day-old ones corresponding approximately to the preschool age, 25-day-old ones with their brain at the level of maturation of prepubertal school age, and adult animals.

Methods

Experiments were performed in five age groups of Wistar albino rats: 7, 12, 18, 25 and 90 days old. Rats were housed under control conditions (12/12 h light/dark cycle with lights on at 6:00 h., temperature 22 \pm 1 °C, humidity 50-60 %) with water and food accessible *ad libitum*. Rat pups were weaned on postnatal day 28. The experimental procedure was approved by the Animal Care and Use Committee of the Institute of Physiology to be in agreement with the Animal Protection Law of the Czech Republic (fully compatible with European Community Council directives 86/609/EEC).

Two neurosteroids were administered: 3 α -hydroxy-5 β -pregnan-20-one (pregnanolone) and triethylammonium 3 α -hydroxy-20-oxo-5 α -pregnan-21-yl hydrogensuccinate (THDOC-conjugate) synthesized in the Institute of Organic Chemistry and Biochemistry. They were freshly put into suspension using a drop of Tween 80; their concentration was 10 mg/ml. The drugs were injected intraperitoneally in doses of 2.5, 5, 10, 20 and 40 mg/kg. Twenty minutes later, pentetrazol (Sigma, MO, freshly prepared water solution at a concentration of 50 mg/ml) was administered subcutaneously in a dose of 100 mg/kg. The animals were then observed in isolation for 30 min and the incidence, pattern and latency of seizures as well as of other behavioral phenomena were registered. Control animals received an injection of physiological saline in a volume corresponding to the highest dose of neurosteroids (i.e. 4 ml/kg) instead of the

neurosteroid. Body temperature of rat pups was maintained by means of a pad electrically heated to 34 °C, i.e. to the temperature in the nest. Individual drugs, age and dose groups consisted of 7-10 animals, control groups were more numerous (up to 16 rats) because they consisted of animals from three different experiments performed in our laboratory at the same time.

An additional experiment was focused on duration of the anticonvulsant action of two neurosteroids (either in the dose of 20 mg/kg i.p.) in 12- and 25-day-old rats. Six intervals between neurosteroid and pentetrazol injections were studied in 12-day-old rat pups – 20, 40, 60, 90, 120 and 180 min. The longest interval was omitted in the 25-day-old group because of the short duration of anticonvulsant action.

Motor seizures were quantified by means of a five-point scale (Pohl and Mareš 1987): 1 – isolated myoclonic jerks; 2 – suspected minimal seizures (only some elements were indicated); 3 – minimal seizures, i.e. clonic seizures of head and forelimb muscles, righting ability preserved with the help of wide abduction of the hindlimbs; 4 – generalized clonic seizures with a loss of righting reflexes; 5 – complete generalized tonic-clonic seizures (tonic phase for at least 5 s) with a loss of righting ability. Animals were scored according to the highest grade exhibited and mean and S.E.M. were calculated for each group.

Incidence of the two types of seizures was statistically evaluated by means of Fischer's exact test; scores and latencies by means of ANOVA with subsequent multiple pairwise comparison by Tukey's test (SigmaStat® SPSS). The level of statistical significance was set at $p=0.05$.

Results

Control animals

The first effect of pentetrazol concerned enhanced activity – locomotion, sniffing. Then isolated myoclonic jerks were observed. Minimal seizures, i.e. clonic seizures of head and forelimb muscles usually lasting 20-40 s with preserved righting ability (hindlimbs widely abducted) appeared regularly in rats 18 days old and older (data not shown). They could be separated from generalized seizures by an interval of up to a few minutes but in some cases they quickly progressed into wild running as the first phase of generalized seizures (lasting only a few seconds). It was followed by a tonic phase (extension of forelimbs and usually flexion of hindlimbs

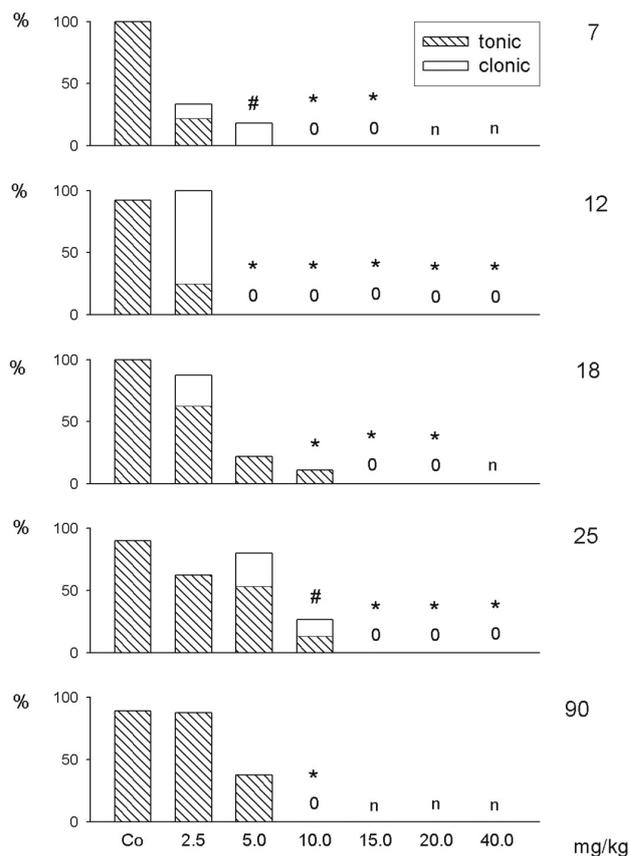


Fig. 1. Incidence of generalized seizures in rats pretreated with pregnanolone. From top to bottom: animals 7, 12, 18, 25 and 90 days old. Abscissa – doses of the neurosteroid, C means control rats; ordinates – percentage of animals exhibiting seizures (hatched parts of columns represent complete tonic-clonic seizures, white parts – generalized clonic seizures without the tonic phase). Asterisks denote significant differences in the incidence of seizures between experimental animals and controls; n – the dose was not tested.

for about 10 s, tonic extension of hindlimbs was observed only exceptionally in our rats) and a clonic phase lasting till the end of the observation period. Righting ability was lost at the beginning of the tonic phase and was not restored during the clonic phase. Rat pups 7 and 12 days old exhibited motor activation, myoclonic jerks and then they progressed directly into generalized tonic-clonic seizures (Fig.1).

Pregnanolone

Motor activation observed in control animals after pentetrazol administration was suppressed by higher doses of pregnanolone in all age groups. If minimal seizures appeared, their motor pattern was not modified. Pretreatment with the two lowest doses of pregnanolone led to an appearance of minimal seizures in a part of 7- and 12-day-old rats, i.e. in age groups where this type of seizures was practically absent in the controls. The three

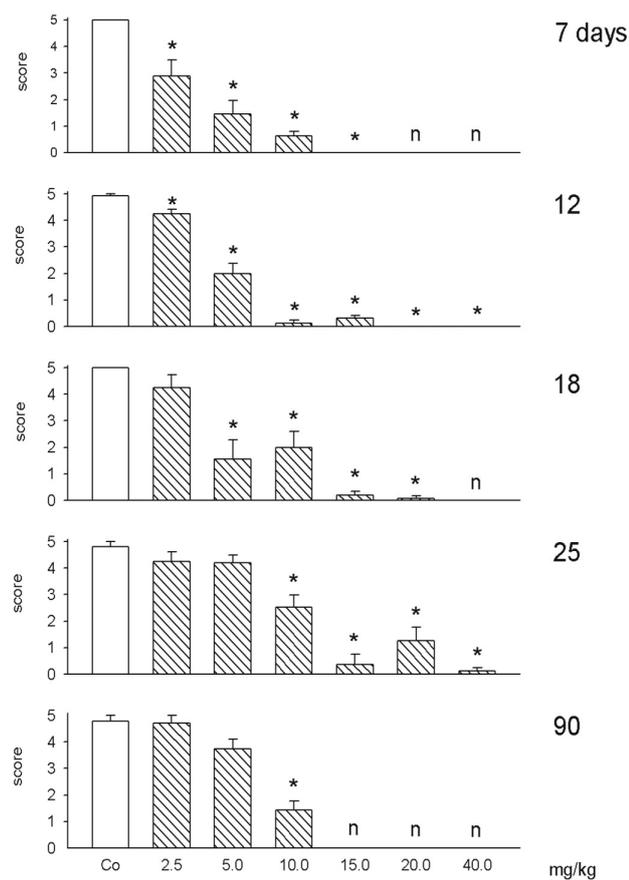


Fig. 2. Severity of seizures in rats pretreated with pregnanolone (mean \pm S.E.M.). Details as in Fig. 1, only ordinates – five-point scale (Pohl and Mareš 1987).

older age groups exhibited a dose-dependent suppression of minimal seizures, a higher sensitivity of 18-day-old rats was evident. Latencies of minimal seizures exhibited a dose-dependent increase in 18-day-old and older animals (data not shown).

Pregnanolone decreased the incidence of generalized tonic-clonic seizures in a dose-dependent manner in all five age groups (Fig. 1). There was a tendency to selective suppression of the tonic phase by lower doses of pregnanolone in all age groups of rat pups but not in adult animals. Latencies of generalized seizures were prolonged by pregnanolone in all age groups with the exception of adults. Statistical evaluation was not possible because of a small number of rats exhibiting generalized seizures.

Seizure severity was decreased by pregnanolone in all five age groups in a dose-dependent manner (Fig. 2). Pregnanolone was more efficient in the three youngest groups than in 25-day-old and adult rats. A statistically significant decrease was found with the dose of 2.5 mg/kg in younger animals whereas the 10 mg/kg dose was necessary in the two eldest groups.

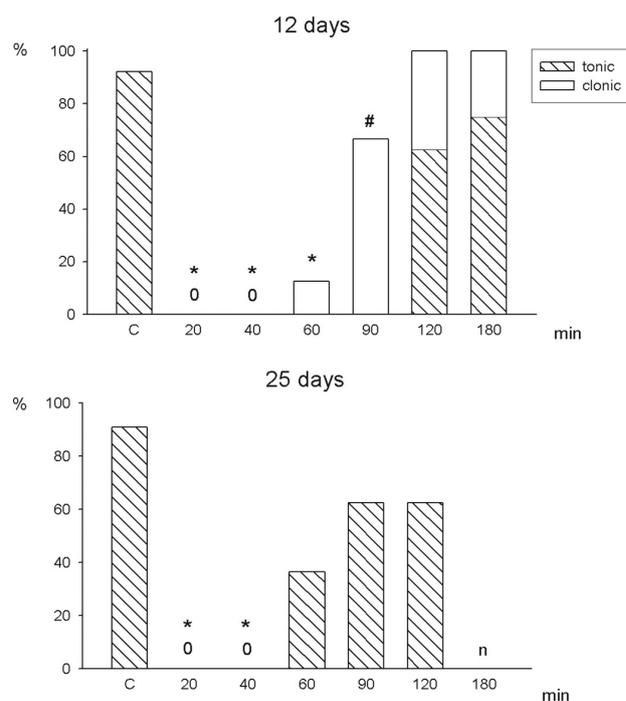


Fig. 3. Incidence of generalized seizures at different intervals after pregnanolone pretreatment (20 mg/kg i.p.). Upper part – 12-day-old rats, lower part – 25-day-old animals. Abscissa – time interval between neurosteroid and pentetrazol injections in minutes, C denotes rats injected only with pentetrazol. Other details as in Fig. 1; 0 means that seizures were abolished; # denoted significant difference in the tonic phase incidence.

Time course of the anticonvulsant action of pregnanolone was different in the two age groups studied. The anticonvulsant effect lasted longer in 12- than in 25-day-old rats. Generalized tonic-clonic seizures were abolished only for 40 min in 25-day-old rats, they reappeared 60 min after the administration. In contrast, there was a difference between suppression of individual phases in 12-day-old animals. The tonic phase was still suppressed 90 min after the administration of pregnanolone, whereas the clonic phase was observed in a majority of rats at this interval (Fig. 3). The dose of pregnanolone used did not significantly affect minimal seizures even at the shortest interval. The action of pregnanolone against generalized seizures and the developmental difference were also reflected in the index of seizure severity (Fig. 4)

The effect of the THDOC-conjugate

Higher doses of this neurosteroid also suppressed motor activation induced by pentetrazol. Minimal seizures only appeared in the youngest group after pretreatment with THDOC-conjugate in doses up to 10 mg/kg. Twelve-day-old rats did not exhibit minimal seizures at all; this type of seizures was dose-dependently

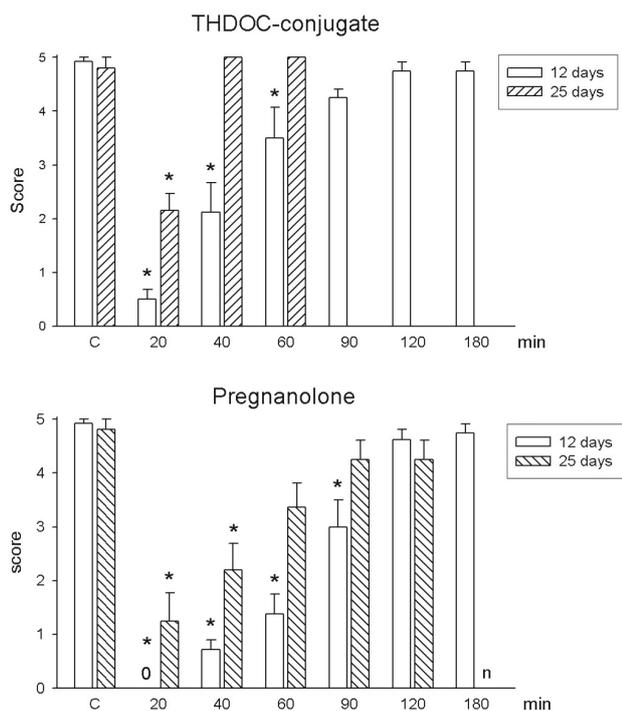


Fig. 4. Severity of seizures at different intervals after pretreatment with neurosteroids. Upper part – pretreatment with THDOC-conjugate; lower part – pretreatment with pregnanolone. White columns denote results in 12-day-old rats, hatched columns – data from 25-day-old animals. Other details as in Fig. 3.

suppressed in 18- and 25-day-old animals. The action of THDOC-conjugate was much better expressed in 18- than in 25-day-old rats. Minimal seizures in adult animals were not suppressed by the doses of THDOC-conjugate used in our experiment. The latency of minimal seizures was increased in a dose-dependent manner in 18- and 25-day-old rats. Unexpectedly, the 5 mg/kg and 10 mg/kg doses of this neurosteroid significantly decreased latencies of minimal seizures in adult rats (data not shown).

The incidence of generalized seizures decreased in a dose-dependent manner in all five age groups (Fig. 5). Higher sensitivity of the tonic than of the clonic phase to the anticonvulsant action of THDOC-conjugate was outlined in 7- and 12-day-old rats, marginally in 18-day-old ones (only one animal exhibited generalized seizures without the tonic phase). Abolition of generalized seizures was reached in all age groups with the exception of adult animals. Latencies of generalized seizures were significantly prolonged by the lowest dose of THDOC-conjugate (2.5 mg/kg) only in 18-day-old rats; in younger animals the dose of 5 mg/kg was sufficient. Twenty-five-day-old rats exhibited a longer latency only after the 15 mg/kg dose. The relation of this

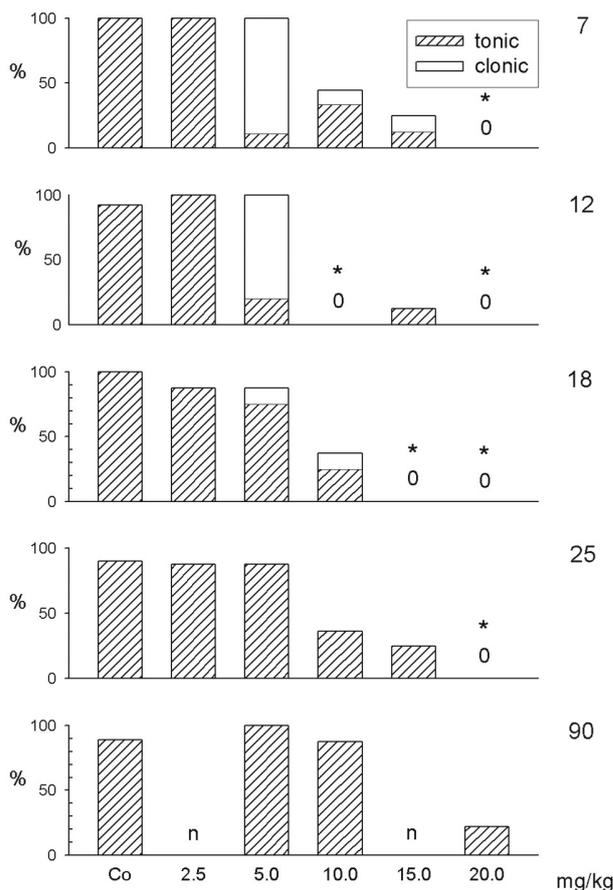


Fig. 5. Incidence of generalized seizures in rats pretreated with THDOC-conjugate. Details as in Fig. 1.

effect to the dose of THDOC-conjugate was at least outlined in all groups of rat pups. No significant difference was observed in adult rats (data not shown).

THDOC-conjugate decreased the severity of seizures dose-dependently in all the age groups studied (Fig. 6). Again the three youngest groups were more sensitive to anticonvulsant action of this drug – the 10 mg/kg dose led to a significant decrease whereas the 15 mg/kg and 20 mg/kg dose was necessary to reach the level of significance in 25-day-old and adult animals, respectively.

Anticonvulsant action of THDOC-conjugate was very short in 25-day-old rats – all rats exhibited generalized tonic-clonic seizures when pentetrazol was injected 40 min after THDOC-conjugate. In contrast, dissociation of the tonic and clonic phases of generalized seizures was observed in 12-day-old rats. The clonic phase appeared in some animals 40 min after the pretreatment in 12-day-old rats whereas only two out of eight rats exhibited complete tonic-clonic seizures 60 and 90 min after the administration (Fig. 7). Seizure severity reflected these differences (Fig. 4).

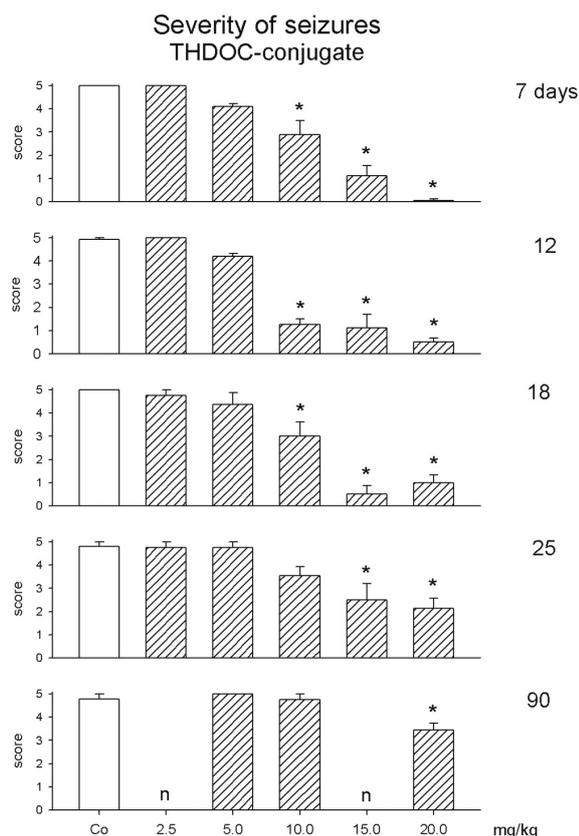


Fig. 6. Severity of seizures in rats pretreated with THDOC-conjugate. Details as in Fig. 2.

Discussion

The action of the two drugs studied in our experiments was similar. There were only some minor differences: activation of minimal seizures in 12-day-old rat pups by pregnanolone but not by the THDOC-conjugate; pregnanolone was effective against both types of seizures in all age groups in lower doses than the THDOC-conjugate. Duration of anticonvulsant action of pregnanolone against generalized seizures was markedly longer than that of THDOC-conjugate in both 12- and 25-day-old rat pups. This finding indicates different pharmacokinetics of the two neurosteroids at least in very immature rats. Unfortunately, there are no published data on pharmacokinetics of any neurosteroid in developing rodents.

From the qualitative point of view the action of the two neurosteroids studied in immature rats is identical with that of allopregnanolone (Mareš *et al.* in press). If effects of individual doses are compared, THDOC is approximately at the same level of efficacy as allopregnanolone, whereas pregnanolone is effective in lower doses. The same relation of anticonvulsant potencies was found in another model of seizures in

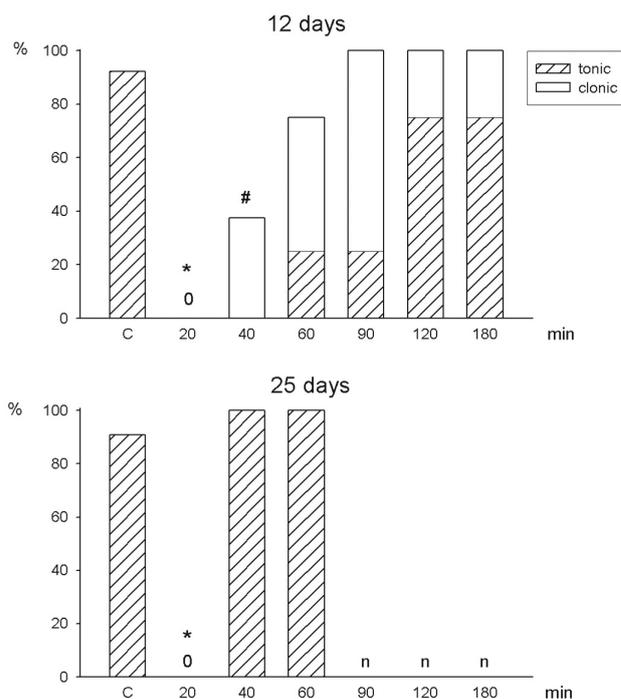


Fig. 7. Incidence of generalized seizures at different intervals after THDOC-conjugate pretreatment (20 mg/kg i.p.). Details as in Fig. 3.

developing rats (12-, 18- and 25-day-old animals were studied) – cortical epileptic afterdischarges (Mareš 2005). Similar difference between pregnanolone and allopregnanolone was also described in behavioral tests in adult mice (Ungard *et al.* 2000).

Both neurosteroids studied in the present experiments as well as allopregnanolone exhibit very short duration of anticonvulsant action in 25-day-old rats. This indicates a short biological half-life. The duration of action is substantially longer in 12-day-old rat pups. This difference might be due to maturation of enzymatic systems metabolizing steroids. The clinically tested neurosteroid, ganaxolone, was synthesized with a clear goal to avoid fast catabolism. Therefore, a methyl group was introduced at the 3-beta position (Carter *et al.* 1997). Modification of the structure used in THDOC-conjugate did not have a similar effect. On the contrary, duration of the effect of this drug is even shorter than that of reference drugs (pregnanolone – this study; allopregnanolone – Mareš *et al.* in press).

It is always necessary to take into account that neuroactive steroids in high (micromolar) concentrations also influence other receptors than GABA_A – nicotinic acetylcholine, glycine and 5-HT receptors (Rupperecht and Holsboer 1999). These actions could play a role in the effects of high doses of neuroactive steroids.

Neurosteroids can have a therapeutic potential

not only in epilepsies but also in anxiety, insomnia, migraine and other neurological and psychiatric disorders (Gasior *et al.* 1999, Wang *et al.* 2001). Therefore, studies of new derivatives are of primary interest.

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Reprint requests

P. Mareš, Institute of Physiology, Academy of Sciences of the Czech Republic, Videňská 1083, 142 20 Prague 4. Czech Republic. E-mail: maresp@biomed.cas.cz