Pergamon

Pharmacology Biochemistry and Behavior, Vol. 52, No. 2, pp. 291–296, 1995

0091-3057/95 $9.50 + .00

0091-3057(95)00115-8

Age-Dependent Anticonvulsant Action of Clonazepam in the *N*-Methyl-D-Aspartate Model of Seizures

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Received 26 October 1994; Accepted 27 February 1995

VELÍŠEK, L. AND P. MAREŠ. *Age-dependent anticonvulsant action of clonazepam in the* N-*methyl*-D-*aspartate model of seizures.* PHARMACOL BIOCHEM BEHAV **52**(2) 291–296, 1995.—Seizures may result from an impaired balance between excitation and inhibition. We tested whether clonazepam [a benzodiazepine that enhances $GABA_A$ inhibitory transmission (0.2 or 1.0 mg/kg, intraperitoneally [IP])] suppresses an age-dependent pattern of *N*-methyl-D-aspartate (NMDA)-induced phenomena in 7-, 12-, 18-, 25-, and 60-day-old rats (10, 40, 100, 100, and 200 mg/kg of NMDA, IP, respectively). There were no effects of clonazepam against the NMDA-induced automatisms and emprosthotonus. In 7-day-old rats, clonazepam was proconvulsant in clonic-tonic seizures (it decreased the latency to onset of seizures), whereas it was anticonvulsant in 25-day-old rats. There was no difference between anticonvulsant effects of clonazepam and its solvent in 12- and 60-day-old rats. Both cortical and hippocampal EEG were extremely suppressed after NMDA, and there was rare specific epileptic activity. The correlation of motor and EEG seizures was extremely poor in this model. There was no improvement of EEG recording after clonazepam. The results demonstrate that impaired excitation cannot be simply balanced by an enhanced inhibition and that the drug effects in young animals cannot be predicted from the effects in adults.

Excitotoxins Rat Seizures Epilepsy Benzodiazepine

EPILEPTIC seizures may result from an impaired balance between excitation and inhibition in the CNS (22). There are models of seizures induced by excitotoxins, drugs that specifically bind to the receptors for excitatory amino acids glutamate and aspartate, thus increasing excitation either in a specific region or throughout the CNS (5,8,13,14,26,40). Seizures induced by the two principal excitotoxins kainic acid (KA) and *N*-methyl-D-aspartic acid (NMDA) have age-dependent features. KA elicits scratching and tonic-clonic seizures in rats during first 3 postnatal weeks, whereas in older rats there are wet dog shakes and clonic seizures of forelimbs and head muscles (1,35,38). NMDA, in addition to tail twisting and clonic-tonic seizures throughout development, produces a special type of flexion seizures (emprosthotonus) in rats until the end of the third postnatal week (21). These developmental features may reflect an age-specific heterogeneity of human epileptic syndromes (39).

In KA- and NMDA-induced seizures, it is possible to restore the altered balance between excitation and inhibition with respective antagonists of the excitatory amino acid receptor. Thus, KA-receptor antagonists block KA-induced seizures and NMDA-receptor antagonists suppress NMDA-induced seizures (32,40). Moreover, there may be a possibility of restoring the impaired excitation–inhibition balance by enhancing inhibitory mechanisms.

GABA is a major inhibitory transmitter throughout the CNS (28). The $GABA_A$ receptor supramolecular complex consists of several binding sites such as the site for GABA, the site for benzodiazepines and additional binding sites inside the chloride channel effector (3). Benzodiazepine binding enhances the function of $GABA_A$ receptor and results in an increased inhibition of the postsynaptic neuron resulting from the hyperpolarizing influx of chloride ions. $GABA_A$ neurotransmission is also age specific. $GABA_A$ receptor number is

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low after birth and steeply increases during the third postnatal week, reaching adult values at approximately 28 days of age in the rat (20). The postnatal development of benzodiazepine receptors (type 1) parallels the development of $GABA_A$ receptors (18).

Therefore, we were interested in whether it is possible to rerestore the balance between the excitation impaired by systemic NMDA administration and inhibition enhanced by benzodiazepine treatment in terms of anticonvulsant effects of the benzodiazepine (12) in the NMDA model of seizures. The experiments were performed in developing rats because of the age-specific features of both NMDA seizures and GABAergic inhibition (20,21,27,34).

METHOD

Male Wistar albino rats (Institute of Physiology breed; n = 159) were used in five age groups: 7, 12, 18, and 25 days old, and young adults (approximately 60 days old, weight 180–200 g). For EEG recordings, we used additional 37 male rats aged 7, 12, 18, and 25 days.

Control rats 7, 12, 18, 25, and 60 days old were injected with NMDA [intraperitoneally (IP), dissolved in normal saline] in doses of 10, 40, 100, 100, and 200 mg/kg, respectively. These doses of NMDA reliably induced seizures in the various age groups (21). Ten minutes before NMDA administration, one additional group was pretreated with a combined solvent (1 ml/kg; propyleneglycol : ethanol : water = 5 : 2 : 3) and two experimental groups with clonazepam [(CZP) Rivotril; Roche, Basle, Switzerland; 0.2 or 1.0 mg/kg in 1 ml/kg, IP]. The rats in solvent and CZP groups received IP NMDA in doses equivalent to their age-matched controls. Immediately after NMDA administration, the rats were put into separate cages (heated to 34°C for pups) and observed for the occurrence and latency to onset of automatisms, flexion seizures, and generalized clonic-tonic convulsions during a 30-min period. Eventually, the mortality of rats was recorded.

For EEG recordings, we operated on 7–25-day-old rats given deep ether anesthesia. After removal of a skin cover of the skull, the parasagital incisurae were cut into the skull with a razor blade. Four cortical electrodes (L-shaped flat silver) were inserted bilaterally into the incisurae above sensorimotor and visual areas. The reference electrode was inserted into the nasal bone. Two twisted wire electrodes were implanted stereotactically into the hippocampus according to the coordinates we determined in our previous experiments (24): for 7-day-old rats, anteroposterior (AP) = 1.8, lateral (L) = 1.5, dorsoventral (DV) = 1.8; for 12-day-old rats, AP = 2.5, L = 1.5, DV = 2.1; for 18-day-old rats, AP = 2.5, L = 1.7, DV = 2.5; and for 25-day-old rats, AP = 2.7, L = 2.0, and DV = 2.7 (all measured in millimeters from bregma). Electrodes were covered with the acrylic, and the rats recovered for at least 2 h. In control rats, we registered baseline EEG and NMDA-induced EEG activity during the 30-min period. In pretreated rats, a baseline EEG, EEG after CZP, and NMDA-induced electrographic changes were recorded. We evaluated the origin, pattern, and propagation of epileptic activity compared to baseline recordings, as well as the correlation of motor and EEG epileptic phenomena induced by NMDA.

The latency to onset of seizures was compared by ANOVA with posthoc Tukey test (25) and the incidence of seizures by Fisher's exact test (16). The level of significance was preset to $p < 0.05$.

RESULTS

Control rats displayed various automatisms. In 12- and 18-day-old rats, there was frequent scratching and tail twisting. In 7- and 25-day-old rats, these automatisms were rare, probably because of the fast development of seizures. In 60-day-old rats, we did recorded no automatisms. There was further development of NMDA effects in 7–18-day-old rats that experienced emprosthotonic (flexion) seizures. This seizure type begins with a loss of righting reflex followed by a body hyperflexion (ball position). Seven- and 18-day-old rats usually regained the righting reflex. However, in 12-day-old pups, we frequently observed a transition from flexion seizures directly to clonic-tonic seizures. Clonic-tonic seizures regularly occurred in all age groups, consisting of a loss of righting reflex (without an eventual recovery), long clonus of all four limbs (several minutes), and a brief tonic phase (several seconds). The rats usually died shortly after the tonic phase (Table 1A).

There were no significant differences in the type, incidence, frequency, and latency to onset of automatisms in solvent- and CZP-treated rats compared to their age-matched controls, except for 12-day-old rats. In this age group, the solvent and both doses of CZP significantly increased the latency to onset of automatisms compared to controls; however, there was no difference between the solvent and CZP pretreatment (data not shown).

The incidence of emprosthotonus in the solvent- and CZP-treated groups of rats did not significantly differ from the incidence of emprosthotonus in controls (50–100% rats). There were only minute effects of either solvent or CZP pretreatment on emprosthotonus. In 7-day-old rats, the solvent increased the latency to emprosthotonus, and the same effect was observed after 1.0 mg/kg of CZP in 12-day-old rats. In 25-day-old rats, we recorded an emprosthotonus in one animal after the solvent pretreatment (data not shown).

CZP pretreatment had mostly anticonvulsant effects against generalized clonic-tonic seizures. In 12-day-old rat pups, the higher dose of CZP (1.0 mg/kg) increased the latency to onset of these seizures [$F(3, 27) = 3.733$; $p < 0.05$]; however there was no difference compared to the solvent-treated group (Fig. 1). In 25-day-old rats, the lower dose of CZP significantly delayed the onset of seizures [$F(2, 8) = 5.037$; $p < 0.05$]. The higher dose of CZP completely abolished clonic-tonic seizures, a significant effect compared to the solvent group (Table 1A). In 60-day-old rats, both doses of CZP and the solvent abolished or suppressed the clonic-tonic seizures. Therefore, there were no data for the statistical evaluation of the latency to onset of clonic-tonic seizures (Table 1A). In contrast, in 7-day-old rats, both doses of CZP accelerated the development of clonic-tonic seizures compared to both controls and solvent-pretreated age-matched rats [$F(3, 25) = 9.579$; $p < 0.05$] (Table 1A and Fig. 1).

Except for 7-day-old rat pups, mortality closely followed the incidence of generalized clonic-tonic seizures. In 12-, 25-, and 60-day-old rats, the higher dose of CZP (1.0 mg/kg) significantly reduced lethal effects of NMDA compared to controls (Table 1B); however, these effects were not significantly different from solvent-treated groups. In 60-day-old rats, the solvent also decreased NMDA-induced lethal effects compared to the age-matched controls.

Changes in Behavior

Control rats (7–18 days old) displayed an intense, unaided biting of surrounding objects as well as self-biting until the

TABLE 1

INCIDENCE OF NMDA-INDUCED GENERALIZED CLONIC-TONIC SEIZURES AND LETHALITY

A: Incidence of Generalized Clonic-Tonic Seizures				
Age	Controls	Solvent	CZP 0.2 mg/kg	CZP 1.0 mg/kg
7 days	7/8	4/8	9/9	9/9
12 days	10/10	6/8	7/8	8/8
18 days	7/8	7/7	8/8	8/9
25 days	5/8	4/7	3/8	0/8*†
60 days	6/8	0/6*	1/6*	0/6*
B: Incidence of NMDA-Induced Lethality				
Age	Controls	Solvent	CZP 0.2 mg/kg	CZP 1.0 mg/kg
7 days	0/8	0/8	0/8	0/8
12 days	10/10	3/8	4/8	2/8*
18 days	5/8	6/7	6/8	6/9
25 days	5/8	2/7	1/8	0/8*
60 days	6/8	0/6*	1/6*	0/6*

Number format x/n shows how many rats (x) of total (n) displayed the phenomenon.

*Statistical significance compared to the age-matched control group ($p < 0.05$), Fisher's exact test.

†Statistical significance compared to the solvent-treated age-matched group ($p < 0.05$) Fisher's exact test.

generalized clonic-tonic seizures occurred after NMDA administration. CZP pretreatment induced an age- and dose-dependent ataxia. The younger the rat, the more pronounced the ataxia was. The higher dose of CZP produced ataxia in all age groups, whereas the lower dose only in 7- and 12-day-old rats. CZP also reduced unaided aggressive and autoaggressive biting.

Electroencephalography

Control EEG recordings consisted of fast, low-voltage activity usually in the β band. In very young 7- and 12-day-old rat pups, the frequency of events was lower than in older age groups. In 7-day-old rats, the incidence of EEG events was also low. Hippocampal recordings usually showed rhythms of higher amplitude and frequency than cortical recordings (Fig. 2, top left).

In 7- and 12-day-old rats following NMDA treatment, there was a substantial depression of the waveform amplitudes. Isolated specific epileptic phenomena (spikes and sharp waves) sometimes interrupted periods of suppressed EEG. In 18- and 25-day-old rats, the NMDA treatment induced > 10-min periods of depressed EEG. The difference from control EEG recorded before the NMDA administration was substantial (Fig. 2, bottom left). This almost isoelectric recording was, however, associated with a variety of motor symptoms from automatisms to generalized clonic-tonic seizures. In 12–25-day-old rats, the limbic type of discharges (serrated waves) occurred exceptionally in both cortical and hippocampal recordings during the late stages of clonic-tonic seizures. In 7-day-old pups during the late stages of tonic clonic seizures, isolated epileptic activity was recorded only in the hippocampus.

In 7- and 12-day-old rats, there were no significant differences in the EEG activity between baseline EEG (or control baseline EEG) and after CZP pretreatment. In contrast, in 18- and 25-day-old rats, pretreatment with CZP increased the amount and amplitude of fast EEG activity before the NMDA application (Fig. 2, top and middle right). However, the combination of CZP and NMDA resulted in more suppressed EEG activity than in age-matched controls after NMDA (Fig. 2, bottom right). There was a reactivation of EEG 5–8 min after the NMDA injection. We observed no specific epileptic activity in CZP-pretreated rats except for a few 12-day-old rats in which high-voltage activity occurred in the hippocampus. In all age groups, the correlation between EEG and motor activity was extremely poor.

DISCUSSION

Our data demonstrate that there is an age-dependent response to NMDA administration and also an age-dependent anticonvulsant activity of CZP. Moreover, the relationships between age and excitation–inhibition are not linear. There is almost no correlation between NMDA-induced motor and EEG effects, and this poor correlation even decreases after the CZP pretreatment.

There were inconsistent effects of CZP pretreatment on the NMDA-induced automatisms and emprosthotonic seizures throughout development. Our previous experiments (35,36) have shown that the automatisms represent a phenomenon originating in a limited brain area, where it may be difficult to suppress the phenotypic expression by enhancing GABAergic inhibition. Emprosthotonic seizures are a unique phenomenon reported only after NMDA pretreatment (21). Their site of origin and relevance to human seizures are unclear. However, the fact that they occur only in developing animals, and their motor expression (flexion seizures) suggest a similarity to hu-

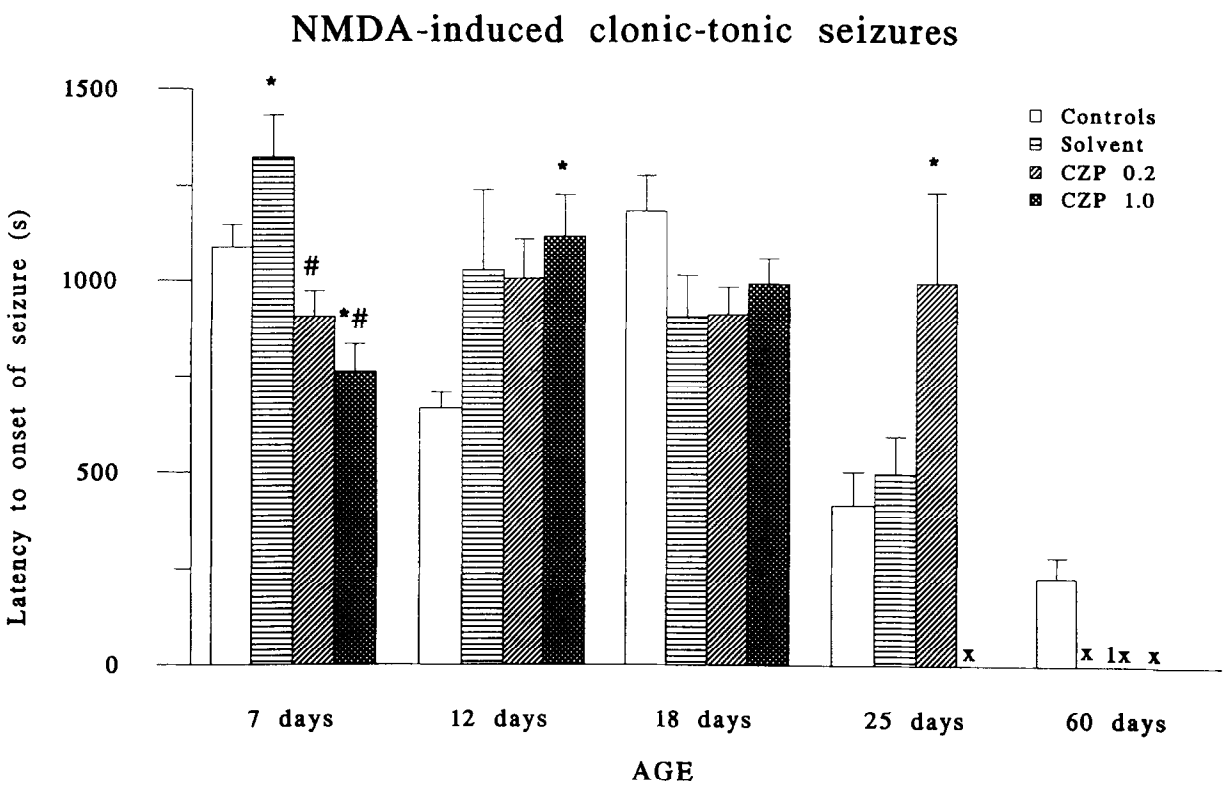

FIG. 1. Latency to onset of clonic-tonic NMDA-induced seizures and the effects of CZP in rats during development. x Axis: age groups (postnatal days); y axis: latency to onset of seizures (in s). *Significant difference compared to age-matched control group (ANOVA with posthoc Tukey test). #Significant difference compared to age-matched solvent group (ANOVA with posthoc Tukey test). [x]Seizures were completely suppressed. [1x]Only one seizure in the subgroup.

man West syndrome (15). This syndrome of flexion seizures appears in early childhood and is extremely resistant to the treatment with antiepileptic drugs. Benzodiazepines are also not an effective treatment in most patients with West syndrome (15). However, this speculation requires further verification.

The effects of CZP on generalized clonic-tonic seizures changed during development. There was a significant proconvulsant action of CZP pretreatment in 7-day-old rat pups compared to both controls and the solvent group. This effect is unclear and may be associated with the low density of $GABA_A$/benzodiazepine type 1 receptors during the first 3 postnatal weeks (2,18,20,33), or it can be mediated by other effects of CZP than at the benzodiazepine site of $GABA_A$ receptor. Alternatively, there may be a different development of $GABA_A$ sites and benzodiazepine sites as well as a developmental difference in their cooperation (20). Finally, there is also a report about excitatory rather than inhibitory $GABA_A$ receptor-mediated effects in some developing neurons (23).

In 12-, 25-, and 60-day-old rats, there were certain anticonvulsant effects of CZP; however, in 12- and 60-day-old rats, the solvent was also anticonvulsant. In 25-day-old rats, there was a clear anticonvulsant action of CZP against clonic-tonic seizures compared to both controls and solvent-treated age-matched rats. In 12- and 60-day-old rats, there were no differences between anticonvulsant effects of CZP and solvent. To determine the precise effects of CZP as distinguished from the anticonvulsant effects of the combined solvent, additional experiments are necessary. These experiments should involve pretreatment with a benzodiazepine antagonist followed by CZP to demonstrate the specific anticonvulsant role of CZP itself. An experiment with a subconvulsive dose of a $GABA_A$ receptor antagonist followed by CZP would highlight the coupling of CZP anticonvulsant effects with the $GABA_A$ site. Unfortunately, the experiments would largely exceed the scope of the present communication. Both active components of the solvent, propyleneglycol and alcohol, may be the anticonvulsants in our model. Alcohol is known to enhance $GABA_A$-ergic transmission. There are also studies showing that alcohol may suppress NMDA receptor transmission and directly interfere with our model (9,17,31). There were no anticonvulsant effects of CZP in 18-day-old rats, probably because of the overwhelming NMDA excitatory neurotransmission especially during the third postnatal week in the rat (4,19,34).

There was suppression of EEG after NMDA treatment, and the EEG did not recover after CZP or during motor seizures. This is in contrast to the KA model of seizures in adult rats, where hippocampal EEG activity precedes motor seizures and always prevails over motor activity (37). However, in KA seizures in the rat pups, the EEG activity often starts in the neocortex and spreads to the hippocampus afterward (6,7). In the NMDA model, we recorded a suppression of EEG seizures in the hippocampus as well. A spreading depression (described after local NMDA) that can mask seizures in the EEG could be an explanation (10,11), although the concurrent onset of the spreading depression in both hemispheres' neocortex and

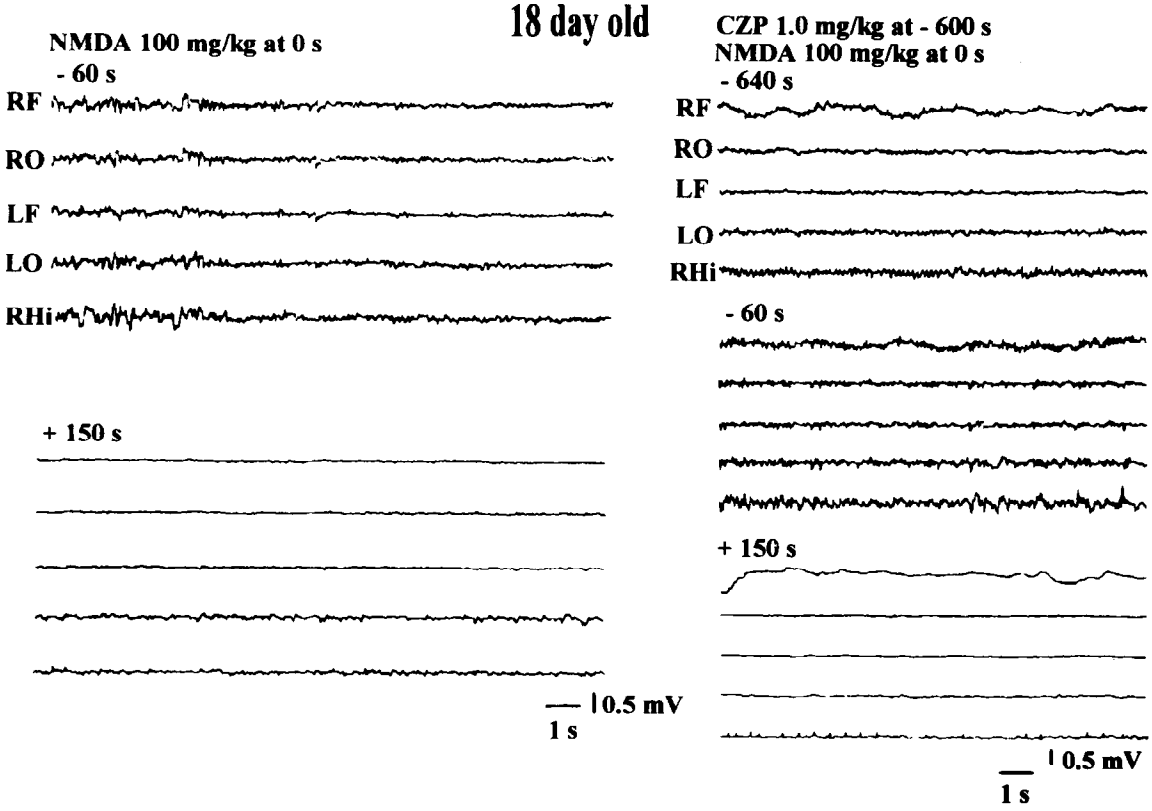

FIG. 2. EEG recordings in control (left) and CZP-pretreated (right) 18-day-old rat. (Left top) Cortical and hippocampal recordings of baseline EEG in the control 18-day-old rat pup 60 s before NMDA administration. RF and RO, right frontal and occipital cortex; LF and LO, left frontal and occipital cortex; RHi, right hippocampus). (Left bottom) Cortical and hippocampal recordings in the same rat 150 s after the administration of 100 mg/kg NMDA. (Right top) Cortical and hippocampal recordings in another 18-day-old rat 40 s before the administration of 1.0 mg/kg CZP (640 s before NMDA). (Right middle) Cortical and hippocampal recordings in the same rat 540 s after the administration of 1.0 mg/kg CZP, but 60 s before the administration of 100 mg/kg NMDA. (Right bottom) Recordings in the same rat 150 s after the administration of 100 mg/kg NMDA. The moderate fluctuation of the RF recording (slow waves) in all three parts on the right was due to the imperfect fixation of the RF electrode. Notice tiny specific epileptic activity (miniature spikes) in the RHi trace in the right bottom part. Time mark 1 s, calibration 0.5 mV.

hippocampi seems bizarre. In any case, epileptiform activity probably spreads fast to the lower brain regions (probably to the brain stem), where it could not be recorded in our setting.

In our model, the effects of CZP were not linear throughout development and changed from proconvulsant to anticonvulsant. This nonlinearity may reflect a different development of inhibitory and excitatory systems (29,30). Early in postnatal life even different mechanisms of action may play a role. The enhanced brain excitation cannot be simply balanced by an increased inhibition, especially early postnatally. In addition, our data suggest that anticonvulsant drug tests should be performed in developing animals, because in early postnatal development the drug effects cannot be predicted from the effects in adult animals.

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