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Age-Dependent Phenytoin Effects on Cortical Stimulation in Rats

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Summary

The effects of phenytoin on threshold intensities of stimulation were studied in cortical epileptic afterdischarges (ADs) in 12-day-old and adult rats with implanted electrodes. Stimulation of the sensorimotor cortical area induced movements directly related to the stimulation as well as EEG afterdischarges (ADs) of the spike-and-wave type and of the limbic type. Rat pups exhibited lower thresholds for stimulation-bound movements and spike-and-wave ADs than adult animals. On the contrary, the limbic type of ADs was elicited with lower current intensity in adult than in immature rats. Phenytoin increased the threshold for stimulation-related movements only in adult rats, whereas threshold intensities for spike-and-wave ADs were increased and thresholds for limbic type of ADs remained uninfluenced in both age groups. The age-dependent effect on stimulation-related movements might be due to a maturation of connectivity in the motor system or to developmental changes in the voltage-gated sodium channels as the main target of phenytoin action.

Key words

Cerebral cortex – Electrical stimulation – Epileptic afterdischarges – Phenytoin – Rat – Ontogeny

Introduction

Phenytoin (PHT) is one of the classical antiepileptic drugs. Clinically PHT is effective mainly against focal epileptic seizures but also against generalized tonic-clonic seizures. Woodbury (1980) classified PHT as a drug suppressing the spread of epileptic activity from the focus. The action of PHT on this spread may be observed in some experimental models of epileptic seizures. More than ten possible mechanisms of action of PHT have been reported but two of them are the most important. They are represented by a decrease of permeability of voltage-gated sodium channels and Ca^{2+} channels (preferentially L-type). Among others, PHT actions exert an influence on both excitatory and inhibitory neurotransmission, and changes in ATPase activity have also been described (DeLorenzo 1995).

Quantitative and even qualitative changes in the action of PHT during ontogenesis were described. This drug even exhibits an excitatory effect during early postnatal development. The net anticonvulsant effect of PHT becomes predominant in rats after the third week of age (Vernadakis and Woodbury 1969). Our previous data demonstrated different effects of PHT in various models of epileptic seizures in developing rats. Its action against the tonic phase of generalised tonic-clonic seizures was found to be stable during ontogenesis (Staňková *et al.* 1992), whereas the convulsant effect of PHT overdose was demonstrated at early developmental stages only (Mareš *et al.* 1987). In addition, cortical interhemispheric (transcallosal) responses in immature and adult rats were differently influenced by PHT (Mareš *et al.* 1993).

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Therefore, 12-day-old and adult animals were used to ascertain if there are developmental changes in PHT action in the observed model of epileptic seizures. This age of rat pups corresponds to early postnatal stages of the development of the human brain (Dobbing 1970), especially as far as the cerebral cortex is concerned (Caley and Maxwell 1968). Changes in the action of PHT have been described at this age (Mareš *et al.* 1993).

In our experiments, we have chosen the model of cortical epileptic afterdischarges (ADs). Rhythmic electrical stimulation of the sensorimotor cortex may elicit two different types of afterdischarges. The first is characterized by spike-and-wave EEG rhythm and is accompanied by clonic seizures of the head and forelimbs (Makal *et al.* 1993, Kubová *et al.* 1996). Similarly, as other spike-and-wave activities (Steriade and Deschenes 1984), it is probably generated in the thalamocortical system. The second type, which appears only when higher stimulation intensities are used (Mareš *et al.* 1996), is characterized by huge delta waves with superimposed spikes (serrated waves) in the EEG. Behavioural changes related to this type of ADs are characterized by automatisms such as face washing, orientation in the home cage and wet dog shakes. The EEG and behavioural manifestations of these ADs are the same as in models of epileptic seizures, generated in the limbic system.

The transition from spike-and-wave type of ADs to the second type of ADs might serve as a model of the spread of epileptic activity from the cortex to deep limbic structures. Therefore we used this phenomenon to test the suppressant effect of PHT on seizure spread. According to Woodbury's classification (Woodbury 1980), an effect of PHT on this type of ADs may be expected. The second question to be answered was whether PHT exhibits a direct effect on the motor system and thus on motor phenomena during stimulation and ADs, as was suggested in earlier reports (Aston and Domino 1961, Blum 1964). The last but not least aim of our study was also to describe possible differences of PHT action in our model at two maturational stages.

Methods

Experiments were performed in 45 adult (90-day-old) and 48 young (12-day-old) male albino rats of the Wistar strain. The animals were kept under standard conditions (temperature 24±1 °C, light/dark cycle 12:12), for adult rats food and water were *ad libitum*. The day of birth of rat pups was counted as day 0, the litters were reduced to eight animals. Surgical preparation was different in adult and young animals. Adult animals were anaesthetized with pentobarbital in a dose of 50 mg/kg i. p. Trephine openings were made with a dental drill. Two stimulation silver ball electrodes were placed epidurally over the right sensorimotor area (AP = −1 and +1, L = 2.5 mm). Flat silver recording electrodes were localized over the contralateral sensorimotor area (AP = 0, L = 2.5 mm) and over occipital visual areas (AP = 6, L = 4 mm) of both hemispheres. An indifferent electrode was inserted into the nasal bone. The electrodes were fixed to the skull by dental acrylic. The animals were allowed to recover for at least one week. Young rats were surgically prepared under ether anaesthesia lasting 15 min on the average. The skull (still imperfectly ossified) was cut with a razor blade and flat silver epidural electrodes (L-shaped with a contact of approximately 1 mm^2) were used for stimulation as well as for recording. The coordinates were recalculated according to the actual bregma-lambda distance taking 8 mm as a value for adult animals. After fixation of electrodes by means of a fast curing dental acrylic, rat pups were allowed to recover for at least one hour, after which we started to stimulate. All animals were placed in plastic boxes during experiments, body temperature of the rat pups was maintained by means of a heating pad. Stimulation was performed by means of a constant current stimulator. Stimulation series lasted 2 seconds and consisted of biphasic rectangular pulses of 1 ms duration and 50 Hz frequency. The intensity of stimulation was changed in the following steps: 0.8 - 1.6 - 2.0 - 3.0 - 4.0 - 5.0 - 6.0 - 8.0 - 10.0 - 12.0 - 15.0 mA in the case of adult rats and 0.8 - 1.0 - 1.2 - 1.4 - 1.6 - 1.8 - 2.0 - 2.5 - 3.0 - 3.5 - 4.0 - 4.5 - 5.0 - 6.0 - 8.0 - 10.0 - 12.0 - 15.0 mA in young rats. These values were chosen on the basis of pilot experiments demonstrating higher thresholds in adult rats. An interval between two stimulation series was at least 10 min. Stimulation was discontinued after the limbic type of afterdischarge had been registered.

Both adult and young animals were divided into three groups:

- The experimental group received an intraperitoneal injection of phenytoin in a dose of 60 mg/kg 10 min before the first stimulation.
- The second, control group was injected with a corresponding volume (i.e. 1.2 ml/kg) of the solvent for phenytoin (a mixture of propylenglycol, ethanol and water in the ratio 5:2:3).
- The third group of animals served as controls without any pretreatment.

EEG (both in reference and bipolar connections) was recorded 15 s before stimulation and 3 min after its end. Motor symptoms were observed and recorded during stimulations as well as during ADs. The following phenomena were recorded: clonic movements accompanying stimulation; electroencephalographic ADs – their pattern and duration; behaviour of animals during ADs and after their termination. The threshold intensities necessary for

elicitation of stimulation-bound movements and the two types of ADs were established and used for statistical evaluation performed by means of two-way analysis of variance (factors treatment and age) with subsequent pairwise comparison (Sigma Stat Jandel). The incidence of the second, limbic type of ADs was evaluated by means of Fisher exact test. The level of statistical significance was set at 5 %.

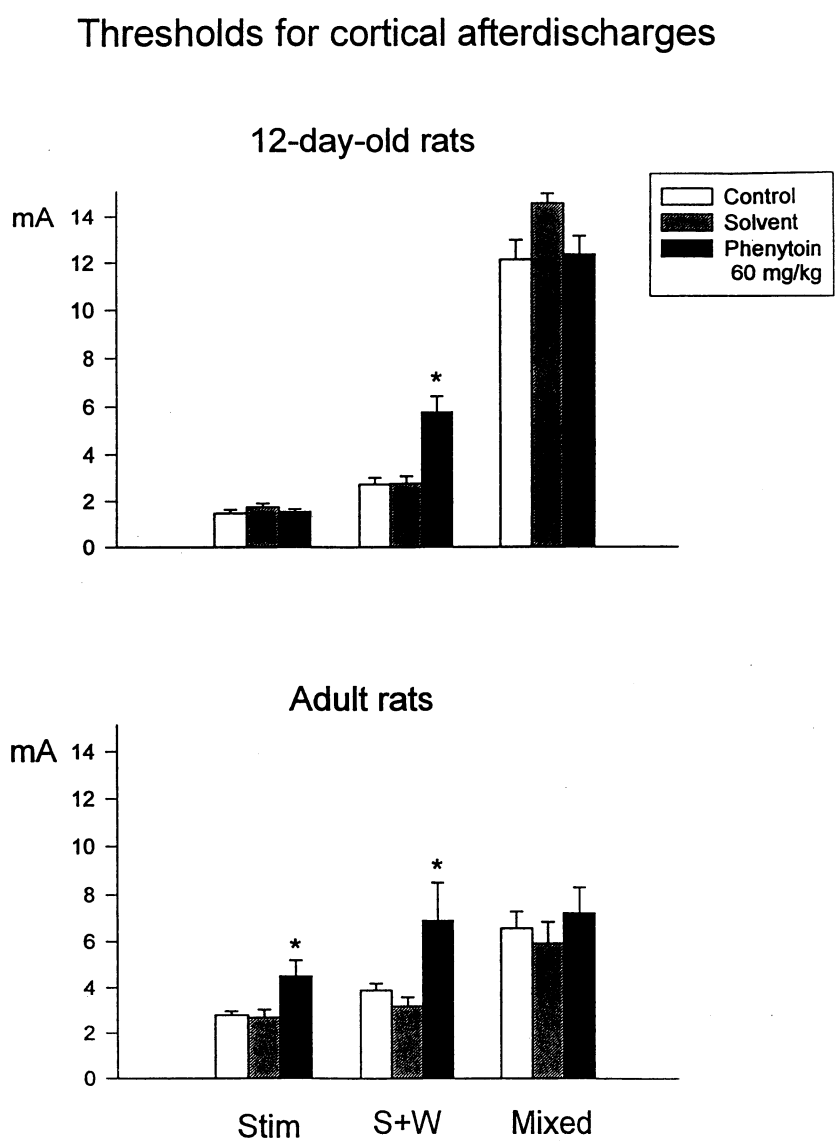

Fig. 1. *Mean threshold current intensities (± S.E.M.) for three phenomena measured in 12-day-old (upper part) and adult (lower part) rats. Abscissae – Stim = stimulation-bound movements, S+W = spike-and-wave type of afterdischarge, Mixed = transition into the limbic type of afterdischarge; ordinates – intensity of currents in mA. Explanation of columns – see inset in the upper right corner. Asterisks denote significant differences in comparison with the solvent group.*

Results

Average thresholds (mean±S.E.M.) for eliciting movements accompanying stimulation (Fig. 1) were lower in the rat pups (1.2±0.2 mA) than in the adults (2.8±0.2 mA). There were differences in the EEG pattern of the spike-and-wave ADs between 12-day-old and adult animals. Young rats were unable to generate this rhythm. They exhibited rhythmic sharp delta waves accompanied by clonic jerks synchronous with these sharp elements similarly as with spikes in older animals (Figs 2 and 3). The same results as for stimulation-bound movements were found for the spike-and-wave type of ADs (2.7±0.3 mA in the youngs and 3.9±0.3 mA in the adults). On the contrary, higher intensities were necessary for eliciting the limbic type of ADs in immature rats (12.±0.84 mA) than in adult rats (6.6±0.7 mA). The EEG pattern was similar in both age groups (Figs 2 and 3).

Non-treated and solvent-injected groups did not significantly differ in the thresholds for all the three phenomena studied. PHT only increased thresholds for movements accompanying stimulation in adult rats (2.8±0.2 vs. 4.5±0.2 mA). The thresholds for spike-and-wave ADs were increased by PHT in both age groups (3.9±0.3 vs. 6.9±1.6 mA in the adults and 2.7±0.3 vs. 5.8±0.6 mA in the youngs), the effect in rat pups was more expressed (Fig. 1). Transition to the

limbic type was not observed in all animals; the incidence was higher in adult than in 12-day-old rats and was not significantly changed by PHT (Table 1). The intensity necessary to elicit the limbic type of ADs was not changed in any age group (6.6±0.7 vs. 7.2±1.1 mA in the adults and 12.1±0.8 vs. 12.3±0.8 mA in the youngs – Fig. 1).

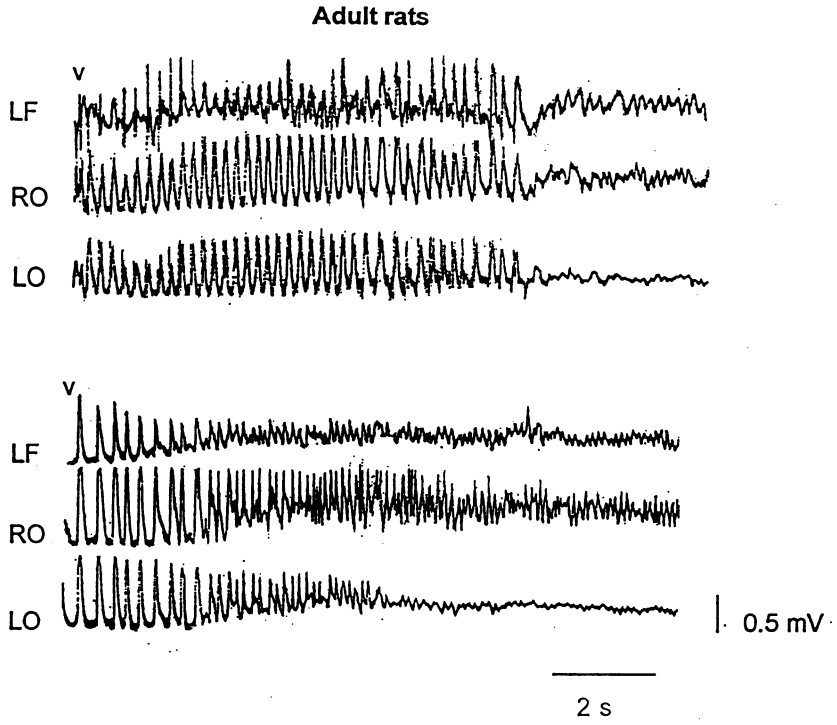

Fig. 2. *Electrocorticographic recordings of spike-and-wave (upper part) and limbic (lower part) afterdischarges in adult rats. In both parts individual leads from top to bottom: LF = left frontal, RO = right occipital, LO = left occipital region in a reference connection. Arrowheads denote the end of stimulation. Amplitude calibration 0.5 mV, time mark 2 s. Note the he presence of spikes in the LF lead in the upper part and progressive decrease of amplitude and uneven termination of the afterdischarge in individual leads of the lower part.*

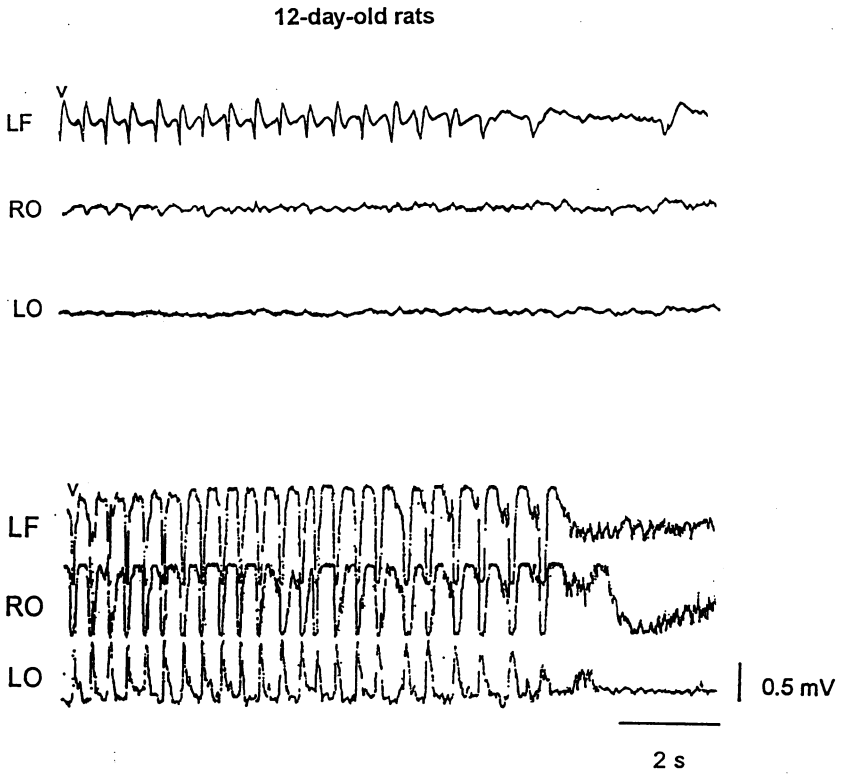

Fig. 3. *Electrocorticographic recordings of spike-and-wave (upper part) and limbic type of afterdischarges (lower part) in 12-day-old rats. Details as in Fig. 2. All graphoelements have longer duration than those corresponding to adult animals. Note that epileptic activity is restricted to the left frontal region in the upper part. Leads LF and RO in the lower part have extremely high amplitude of graphoelements, so that a similarity to the adult recording may be observed in the LO lead in the lower part.*

Table 1. Incidence of the limbic type of afterdischarges

	Controls	Solvent	Phenytoin
12-day-old	9/12	7/13	9/21
90-day-old	19/21	9/10	10/12

Fractions denote number of rats exhibiting limbic afterdischarge/number of rats studied

Discussion

The two age groups studied exhibited markedly different thresholds for all three phenomena evaluated. Higher current intensities were necessary for induction of movements accompanying stimulation and the spike-and-wave type of ADs in the adults than in rat pups. This is in agreement with our previous data obtained with low frequency stimulation (Makal *et al.* 1993, Mareš *et al.* 1996) demonstrating significantly higher thresholds for adult animals in comparison with rat pups using an identical stimulation paradigm. There are two possible sources of errors: one is a size of the brain, the other the difference in methods. Were the size of the brain a decisive factor, then there must be a progressive increase in threshold intensities with growth. In contrast to this presumption, the lowest threshold for movements as well as spike-and-wave afterdischarges was found in 18-day-old rats with both 8-Hz and 50-Hz stimulation (Makal *et al.* 1993, Haugvicová *et al.* in preparation). The difference in methods has to be taken into account but the finding that the threshold in 35-day-old rats is significantly higher than that in 25-day-old animals (Haugvicová *et al.* in preparation) speaks against an important role of this factor. Our results might thus reflect higher excitability of the immature brain and might be taken as a sign of a higher seizure susceptibility of the developing brain. The inability of the immature cortex to generate a spike-and-wave rhythm may be due to immaturity of reciprocal thalamo-cortical connections (Scheibel *et al.* 1976) as well as cerebral cortex (Caley and Maxwell 1968). Rhythmic delta waves represent correlates of spikes in the mature spike-and-wave rhythm; there is no correlate of the wave component in 12-day-old rats. On the other hand, immature rats exhibited higher thresholds for evoking the limbic type of ADs, i. e. transition from spike-and-wave rhythm to delta waves in the EEG with an appropriate change of behaviour in both the present and previous experiments. The routes of spread of epileptic activity from the cortex to the limbic system must be taken into account in explaining this result. There are no direct connections between the sensorimotor cortex and the limbic system (Lopes da Silva *et al.* 1990). Polysynaptic neuronal paths have to be used. Three most probable pathways of spread are mediated by the thalamus: anterior nuclei, mediodorsal nucleus and posterior nucleus have direct connections with different limbic structures (Price 1995). The immaturity of the connections between the cortex, thalamus and the limbic structures might impede the spread of epileptic activity and thus raise the thresholds for the limbic type of ADs in 12-day-old animals. The spread through entorhinal cortex cannot be excluded, but it is less probable. Entorhinal cortex receives fibres from neocortical association areas (Lopes da Silva *et al.* 1990), so that here is again a polysynaptic pathway from the sensorimotor cortical area to the limbic system.

There are two different levels in explanation of our findings as concerns the action of PHT: 1. structures of the central nervous system, which might represent targets for PHT, and 2. the molecular level of effects.

PHT affected the direct activation of the motor system by stimulation of the sensorimotor cortex in adult but not in immature rats. This effect can be explained by a direct change in the cortex (a decrease of excitability) or by an activation of some structures influencing the motor system. Changes in the action of PHT on the cerebral cortex were described in our previous study (Mareš *et al.* 1993). Some former findings also suggest the importance of the cerebellum. Julien and Halpern (1972) hypothesized that PHT primarily affects cerebellar neurones and thus exhibits its anticonvulsant action through the inhibitory output of the cerebellum. This hypothesis cannot fully explain PHT anticonvulsant effects (Buřitová *et al.* 1994). Other target structures responsible for the influence of PHT on the motor system have to be taken into account (e.g. the spinal cord – Raines and Standaert 1967). Our data from cerveau isolé rats demonstrated that at least a part of PHT action is exerted through structures below the midbrain level (Mareš 1994).

PHT did not show an effect on the motor system of 12-day-old rats. It suggests that the target structure of the PHT action is immature at this age. This excludes the spinal level and supports the possible involvement of the cortex and/or cerebellum. Maturation of the cerebellum takes place at least up to the fourth postnatal week. The external granular layer as a source of precursor cells for the cerebellar cortex is present till the end of the third postnatal week in small laboratory rodents (Mareš *et al.* 1970).

As far as the subcellular level is concerned, the development of voltage-gated sodium channels as the main targets of PHT action should be taken into consideration. Neurones in the central nervous system exhibit at least five different isoforms of voltage-gated sodium channels, with a different course of maturation, regional representation, kinetics and voltage dependence (Barres *et al.* 1989, Catterall 1995).

Individual subtypes also differ in their sensitivity to antiepileptic drugs (Song *et al.* 1996). Sodium channel II is the most abundant subtype at all developmental stages. An especially high level of expression of sodium channel II messenger RNAs was found in the granular layer of the adult cerebellum. This is in strict contrast to the low levels in the developing cerebellum (Brysch *et al.* 1991). These results support the hypothesis on the importance of the cerebellum in PHT action (Julien and Halpern 1972). The absence of the PHT effect on the threshold intensity current for movements elicited by direct activation of the motor system in immature rats might be due to the low expression of voltage-gated sodium channels in the cerebellum at this age.

The effect of PHT on the spike-and-wave type of ADs seems to be in accordance with former reports on monkeys and cats (Aston and Domino 1961, Blum 1964). These authors described a PHT-induced increase of thresholds of ADs but a detailed description of ADs is missing. Our results allow to hypothesize that PHT blocked the spread of epileptic activity from the cortex to thalamus (responsible for the generation of the spike-and-wave rhythm) as well as to motoneurones for the muscles of the head, neck and forelimbs by affecting the action potentials by acting on voltage-gated sodium channels. A failure of this mechanism in the case of the limbic type of the ADs remains to be analyzed.

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