Anticonvulsant Action of 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline in Immature Rats: Comparison with the Effects on Motor Performance¹

P. MAREŠ, A. MIKULECKÁ and M. POMETLOVÁ
Institute of Physiology, Academy of Sciences of the Czech Republic (P.M., A.M.) and Department of Pathophysiology, 3rd Medical School, Charles University (M.P.), Prague, Czech Republic

Accepted for publication February 24, 1997

ABSTRACT
Anticonvulsant action of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX), a competitive antagonist at non-N-methyl-D-aspartate receptors for excitatory amino acids, was studied in a model of cortical epileptic afterdischarges (ADs) in 12-, 18- and 25-day-old rat pups with implanted electrodes. Electrical stimulation of sensorimotor cortex was repeated four times with 20-min intervals, NBQX (in doses of 10, 30, 60 or 90 mg/kg i.p.) or solvent (dimethyl sulfoxide, 1 ml/kg i.p.) were injected 10 min after the first afterdischarge. Dimethyl sulfoxide did not change the phenomena recorded; NBQX shortened ADs or at least blocked progressive prolongation observed under control conditions. Intensity of movements accompanying stimulation decreased after NBQX, and clonic movements accompanying ADs were suppressed in a dose-dependent manner. The highest dose of NBQX disabled the animals; therefore, the action of this drug on motor skills was studied in another group of animals. Even the dose of 30 mg/kg NBQX interfered with motor performance in 12- and 18-day-old rat pups, 25-day-old rat pups were more resistant to this action. NBQX exhibited only moderate antiepileptic action (suppression of progressive lengthening of ADs) at doses where unwanted side effects were absent.

Most types of epileptic seizures are caused by a disturbance of equilibrium between excitation and inhibition in the central nervous system (Heinemann and Jones, 1991). Drugs, which restore this equilibrium, may be effective anticonvulsants. There are two possible ways to restore this equilibrium: potentiation of inhibition or suppression of excitation (Mutani et al., 1991; Heinemann et al., 1994). Both possibilities are studied extensively in adult animals (Meldrum, 1989; Chapman, 1991). Practically all known antagonists of excitatory amino acids exhibit anticonvulsant action (for review see Dingledine et al., 1990; Chapman, 1991). For a long time attention was focused on antagonists of NMDA type of receptors because competitive (2-amino-5-phosphonovaleric acid, 2-amino-7-phosphonoheptanoic acid and CPP; Watkins, 1991) as well as noncompetitive antagonists binding to the ion channel regulated by NMDA receptor (e.g., ketamine and dizocilpine, i.e., MK-801; Foster, 1991) were at disposal (Watkins et al., 1990). Unwanted side effects stopped the clinical trials of dizocilpine in spite of its marked anticonvulsant action (Troupin et al., 1986). With the discovery of specific nonNMDA receptor antagonists, competitive NBQX (Sheardown et al., 1990) and noncompetitive GYKI 52466 (Tarnawa et al., 1990), the attention was shifted to this class of drugs. Both these antagonists exhibit clear-cut anticonvulsant effect in various models of epileptic seizures in adult rats (Chapman et al., 1991; Smith et al., 1991; Meldrum et al., 1992; Löschter et al., 1993; Yamaguchi et al., 1993; Dürmüller et al., 1994; Lallement et al., 1994; Löschter and Honack, 1994), but nothing is known about their possible action in immature brain. There are developmental changes in concentration of nonNMDA receptors in rat brain (Miller et al., 1990), which suggests a possibility of changing sensitivity. Because of this possibility we started ontogenetic studies in rats. Our first study demonstrated a moderate action of NBQX against pentylenetetrazol-induced motor seizures at all developmental stages (7-, 12-, 18- and 25-day-old rats). This action was rather specific against the tonic phase of generalized tonic-clonic seizures leaving minimal, clonic seizures untouched (Veříšek et al., 1995). We continued our study of NBQX anticonvulsant action in another model of epileptic seizures: epileptic ADs elicited by electrical stimulation of sensorimotor cortex and accompanied by clonic seizures similar to those induced by pentylenetetrazol (Kubová et al., 1993). Recent results with a noncompetitive nonNMDA

ABBREVIATIONS: Ads, afterdischarges; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline; NMDA, N-methyl-D-aspartate; EEG, electroencephalogram.
antagonist GYKI 52466 demonstrated an anticonvulsant action in all age groups studied (12-, 18- and 25-day-old rats; Kubová, H., Vilagi, I., Mikulecká, A. and Mareš, P., submitted) and our present study demonstrates similar action of NBQX. Action of NBQX on motor performance of immature rats was studied in identical age groups as the anticonvulsant action. This part of our study was based on developmental studies of Altman and Sudarshan (1975), in which a battery of tests with basic ontogenetic data is described. It was thus possible to choose tests adequate for our age groups. The tests of motor skills do not stress rat pups, because they represent a play rather than a task for the animals.

Methods

Experiments were performed in Wistar albino rats (Charles River) in three age groups (12, 18 and 25 days old) raised in our laboratory. The environmental temperature was 21–23°C. The colony room was naturally illuminated; food and water were available ad libitum. All experiments were performed between 9 A.M. and 3 P.M.

Electrophysiology

Experiments were performed in 119 rats. Cortical electrodes were implanted epidurally under ether anesthesia: two stimulation electrodes were placed over the right sensorimotor area, and recording electrodes were placed over the left sensorimotor area and visual areas of both hemispheres. An indifferent electrode was localized in the nasal bone (for details see Kubová et al., 1993). Surgical preparation lasted less than 15 min. After interruption of ether anesthesia the animals were allowed to recover for at least 1 hr, after which they were neurologically examined (righting and placing reflexes) and fed by sucrose solution. Only then were the experiments started.

Stimulation was performed by means of a constant current stimulator of our own construction. Stimulation series lasted 15 sec and were neurologically examined (righting and placing reflexes) and fed by sucrose solution. Only then were the experiments started.

The first AD always served as a control. Ten minutes after its end the animals were injected intraperitoneally either with NBQX (freshly dissolved in dimethyl sulfoxide so that the volume of 1 ml/kg was always administered) in one of the following doses: 10, 30, 60 or 90 mg/kg or with dimethyl sulfoxide in a volume of 1 ml/kg. Each age and dose group consisted of seven to nine animals.

Electrocorticogram was recorded before and during stimulation, during the AD and 1 min after its end. Behavior of animals was marked into EEG recordings. Type and duration of ADs were evaluated. Racine’s five-point scale (Racine, 1972), modified only in point 1, was used for quantification of behavior: 0, no activity; 1, activities asynchronous with stimuli or sharp elements of ADs; 2, head jerks; 3, clonic forelimb movements; 4, clonic forelimb movements + rearing; 5, clonic forelimb movements + rearing + falling. Activities under points 2 to 5 were synchronous with stimuli or sharp elements in the electrocorticogram during ADs. The most severe behavioral phenomenon was taken as a score, then the average and standard error of the mean were calculated for each group. Statistical evaluation of duration of ADs was done by means of three-dimensional analysis of variance (factors age, dose and number of stimulation) with post hoc comparison of individual groups by Holm’s method (Holm, 1979). Behavioral scores were evaluated with Friedman’s and Kruskal-Wallis nonparametric analysis with multiple comparison tests (Dixon, 1988). Five percent level was taken as statistically significant.

Motor Skills

Motor skills were tested in 280 rats in the same three age groups, i.e., 12, 18 and 25 days old. Entire litters (eight pups) were tested. In each litter two rat pups served as naive controls, six rats were injected intraperitoneally with NBQX or dimethyl sulfoxide. The NBQX solution was prepared similarly as for the electrophysiological experiments. The 30 mg/kg dose of NBQX was administered to all age groups. The second dose of NBQX was chosen according to the results with the 30 mg/kg dose in individual age groups to find the dose which does not interfere with motor performance. Twenty-five-day-old animals received the 60 mg/kg dose, the two younger groups the 10 mg/kg dose. Solvent control group was injected with dimethyl sulfoxide in a volume of 1 ml/kg. The rats were tested between 10 and 40 min after the drug or solvent administration.

The following five tests, slightly modified from the battery described by Altman and Sudarshan (1975), were used.

Surface righting reflex. Pups were placed on a laboratory desk in a supine position. After 30 sec at maximum. Time of righting and consistent placement of hindlimbs along the abdomen was recorded. The test was repeated at the end of the session, i.e., approximately 40 min after NBQX administration.

Negative geotactic reaction. Pups were placed on an inclined (30°) rough surface with the head facing downward (0°). The ability of pups to turn to 90° and consequently to 180° as well as time to turn were recorded. The animals were tested for maximum time of 90 sec.

Bar holding. Pups were held so that their forepaws touched a 25-cm-long wooden rod with a diameter of 1 cm, hanging 25 cm above a padded surface. Time of grasping the bar was recorded, time limit was 120 sec. In addition, the hindlimb support of the body was also recorded.

Wire-mesh ascending. A surface consisting of 10-mm wire mesh, 45 cm high and 15 cm wide, was placed at an angle of 70° in contact with a platform on the top and with an edge of laboratory desk at the bottom. The wire mesh was divided into five sections. To promote the ascending of a rat pup, its littermates were placed on the top platform while the tested rat was placed at the bottom of the wire mesh. Distance of climbing up for rejoining the siblings was measured up to 120 sec.

Crossing a bridge. The test consisted of two elevated platforms (start and goal) connected by a plywood bridge (30 cm long and 3 cm wide) divided into five sections. A litter of animals was placed on the goal platform, and one pup at a time was removed and placed on the

Fig. 1. Cortical epileptic AD in a 25-day-old rat. Individual leads from top to bottom: left frontal (LF), right occipital (RO) and left occipital (LO) cortical regions in reference connection. An arrow denotes the end of stimulation. Time mark, 1 sec; amplitude calibration, 0.5 mV.
start platform. During the 120-sec observation period distance traversed to join littermates or to fall was measured.

In the tests of bar holding, wire mesh and crossing bridge a box with a soft cover at the base served as a protection for the falling pups.

Nonparametric methods were used for statistical analysis. Differences among individual measurements were evaluated with the Kruskal-Wallis One Way Analysis of Variance on Ranks test. The level of statistical significance was set again at 5%.

Results

Electrophysiology. The first predrug stimulation always induced an EEG AD formed by spike-and-wave rhythm in 25- and 18-day-old rats or rhythmic sharp waves in 12-day-old rat pups. Stimulation elicited clonic movements of head and forelimbs synchronous with individual stimuli; the ADs were accompanied by similar movements (clonic seizures) synchronous with sharp elements in the EEG. The first AD was longest in 12-day-old rat pups (10.9 sec on the average) and its duration decreased with age to 9.3 and 7.2 sec in 18- and 25-day-old rats, respectively. The differences between individual age groups were significant.

Progressive prolongation of ADs with repeated stimulations was present in 12-day-old rat pups. Rats 25 days old exhibited the prolongation of the second AD, but the duration of the third and fourth ADs tended to be shorter than the second AD. The changes in 18-day-old animals did not reach the level of statistical significance. Intensity of movements accompanying stimulation as well as of clonic seizures remained stable during repeated stimulations.

Dimethyl sulfoxide did not change any parameter of ADs or motor phenomena in any age group when compared with naive age-matched controls. NBQX did not change the pattern of ADs, but shortened the duration of ADs in a dose-dependent manner in all age groups (fig. 2). In 12-day-old rat pups the two lower doses suppressed, at least temporarily, the increase in duration of ADs with repeated stimulations. The 60-mg/kg dose resulted in a complete block of ADs in four out of eight animals, and ADs could be recorded only exceptionally after the 90-mg/kg dose. In 18-day-old rats the 10-mg/kg dose did not have significant effect, the 30- and 60-mg/kg doses shortened ADs temporarily and the highest dose was necessary for reliable suppression of ADs. Similar results were found in 25-day-old rats with the only exception that the increase in duration was reliably blocked by all doses of NBQX (the postdrug ADs were significantly shorter than the corresponding ADs in the control, dimethyl sulfoxide group).

Movements accompanying stimulation as quantified by the five-point scale remained mostly uninfluenced by NBQX (fig. 3). The only exception was the lower intensity of these movements after the highest dose of NBQX in 12- and 18-day-old rats. In contrast to the negative results of the quantification, there was a marked qualitative change. Movements started as minute jerks of digits. Their intensity increased during stimulation, reaching the pattern of marked forelimb jerks only toward the end of the 15-sec stimulation series, a phenomenon never seen in control animals in which the intensity of movements was stable from the very beginning. Most of the 12-day-old rat pups exhibited minute movements during the whole stimulation period after 30-, 60- and 90-mg/kg doses of NBQX.

Intensity of clonic seizures (fig. 4) was decreased in all age groups. Most marked changes were observed in 12-day-old rat pups with complete suppression of seizures by the two highest doses of NBQX in some animals, so that ADs were without any motor counterpart. Significant changes in the intensity of seizures with an outlined tendency to a dose-
dependent manner were also found in other age groups. On the other hand, the highest dose used (90 mg/kg), which exhibited marked anticonvulsant effects, resulted in an extremely slow righting reaction in all age groups; some of the 25- and 18-day-old and nearly all 12-day-old rat pups lost their righting ability after this dose.

**Motor skills.** In all five tests, there were no differences between the control noninjected and dimethyl sulfoxide-treated groups, but there were differences in the bar-holding test in 12-day-old rat pups, where solvent shortened the time of grasping the bar.

In the righting ability test performed at the beginning of testing (fig. 5), the dose of 10 mg/kg NBQX did not significantly lengthen the latency of turning from a supinal to normal position. The dose of 30 mg/kg significantly increased the latency of turnings in 12- and 18-day-old but not in 25-day-old rats. The dose of 60 mg/kg also led to the increased latency of turning in 25-day-old rats. The differences between control and NBQX-treated rats failed to reach statistical significance when the test was repeated 40 min after NBQX administration.

---

**Fig. 3.** Intensity of movements accompanying stimulations. Details as in figure 2, only ordinate; intensity expressed in the five-point scale was described under “Methods.”

**Fig. 4.** Intensity of clonic seizures accompanying ADs. Details are described in figure 2, except here the abscissa showed the five-point scale.
mg/kg shortened the time of successful grasping in all age groups; in 25-day-old rats the 60-mg/kg dose had the same effect. Ataxia of hindlimbs (expressed as an inability to place the hindlimbs on the bar) was observed in 12- and 18-day-old rats after the 30-mg/kg dose and in 25-day-old animals after the 60-mg/kg dose.

There were no significant differences in the wire-mesh ascending test (fig. 6) in the distance for which the young rats were able to climb up with a dose of 10 mg/kg. When the 30-mg/kg dose was administered, the climbing distance decreased significantly in all groups tested. The same was true in the 25-day-old group treated with the 60-mg/kg dose.

Similarly, no differences were found between the control and experimental groups treated with the 10-mg/kg dose in the bridge-crossing test (fig. 6). The distance crossed decreased significantly in 12- and 18-day-old rats treated with the 30-mg/kg dose. There was no change in 25-day-old rats treated with the 30-mg/kg dose, but the higher dose led to a significant shortening of the traversed distance.

Fig. 5. Results of three tests of motor skills as described under “Methods.” In all graphs, the abscissa shows different groups: co = naive noninjected animals; dms = rats injected with dimethyl sulfoxide, i.e., solvent used; 10, 30 and 60 mg = three different doses of NBQX. Individual columns denote various age groups (see description on the right margin). Ordinates for “Surface righting” and “Negative geotaxis” – average time (± S.E.M.) in seconds, necessary for completion of the task. The righting in both control groups and in 25-day-old rats injected with the 30-mg/kg dose was immediate, so that one second as the shortest measurable time was given to all animals. Ordinate for “Bar holding” means time spent on the bar. Asterisks denote significant differences in comparison with the age-matched dms group.

Results of the negative geotaxis test (fig. 5) were similar to those observed in the first righting ability test. In the bar holding test (fig. 5), results with the dose of 10 mg/kg did not differ from those with solvent. The dose of 30 mg/kg shortened the time of successful grasping in all age groups; in 25-day-old rats the 60-mg/kg dose had the same effect. Ataxia of hindlimbs (expressed as an inability to place the hindlimbs on the bar) was observed in 12- and 18-day-old rats after the 30-mg/kg dose and in 25-day-old animals after the 60-mg/kg dose.

There were no significant differences in the wire-mesh ascending test (fig. 6) in the distance for which the young rats were able to climb up with a dose of 10 mg/kg. When the 30-mg/kg dose was administered, the climbing distance decreased significantly in all groups tested. The same was true in the 25-day-old group treated with the 60-mg/kg dose.

Similarly, no differences were found between the control and experimental groups treated with the 10-mg/kg dose in the bridge-crossing test (fig. 6). The distance crossed decreased significantly in 12- and 18-day-old rats treated with the 30-mg/kg dose. There was no change in 25-day-old rats treated with the 30-mg/kg dose, but the higher dose led to a significant shortening of the traversed distance.

Fig. 6. Results of the “Wire mesh” and “Bridge crossing” tests. Details as in figure 5, only ordinates mean distance (in centimeters) traversed by the rats.
Three different phenomena could be evaluated in our electrophysiological experiments: movements accompanying stimulation (i.e., the direct activation of the motor system), generation and progressive augmentation of ADs and motor seizures accompanying ADs (i.e., the spread of epileptic activity into the motor system).

In agreement with results of Jensen et al. (1995) obtained in hypoxia-induced epileptogenesis in 10-day-old rat pups, NBQX exhibited a marked anticonvulsant action in immature rats in our model. This action was expressed in shortening of the duration of ADs up to the abolition of ADs after the highest dose, as well as in a decrease of intensity of clonic seizures accompanying cortical ADs. Dimethyl sulfoxide, used as a solvent for NBQX, did not possess these actions. Three different anticonvulsant actions could be hypothesized based on changes found: 1) the action against progressive epileptogenesis as the most marked effect is suggested by a block of progressive increase of duration of ADs with low doses of NBQX; 2) the action against generation of epileptic ADs is demonstrated by complete suppression of ADs by higher doses of NBQX; 3) the action against spread of epileptic activity at least into the motor system is demonstrated by the appearance of ADs without any motor correlate after high doses of NBQX. Our data on AD duration differ from those of Dürmüller et al. (1994), which describe the reduction of seizure score but not shortening of ADs in fully kindled rats (amygdala kindling) and no significant effect on kindling development. The suppression of ADs in our experiment is caused by the higher doses used. The difference in the effect on progressive prolongation of ADs, which was found even with very low dose (10 mg/kg), might be caused by two factors: cortical ADs are more sensitive to NBQX action than amygdala ADs, and immature rats exhibit higher sensitivity to NBQX than adult animals. Both explanations are plausible. In addition, a possible difference between classical kindling in the paper of Dürmüller et al. (1994) and partial, initial kindling (Racine et al., 1973) in our study has to be taken into account. This possible difference is suggested by figure 6 in Dürmüller’s study which demonstrated transient suppression of kindling development on the first day of NBQX administration, i.e., under the conditions similar to our paradigm of partial kindling when stimulations were repeated with much shorter intervals after a single injection of NBQX.

Effects of NBQX on the motor system were demonstrated not only as a modification of movements elicited by stimulation of the sensorimotor cortical area by high doses but also as worsening of performance in motor skill tests where even low doses led to significant results. Action on the motor system had to be expected because of physiological role of excitatory amino acids in this system. At least the corticosubcortical pathway (i.e., the first neuron in the extrapyramidal system), as well as the corticorubral pathway, uses glutamate as its transmitter (Headley and Grillner, 1990). In addition, the effects of NBQX on spinal cord ventral root reflexes were demonstrated (Farkas and Ono, 1995).

The effects of NBQX on the motor system are markedly expressed so that only the first of the three anticonvulsant actions enumerated above is exhibited by doses not derranging motor performance of rat pups. The other anticonvulsant effects were observed only after doses inducing marked side effects. These results demonstrate a narrow therapeutic range of NBQX in immature rats. In addition, the difference between anticonvulsant and toxic effects is related to the age of animals; it increases with maturation, i.e., the therapeutic index increases during development. Our results from 25-day-old rats are in agreement with data from adult mice in which anticonvulsant effects were always more marked than motor-impairing effects (Swedberg et al., 1995).

Higher sensitivity of younger animals in comparison with older ones, which was seen in both electrophysiological and motor skills experiments, might be caused by the larger amount of AMPA receptors in most brain regions at the end of the third postnatal week in comparison with older animals (Insel et al., 1990), but the published data demonstrate the same concentration of AMPA receptors in 14- and 28-day-old rats with the only exception of CA3 hippocampal field where the 14-day-old pups have more AMPA receptors than older rats (Insel et al., 1990). On the other hand, Blue and Johnston (1995) found the peak of quisqualate receptors in the rat somatosensory cortex at postnatal day 10, but they did not differentiate between metabolic and AMPA receptors with the exception of localization in relation to barrels in layer IV on postnatal day 10 only. Regional differences were found during development also in an in situ hybridization of GluR-1, GluR-2, GluR-3 and GluR-4 genes (Pellegrini-Giampietro et al., 1991; Bettler et al., 1990). Developmental changes in the composition of subunit proteins of the AMPA-prefering glutamate receptor which were demonstrated in individual thalamic nuclei (Spreafico et al., 1994) and in the whole telencephalon (Hall and Bahr, 1994) might also form a background for high sensitivity of rat pups. Unfortunately, there are no available data on the binding of NBQX to different subunits during brain maturation. In addition, data of Insel et al., (1990) that K_g remains the same in 21-day-old and adult rats speak against this explanation, but these authors did not publish K_g data for younger animals.

Our recent data demonstrated anticonvulsant action of NBQX against the tonic phase of generalized tonic-clonic seizures elicited by pentylentetrazol in rat pups (Velislé et al., 1995), which indicates possible clinical effects against generalized tonic seizures. Cortical epileptic ADs used in the present paper may be taken as a model of myoclonic seizures (Kubová et al., 1996; Polášek et al., 1996); therefore, the action of NBQX in this model might predict efficacy against human myoclonic seizures. Marked anticonvulsant action of NBQX is accompanied by an impairment of motor functions as demonstrated with doses effective in maximal electroshock model in adult mice (Yamaguchi et al., 1993). Developmental changes of these unwanted effects shown in our present study indicate higher sensitivity of the immature brain. This fact might impede clinical use of NBQX and probably also other nonNMDA antagonists at least in pediatric epileptology.

References


Send reprint requests to: P. Maresˇ, M.D., D.Sc., Institute of Physiology, Academy of Sciences of the Czech Republic, Vı ´den ˇ ska ´ 1083, CZ 142 20 Prague 4, Czech Republic.