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Research Report

The Na^+/K^+ATPase activity is increased in the hippocampus after multiple status epilepticus induced by pilocarpine in developing rats

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ABSTRACT

The effects of repetitive pilocarpine-induced *status epilepticus* (SE) in the hippocampal Na^+/K^+ATPase activity were studied in developing rat. Na^+/K^+ATPase is a membrane-bound enzyme responsible for the active transport of sodium and potassium ions through the membrane. It is necessary to maintain neuronal excitability. The malfunction of this enzyme has been associated with neuronal hyperexcitability. The pilocarpine-induced *status epilepticus* in developing rats leads to neuronal hyperexcitability and brain damage. We examined the activity of the Na^+/K^+ATPase enzyme in hippocampus of rats submitted to 1 episode of *status epilepticus* on postnatal day 9 and to 3 episodes of pilocarpine-induced *status epilepticus* on postnatal days 7, 8 and 9. Our findings showed that one *status epilepticus* episode does not modify the Na^+/K^+ATPase activity in hippocampus of rats studied 7 or 30 days later (at P16 or P39). However, an increase in the Na^+/K^+ATPase activity was detected in hippocampus of rats submitted to three consecutive *status epilepticus* during the development studied 7 (+142%) and 30 (+400%) days following the injections. In addition, a significant reduction in the Na^+/K^+ATPase activity was observed in control rats at P39 compared to P16. Our data suggest that multiple pilocarpine-induced *status epilepticus* in developing rats induce long-lasting increase in the Na^+/K^+ATPase activity in the hippocampus, reflecting hyperexcitability.

1. Introduction

Clinical studies show that the incidence of epileptic seizures during the childhood is higher than in the adult life (Holmes, 1997). There are many evidences suggesting that the neuroplastic potential of the immature brains differs from the adult. The differences may affect the threshold and the propagation of epileptic discharges (Freeman, 1995).

Experimental studies both in vivo and in vitro have shown that the immature limbic system is an epileptogenesis site very frequent during the beginning of postnatal life (Moshe, 1987; Swann et al., 1993). Studies on different epilepsy models

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have shown an increase in the susceptibility to seizures during the second and third postnatal week in rats (Gottieb et al., 1977). The increase has been also described in amygdala kindling (Moshe, 1981), hippocampal kindling (Michelson et al., 1989; Haas et al., 1990) and hippocampal electrical stimulation (Velísek and Marés, 1991).

The administration of high doses of pilocarpine induces the main characteristics of the human temporal lobe epilepsy in rats (Cavalheiro, 1995). Ontogenetic studies have shown that the consequences of SE induced by pilocarpine are age-dependent (Priel et al., 1996). Chronic seizures and brain damage can be observed only when pilocarpine is administered after the 18th day of life. Younger rats do not present damage, behavioral or electroencephalographic alterations as late consequences of pilocarpine-induced SE (Priel et al., 1996). However, animals suffering multiple pilocarpine-induced SE at P7–9 (postnatal day) exhibit important electrographic, behavioral and morphological changes that persist throughout adult life, despite the absence of chronic epilepsy (Santos et al., 2000a). A large number of apoptotic cells are observed in the hippocampus and thalamus, while the electrophysiological study reveals a chronic hyperexcitability in the hippocampus of these animals (Santos et al., 2000b). Taken together, these data show that multiple pilocarpine-induced SE in immature rats causes long-lasting changes in the brain, but the mechanisms are not yet completely unveiled.

The enzyme Na^+/K^+ATPase (E.C.3.6.1.3) is responsible for the active ionic exchange through the cell. It provides the efflux of three Na^+ ions and the influx of two K^+ ions per spliced molecule of ATP in several cell types, including neurons and glia (Grisar et al., 1992). Due to its high importance in the maintenance of resting membrane potential and the propagation of neuronal impulse, the malfunction of this enzyme has been associated with the neuronal hyperexcitability (Donaldson et al., 1971).

Reduced Na^+/K^+ATPase activity has been found in cerebral tissue of epileptic patients (Rapport et al., 1975) as well as in the hippocampus of kainate-injured rats (Anderson et al., 1994), although higher enzyme activity levels were observed in pilocarpine-lesioned rats (Fernandes et al., 1996). In addition, ouabain, an irreversible inhibitor of the Na^+/K^+ATPase pump, induces focal spikes discharges as well as contralateral focal seizures when applied locally on the cortical surface of rats (Lewin, 1971).

In this study, we investigated the Na^+/K^+ATPase activity in hippocampus of rats submitted to 1 or 3 consecutive episodes of pilocarpine-induced SE on postnatal days 9 and 7, 8 and 9, respectively. Hippocampus tissues were analyzed 7 and 30 days after pilocarpine-induced SE.

2. Results

2.1. *Pilocarpine-induced status epilepticus*

A few minutes after pilocarpine administration, all animals presented continuous scratching, strong body tremor, mastication, clonic movements of forelimbs and head bobbing culminating in SE, which lasted about 6 h. The tonic seizures were more frequent during the second and third SE inductions. The mortality rate after SE was 16% (3/18 for 1SE group and 3/14 for 3SE).

The control animals did not present any of the alterations described bellow.

2.2. *Enzyme activity*

The hippocampal activities of Na^+/K^+ATPase (μmol Pi/mg protein/h) are shown in Fig. 1. An increase in the activity of Na^+/K^+ATPase was observed in rats submitted to the 3 episodes of SE (G-3SE), studied 7 and 30 days after pilocarpine treatment. Following 7 days after pilocarpine injection (P16), Na^+/K^+ATPase activity increased 142% ($p<0.05$) when compared to the G-CT, and 141% ($p<0.05$) when compared to the G-1SE. A higher increase in the magnitude of the enzyme activity was observed in the G-3SE studied 30 days later (P39). In this experimental condition, Na^+/K^+ATPase activity increased 400% ($p<0.001$) when compared to the G-CT, and 328% ($p<0.001$) when compared to G-1SE. There was no significant difference in the Na^+/K^+ATPase activity of G-1SE when compared to the G-CT. However, when the enzyme activities of the saline-treated controls at different ages were compared, we found that the animals at P39 presented a significant Na^+/K^+ATPase activity reduction (−76%, $p<0.001$) than the P16.

3. Discussion

The present study showed that one episode of pilocarpine-induced SE in developing rats does not cause long-lasting alteration in the Na^+/K^+ATPase activity in the hippocampus. However, consecutive episodes of SE induce a significant long-term increase in the enzyme activity 7 and 30 days after the SE inductions. The alteration may reflect the hippocampal hyperexcitability previously shown by Santos et al. (2000b).

Electrophysiological studies by Santos et al. (2000b) showed that the hippocampus of rats submitted to multiple SE by pilocarpine during the development exhibits lower threshold to convulsant agents when compared to control or 1SE rats that were electrically stimulated. The hippocampus is considered to be an epileptogenic area and according to several authors this vulnerability is due to its ability of accumulating potassium in the extracellular space (Green, 1964; Zuckermann and Glaser, 1968; Ogata et al., 1976; Cowan and Cavalheiro, 1980; Rutecki et al., 1985). The hippocampal hyperexcitability could result in the elevation of the extracellular levels of K^+ and, in this case, the increased Na^+/K^+ATPase activity may reflect a compensatory mechanism to reduce the extracellular potassium content and block epileptiform discharges in the hippocampus (Rutecki et al., 1985; Bragdon et al., 1986; Korn et al., 1987). It has been demonstrated that discharges produced by electrical stimulation are followed by an increase in the Na^+/K^+ATPase activity, which agrees with the theory that an increase in the electrical activity of the brain produces an enzymatic activation to compensate the changes in the distribution of the electrolytes (Bignami et al., 1966; Harmony et al., 1968).

In addition to hyperexcitability, other alterations such as increased hippocampal/thalamic apoptosis and severe cognitive impairment in adulthood are observed in rats submitted

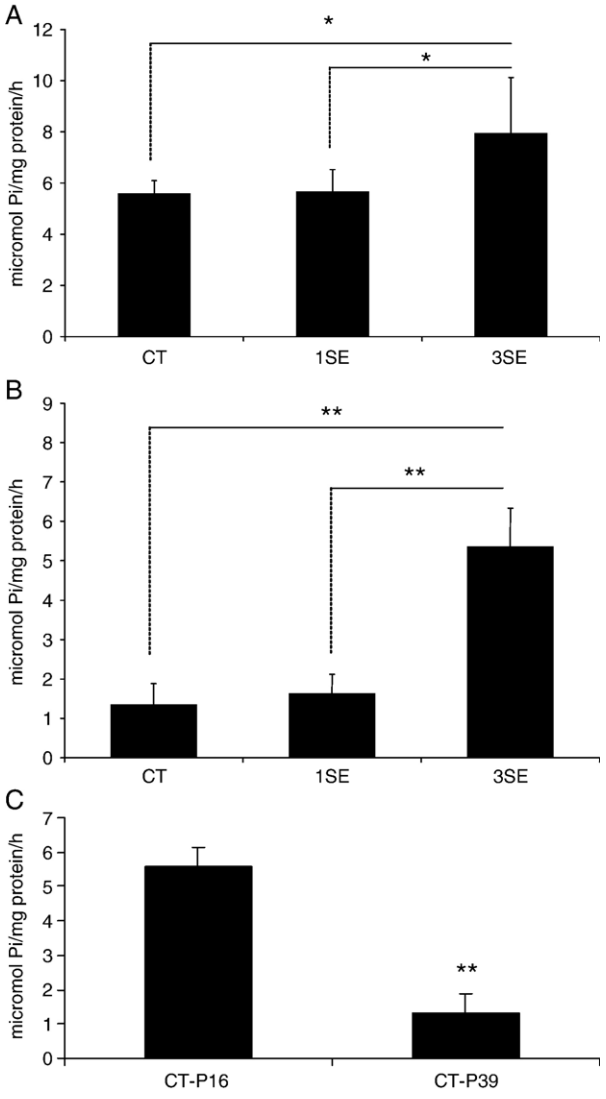

Fig. 1 – Comparison of the means of hippocampal Na^+/K^+ATPase activities among saline, 1SE and 3SE groups, studied 7 (A) and 30 days (B) after the injections of pilocarpine. (A) A significant increase in the enzyme activity was observed in the 3SE group, studied 7 days after the injections of pilocarpine (P7–P9), compared to control and 1SE groups evaluated at the same period. (B) Persistent increase in the Na^+/K^+ATPase activity was observed in the 3SE group compared to the other groups, 30 days after successive injections of pilocarpine at P7–P9, reflecting the irreversible effects of repeated SE during the development. (C) Na^+/K^+ATPase activity of control rats studied at 7 and 30 days after the injection of NaCl (P7–P9) showing a significant decrease in the enzyme activity in older animal. Values are expressed as mean ± S.D. (*) $p<0.05$; (**) $p<0.001$ (ANOVA followed by Tukey's test).

to the pilocarpine-induced SE during the development (Santos et al., 2000a,b). These authors showed that repetitive SE episodes in developing rats induce electrographic epileptic features and cognitive disorders that are not accompanied by gross neuronal damage. Similar alterations were also demonstrated in the lithium-pilocarpine model (Kubová et al., 2004). Thus, Silva et al. (2005) recently reported that the occurrence of pilocarpine-induced SE episodes during development induces complex cellular changes that probably affect the maturation of neocortical and hippocampal circuits.

It has been shown that neonatal hypoxia is a risk factor for the later development of epilepsy (Bergamasco et al., 1984; Connell et al., 1989; Volpe, 1989; Holmes and Kull, 1990). Jensen and Wang (1996) reported that hippocampal slices prepared from adult rats that underwent hypoxia at P10 show significantly lower thresholds to convulsant conditions compared with slices from controls. Clinical observations also suggest that the acute and chronic epileptogenic effects of hypoxia might be age-dependent, being that the immature brain is more susceptible than the adult (Aicardi and Chevrie, 1970).

Although hypoxia was not monitored in our study, we noted the presence of episodes of hypoxia between the sequence of behavior alterations during SE. Seizure-induced hypoxia may be a mechanism by which seizure can injure the developing brain, contributing to the alterations that led to the hyperexcitability, reflected by an increase of the Na^+/K^+ATPase activity. Although developing rats do not show evident neuronal damage and mossy fiber sprouting after SE induction, the SE-associated hypoxia may lead to important plastic changes in critical periods of brain maturation that can become apparent through a variety of epileptic manifestations (Santos et al., 2000a).

This work showed a higher Na^+/K^+ATPase activity in younger rats. In fact, some authors have already shown that the activity of this enzyme is increased in the developing rat brain and it decreases during aging (De Souza et al., 1978). Conversely, Kennedy et al. (1985) and Torlinska and Grochowalska (2004) demonstrated that the Na^+/K^+ATPase activity remains almost unchanged with age. A proposed mechanism to explain the decreased enzyme activity found in these animals is the depression of neuronal excitability and the decrease in metabolic energy observed in older animals, as described by Tsakiris et al. (1998).

In conclusion, our data show that consecutive SE causes an increase in the Na^+/K^+ATPase activity.

4. Experimental procedure

4.1. *Animals*

Animal protocols were conducted in accordance with national and international legislation (guidelines of the Brazilian College of Animal Experimentation-COBEA and NIH guide for the use and care of Laboratory animals), and with the approval of the ethical committee of the University. Adequate measures were taken to minimize pain or discomfort.

A total of 32 Wistar rats at postnatal days 7–9 (P7–9) were used in this study. The colony room was kept at 21 ± 2 °C with a 12 h light/dark schedule. The rats were bred in our laboratories and the birth date was considered to be day 0. The animals were selected randomly into the experimental (SE) or control (saline) groups. To avoid interliter variations, different treatment groups were represented in each litter. SE was induced at

different ages (P7–9) by pilocarpine (Sigma, St. Louis, MO) administration (380 mg/kg, i.p.). Control rats were injected with the same volume of 0.9% saline instead of pilocarpine.

According to the treatment, three groups were obtained:

- G-1SE: rats submitted to one episode of SE with pilocarpine at postnatal day 9 ($N=15$);
- G-3SE: rats treated with pilocarpine during three consecutive days (P7–P9) ($N=11$);
- G-CT: rats treated with saline during one or three consecutive days (P7–9) ($N=12$).

All treated animals were carefully warmed for a system of controlled heating (37 °C) and the surviving animals were returned in the cages with their mothers. The rats were studied 7 and 30 days after pilocarpine or saline injection.

4.2. *Enzyme determination*

The animals were sacrificed by decapitation. Their hippocampi were dissected on an ice plate and kept frozen at −80 °C until assay. For enzymatic assay, the tissues were homogenized in Tris–HCl 50 mM (pH 7.4) buffer containing 0.5 mM EDTA (40 μL/mg tissue). Homogenate fractions were used for protein determination using the Lowry method modified by Hartree (1972). The Na^+/K^+ATPase activity was performed according to the methods of Mishra and Delivoria-Papadopoulos (1988) and Taussky and Shorr (1953), with a few modifications. In summary, the samples (200 μL) were incubated for 20 min at 37 °C in medium containing 100 mM NaCl, 20 mM KCl, 6 mM ATP and 6 mM $MgCl_2$ in the presence or in the absence of 1 mM ouabain. The samples containing ouabain were incubated in the absence of KCl and the final volume of each tube was 1 mL. These reactions were stopped using 500 μL of trichloroacetic acid (TCA; 12.5%) and placed in an ice bath for 10 min. The samples were then centrifuged at 2.500 rpm at 4 °C for 10 min and the supernatant was taken for inorganic phosphate (Pi) determination using the Taussky and Shorr (1953) method. Aliquots of the supernatant (750 μL) were incubated for a few minutes with 500 μL of a chromogenic solution "freshly prepared" and obtained through the addition of 10 mL of a stock solution composed of ammonium molybdate 10% (prepared with sulfuric acid 10N), 50 mL of distillated water and 5 g of ferrous sulphate (final volume 100 mL) at room temperature. Reactions were quantified using a spectrophotometer at 660 nm.

A standard curve using phosphate monosodium was obtained with the same chromogenic solution used to quantify the inorganic phosphate in the samples. Na^+/K^+ATPase activity was obtained by calculating the difference between the values obtained in the presence and in the absence of ouabain and expressed in μmol of Pi released per mg of protein per hour.

4.3. *Statistical evaluation*

Hippocampal Na^+/K^+ATPase activity of the three groups (G-1SE, G-3SE and G-CT) was assessed by One-way Analysis of Variance (ANOVA) and the Tukey's test. Values of $p<0.05$ were accepted as significant.

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REFERENCES

Aicardi, J., Chevrie, J.J., 1970. Convulsive status epilepticus in infants and in children: a study of 239 cases. Epilepsia 11, 187–197.

Anderson, W.R., Franck, J.E., Sthal, W.L., Maki, A.A., 1994. Na^+/K^+ ATPase is decreased in hippocampus of kinate-lesioned rats. Epilepsy Res. 17, 221–231.

Bergamasco, B., Penna, P., Ferrero, P., Gavinelli, R., 1984. Neonatal hypoxia and epileptic risk: a clinical prospective study. Epilepsia 25, 131–146.

Bignami, A., Palladini, C., Venturini, G., 1966. Effect of cardiazol on sodium-potassium adenosinetriphosphatase of the rat brain in vivo. Brain Res. 1, 413–414.

Bragdon, A.C., Taylor, D.M., Wilson, W.A., 1986. Potassium-induced epileptiform activity in area CA3 varies markedly along the septotemporal axis of the rat hippocampus. Brain Res. 378, 169–173.

Cavalheiro, E.A., 1995. The pilocarpine model of epilepsy. Ital. J. Neurol. Sci. 16, 33–37.

Connell, J., Oozeer, R., De Vries, L., 1989. Continuous EEG monitoring of neonatal seizures: diagnostic and prognostic considerations. Arch. Dis. Child. 64, 459–464.

Cowan, C.M., Cavalheiro, E.A., 1980. Epilepsy and membrane Na^+/K^+ATPase: changes in activity using an experimental model of epilepsy. Acta Physiol. Latinoam. 30, 253–258.

De Souza, B.N., Kendrick, Z.V., Roberts, J., Baskin, S.I., 1978. Na^+, K^+ ATPase in brain and spinal cord during aging. Adv. Exp. Med. Biol. 97, 255–258.

Donaldson, J., St-Pierre, J., Minich, J., Barbeau, A., 1971. Seizures in rats associated with divalent cation inhibition of Na^+/K^+ATPase. Can. J. Biochem. 49, 1217–1224.

Fernandes, M.J.S., Naffah-Mazzacoratti, M.G., Cavalheiro, E.A., 1996. Na^+/K^+ATPase activity in the rat hippocampus: a study in the pilocarpine model of epilepsy. Neurochem. Int. 28, 497–500.

Freeman, J.A., 1995. Clinician's look at the developmental neurobiology of epilepsy. In: Schwartzkroin, P.A., Moshé, S.L., Noebels, J.L., Swann, J.W. (Eds.), Brain Developmental and Epilepsy. Oxford Univ. Press, New York, pp. 9–33.

Gottieb, A., Keydor, I., Epstein, H.T., 1977. Rodent brain growth stages. An analytical review. Biol. Neonate 32, 166–167.

Green, J.D., 1964. The hippocampus. Physiol. Rev. 44, 561–608.

Grisar, T., Guillaume, D., Delgado-Escueta, A.V., 1992. Contribution of Na^+/K^+ATPase to focal epilepsy: a brief review. Epilepsy Res. 12, 141–149.

Haas, K.Z., Sperber, E.F., Moshé, S.L., 1990. Kindling in developing animals: expression of severe seizures and enhanced development of bilateral foci. Dev. Brain Res. 56, 275–280.

Harmony, T., Urbá-Holmgren, R., Urbay, C.M., Szava, S., 1968. (Na–K) ATPase activity in experimental epileptogenic foci. Brain Res. 11, 672–680.

Hartree, E.F., 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. 48, 422–427.

Holmes, G.L., 1997. Epilepsy in developing brain: lessons from the laboratory and clinic. Epilepsia 38, 12–30.

Holmes, G.L., Kull, L.L., 1990. Neonatal seizures. Am. J. EEG Technol. 30, 281–308.

Jensen, F.E., Wang, C., 1996. Hypoxia-induced hyperexcitability in

vivo and in vitro in the immature hippocampus. Epilepsy Res. 26, 131–140.
Kennedy, R.H., Akera, T., Katano, Y., 1985. Aging: effects on sodium- and potassium-activated triphosphatase activity and ouabain binding sites in rat brain. J. Gerontol. 40, 401–408.
Korn, S.J., Giacchino, J.L., Chamberlin, N.L., Dingledine, R., 1987. Epileptiform burst activity induced by potassium in the hippocampus and its regulation by GABA-mediated inhibition. J. Neurophysiol. 57, 325–340.
Kubová, H., Mares, P., Suchomelova, L., Brozek, G., Druga, R., Pitkanen, A., 2004. Status epilepticus in immature rats leads to behavioural and cognitive impairment and epileptogenesis. Eur. J. Neurosci. 19, 3255–3265.
Lewin, E., 1971. Epileptogenic cortical foci induced with ouabain sodium, potassium, water content and sodium–potassium-activated ATPase activity. Exp. Neurol. 30, 172–177.
Michelson, H.B., Williamson, J.M., Lothman, E.W., 1989. Ontogeny of kindling: the acquisition of kindled responses at different ages with rapidly recurring hippocampal seizures. Epilepsia 30, 672.
Mishra, O.P., Delivoria-Papadopoulos, M., 1988. Na^+/K^+ATPase in developing fetal guinea pig brain and the effect of maternal hypoxia. Neurochem. Res. 13, 765–770.
Moshe, S.L., 1981. The effects of age on the kindling phenomenon. Dev. Psychobiol. 14, 75–81.
Moshe, S.L., 1987. Epileptogenesis and the immature brain. Epilepsia 28 (Suppl. 1), S3–S15.
Ogata, N., Hori, N., Katsuda, N., 1976. The correlation between extracellular potassium concentration and hippocampal epileptic activity in vitro. Brain Res. 110, 371–375.
Priel, M.R., Santos, N.F., Cavalheiro, E.A., 1996. Developmental aspects of the pilocarpine model of epilepsy. Epilepsy Res. 26, 115–121.
Rapport, R.L., Harris, A.B., Friel, P.N., Ojemann, G.A., 1975. Human epileptic brain. Arch. Neurol. 32, 549–554.
Rutecki, P.A., Lebeda, F.J., Johnston, D., 1985. Epileptiform activity induced by changes in extracellular potassium in hippocampus. J. Neurophysiol. 54, 1363–1374.
Santos, N.F., Arida, M.R., Trindade, E.M., Priel, M.R., Cavalheiro, E.A., 2000a. Epileptogenesis in immature rats following recurrent status epilepticus. Brain Res. Rev. 32, 269–276.
Santos, N.F., Marques, R.H., Correia, L., Sinigaglia-Coimbra, R., Calderazzo, L., Sanabria, E.R.G., Cavalheiro, E.A., 2000b. Multiple pilocarpine-induced status epilepticus in developing rats: a long-term behavioral and electrophysiological study. Epilepsia 41 (Suppl. 6), S57–S63.
Silva, A.V., Regondi, M.C., Cipelletti, B., Frassoni, C., Cavalheiro, E.A., Spreafico, R., 2005. Neocortical and hippocampal changes after multiple pilocarpine-induced status epilepticus in rats. Epilepsia 46, 636–642.
Swann, J.W., Smith, K.L., Brady, R.J., Pierson, M.G., 1993. Neurophysiological studies of alterations in seizure susceptibility during brain development. In: Schwartzkroin, P. (Ed.), Epilepsy. Models, Mechanisms and Concepts. Cambridge Univ. Press, pp. 209–243.
Taussky, H.H., Shorr, E., 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202, 675–685.
Torlinska, T., Grochowalska, A., 2004. Age-related changes of Na^+/K^+-ATPase, Ca^{+2}-ATPase and Mg^{+2}-ATPase activities in rat brain synaptosomes. J. Physiol. Pharmacol. 55, 457–465.
Tsakiris, S., Angelogianni, P., Stavridis, J.C., 1998. Effect of aging on the activity of acetylcholinesterase, Na+, K(+)-ATPase and MG (2+)-ATPase in rat pituitary and hypothalamus. Z. Naturforsch. 53, 168–172.
Velísek, L., Marés, P., 1991. Increased epileptogenesis in the immature hippocampus. Exp. Brain Res. Ser. 20, 183–185.
Volpe, J.J., 1989. Neonatal seizures. Pediatrics 84, 422–428.
Zuckermann, E.C., Glaser, G.H., 1968. Hippocampal epileptic activity induced by localized ventricular perfusion with high-potassium cerebrospinal fluid. Exp. Neurol. 20, 87–110.