Neuroprotection by lamotrigine in a rat model of neonatal hypoxic-ischaemic encephalopathy

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Abstract

Hypoxic-ischaemic (HI) encephalopathy is a severe complication of perinatal asphyxia and remains a frequent cause of a variety of brain disorders with long-term effects on the patients’ life. The associated brain damage is strongly related to the toxic action of excitatory amino acids, especially glutamate and aspartate. Lamotrigine is an anti-epileptic drug that blocks the voltage-gated sodium channels of the presynaptic neuron and inhibits the release of glutamate. In the present study a well-established model of perinatal asphyxia in 7-d-old rats was used to investigate the effect of lamotrigine on HI-induced damage to different hippocampal brain structures, since disruption of this brain area is thought to play a key role in schizophrenia and epilepsy. Therefore, a combination of ischaemia, induced by unilateral occlusion of the left common carotid artery, followed by exposure to a 1-h period of hypoxia, was carried out in neonatal 7-d-old rats. Immediately after the insult, lamotrigine was given i.p. The histological outcome in the hippocampus was conducted and the tissue levels of glutamate, aspartate, GABA, and glutamine in the same area were determined. A remarkable reduction of HI-evoked damaged neurons in most of the investigated hippocampal regions was noted after lamotrigine administration. Furthermore, lamotrigine decreased the asphyxia-induced hippocampal tissue levels of glutamate and aspartate. Immediately after perinatal asphyxia GABA levels were enhanced, while levels of glutamine were decreased. Lamotrigine administration did not affect either GABA or glutamine levels. These results suggest a neuroprotective effect of lamotrigine in this particular animal model of neonatal HI encephalopathy.

Introduction

Perinatal asphyxia is one of the main causes of morbidity and mortality in infants and children. The incidence of asphyxia is about 0.2–0.4% in full-term infants and approaches 60% in premature newborns. Around 20–50% of asphyxiated newborns expire during their first period of life and 25% of the survivors exhibit severe neuropsychiatric deficits (Vannucci et al., 1999). Perinatal asphyxia exposes the immature brain to hypoxic and ischaemic conditions that cause permanent damage known as hypoxic-ischaemic (HI) encephalopathy. HI encephalopathy remains a frequent cause of a variety of brain disorders including cerebral palsy, epilepsy and mental retardation (Esclapez et al., 1999; Hill, 1991; Johnston et al., 1995). Long-term effects on cognition, memory, learning and behaviour have also been observed in various studies (Altemus and Almli, 1997; de Haan et al., 2006; Kofman, 2002; Weitzdoerfer et al., 2004). Furthermore, anoxic insults to neonates may play a role in increasing vulnerability to a spectrum of neuropsychiatric conditions such as attention deficit hyperactivity disorder, and autism (Hultman et al., 2002; Zappitelli et al., 2001). Numerous studies have also focused on neonatal damage of the hippocampus in rats as a potential model of schizophrenia as various functional and structural changes in the hippocampus have been consistently implicated in schizophrenia in...
humans (Boksa, 2004; Dalman et al., 2001; Jones et al., 1998; Zornberg et al., 2000).

Asphyxia induces neuronal injury in several regions of the developing brain, especially in the hippocampus. The neuronal injury is partly attributed to oxidative stress due to the toxic action of oxygen-derived free radicals and is also strongly related to the toxic action of the excitatory amino acids (EAs) glutamate and aspartate, a process called excitotoxicity. During hypoxia-ischaemia (HI) excessive amounts of glutamate, aspartate and other neurotransmitters are released into the extracellular space and contribute to cell death mediated by overactivation of the EAA receptors (Barks and Silverstein, 1992; Choi and Rothman, 1990; du Plessis and Johnston, 1997; Johnston et al., 2001). The excitatory glutamatergic innervation in the hippocampus is modulated by the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Therefore the GABAergic system may be of particular importance because it functions in opposition to that of glutamate. Compared to glutamate, the role of inhibitory neurotransmission in asphyxia-induced neuronal death has received relatively little attention. Some results from investigations in ischaemic brain conditions show an early disruption in GABA neurotransmission (Schwartz-Bloom and Sah, 2001).

Lamotrigine (LTG; lamotrigine isethionate, Glaxo/Wellcome, Research Triangle Park, NC, USA) is an anti-epileptic agent effective in partial and secondary generalized tonicoclonic seizures and is used either as adjunctive therapy or as monotherapy (Bazil, 2002; Kwan et al., 2001). It is also used as a well-tolerated maintenance treatment for bipolar I disorder primarily effective against depression (Calabrese et al., 2003). LTG’s mechanism of action involves the stabilization of the presynaptic membrane through blockade of voltage-sensitive sodium channels resulting in inhibition of the release of EAs, particularly glutamate (Calabresi et al., 2000; Goa et al., 1993; Lees and Leach, 1993; Wang et al., 2001). It has also been proposed that LTG inhibits high voltage-activated calcium currents that consequently interact with the vesicular release of neurotransmitters (Grunze et al., 1998; Wang et al., 2001). LTG has been found to improve brain damage after focal or global brain ischaemia in several experimental adult animal models, reducing the histological damage or the behavioural deficits (Shuaib et al., 1995; Smith and Meldrum, 1995; Wiard et al., 1995).

However, to the best of our knowledge, there are no published studies that have evaluated the effects of LTG in experimental HI conditions mimicking the ones that prevail during perinatal asphyxia. The aim of our study was to investigate the possible neuroprotective effects of LTG treatment in the developing brain after induction of HI in a widely used animal model of perinatal asphyxia (Rice et al., 1981). For that purpose we examined the histological outcomes and determined the changes in amino-acid levels in the hippocampus of the asphyxiated newborn rat after intraperitoneal (i.p.) administration of LTG.

**Methods**

Attention was paid to minimize pain and discomfort to the animals. Experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the ‘Principles of laboratory animal care’ (NIH publication no. 85-23, revised 1985) and were approved by the Ethical Committee of the School of Medicine of Aristotle University, Thessaloniki.

Seven-day-old Wistar rats, of either sex, delivered spontaneously and weighing 12–15 g were used for these experiments. The animals were maintained on a 12 h light/dark cycle (lights on at 07:00 hours) at a controlled temperature of 22 °C throughout the study. They had ad libitum access to food and water.

For the induction of brain HI a well-established model of perinatal brain damage in 7-d-old rats was used (Rice et al., 1981). This age is approximately equivalent to the 34-wk gestation in human infants. In this experimental procedure, brain injury is largely restricted to the cerebral hemisphere ipsilateral to the common carotid artery occlusion (Vannucci et al., 1999).

Ischaemia was induced to the left cerebral hemisphere by left common carotid artery ligation. In order to achieve this the rat pups were anaesthetized with diethyl ether and the midline of the neck was incised at the longitudinal plane. The left common carotid artery was exposed, dissected from the vagus nerve and doubly ligated with 4-0 silk suture to ensure cessation of blood flow. Total time of surgery was <6 min. The rats were then allowed to recover without the dams and ~2–3 h after surgery they were exposed to a 1-h period of hypoxia (8% oxygen, 92% nitrogen) by placing them in an airtight plastic jar which was partially submerged in a 37 °C water bath to provide a stable thermal environment. Rats were divided into five groups. Rats in group 1 (n = 14) did not undergo either ischaemia nor hypoxia and were used as the control group. Half were sacrificed on day 7 and the remainder on day 14. Rats in group 2 (n = 14) underwent the HI procedure; half were sacrificed on day 7 immediately after hypoxia and half
on day 14 of life (7 d after the insult). Rats in group 3 \((n = 14)\) and group 4 \((n = 14)\) received a single i.p. dose of LTG immediately after hypoxia at 10 mg/kg and 20 mg/kg, respectively. LTG was dissolved in water for injection and the injection volume was 0.01 ml/g body weight. Rats in group 5 \((n = 14)\) received the same volume of normal saline. After drug administration the rats of groups 3–5 were returned to their dams until day 14 of life when they were sacrificed.

Doses of LTG were chosen to be near the threshold for observation of neurochemical pharmacodynamic events based upon previous studies. The 10 mg/kg dose results in plasma concentrations that are within the anticonvulsive range both in rats and in humans. Doses of >20 mg/kg LTG may be associated with undesirable cardiovascular affects in some species (Castel-Branco et al., 2003, 2005; Morris et al., 1998). Thus, the doses used in the present study are well within the therapeutic index for the drug.

To investigate the possible neuroprotective effect of LTG, the histological outcomes were examined. Further, to define the possible mechanism of neuroprotection the concentration changes of the neurotransmitting amino acids were determined in homogenates of the hippocampus of asphyxiated newborn rats (Jabaudon et al., 2000; Kohlhauser et al., 1999; Rossi et al., 2000).

**Histological evaluation**

Seven days after recovery from HI, the rats were anaesthetized with halothane and perfused with PBS followed by 10% buffered formalin. After decapitation the brains were removed, post-fixed in 10% buffered formalin for at least 3 d and processed for paraffin sectioning. Brain sections were taken at the coronal plate according to Paxinos and Watson coordinates (Paxinos and Watson, 2002). Coronal sections \((8 \mu m)\) were obtained from each brain at the level of the anterior hippocampus and stained with haematoxylin and eosin. Damage to CA1, CA3, CA4 and dentate gyrus (DG) neurons was quantified by counting the viable cells within a 250 \(\mu m\) length of each region. Four counts were averaged from each animal. Seven animals per group were used for the histological evaluation. Perinatal asphyxia results in cells with features of necrosis and apoptosis and also in hybrid neurons with mixed features of both types of cell death (Johnston et al., 2001). Therefore in the damaged neurons we included all the neurons that displayed morphological characteristics of cell death (either necrosis or apoptosis, or both). The parameter used in the evaluation of the results was the total percentage of damaged neurons. Statistical comparisons of histological data were undertaken using ANOVA, followed by post-hoc comparisons by LSD test.

**Determination of amino acids in brain tissue**

The hippocampus was punched out from brains \((n = 7\) from each group) by a cannula with an outer diameter of 0.9 mm and an internal diameter of 0.6 mm on a refrigerated \((-80^\circ C)\) plate. The punches were immediately placed into 0.1 N HCl, weighed, homogenized and subsequently kept frozen at \(-80^\circ C\). Samples were thawed prior to use at 4 \(^\circ C\) and spun down in a vacuum centrifuge for 20 min at 193000 \(g\); the supernatant was taken for HPLC determinations. Determination of glutamate, aspartate, glutamine and GABA, were performed by a HPLC technique (Shimadzu, Europa GmbH, Duisburg, Germany) with fluorimetric detection after derivatization with o-phthalodialdehyde (OPA) (Singewald et al., 1995). The recordings and data analysis were performed using chromatography software (DataApex Clarity, Prague, Czech Republic). Data were analysed using the ANOVA test followed by post-hoc multiple comparisons by Dunnett’s test.

**Results**

**Effect of LTG on HI-evoked damage to hippocampal neurons in the CA1, CA3, CA4, and DG regions in neonatal rats**

Measurement of the percentage of affected neurons in various regions of the neonatal hippocampus of the left hemisphere caused by HI revealed that the CA1 region suffered the greatest damage followed by DG and CA3, while the CA4 region displayed the least damage (Figure 1). Administration of 10 mg/kg LTG led to a distinct reduction in the percentage of disrupted neurons in the CA1 region (Figure 1). In the CA3, CA4, and DG regions administration of 10 mg/kg LTG tended only to reduce asphyxia-evoked destruction (Figure 1). Administration of 20 mg/kg LTG decreased the percentage of damaged neurons in the CA1, CA3 and DG regions, while in the CA4 region only a slight amelioration was observed (Figures 1, 2).

**Effect of LTG on HI-evoked tissue levels of the amino acids glutamate, aspartate, GABA, and glutamine in the hippocampus of neonatal rats**

Hippocampal tissue levels of glutamate, aspartate, and GABA in the damaged left cerebral hemisphere immediately after HI (day 7) were increased compared
to the control group (non-HI, day 7), while the levels of glutamine were decreased (Figure 3).

Seven days after HI (day 14) the tissue levels of glutamate and aspartate remained elevated (Figure 3). However, levels of GABA, taken 7 d after HI, returned to control values (Figure 3). Moreover, hippocampal tissue levels of glutamine were similar to control values on day 14 (Figure 3).

Administration of 10 mg/kg LTG tended to decrease HI-evoked hippocampal tissue levels of glutamate and aspartate (Figure 4). A dose of 20 mg/kg LTG abolished asphyxia-induced elevation of tissue levels of both glutamate and aspartate (Figure 4). GABA and glutamine levels were not affected by LTG administration at doses of either 10 mg/kg or 20 mg/kg (Figure 4). Injection of saline did not influence any investigated tissue levels (Figure 4).

Discussion

Histological evaluation revealed that the most extensive damage caused by asphyxia was in the CA1 region of the neonatal hippocampus. The vulnerability of this region was also observed in experiments in asphyxiated human neonates (de Haan et al., 2006). The second highest percentage of neuronal loss was found in the DG region followed by the CA3 region. A significant damage in granule cells of the inner layers of the DG and similar damage in the CA1 region has been observed in a rat model of perinatal asphyxia with bilateral permanent carotid ligation (Liu et al., 2004; Pulera et al., 1998). Some authors support the view that in asphyxiated humans the extent of gliosis following DG damage is an index of the severity of the insult (Del Bigio and Becker, 1994). In our study the CA4 region was less influenced by HI. This may possibly be related to the morphogenesis of the different hippocampal regions in the first days after birth as more differentiated cells might be more resistant to HI. Pyramidal cell morphogenesis does not occur synchronously throughout the CA hippocampal regions, but cells in the CA4 region undergo morphological differentiation at earlier stages, than CA3, and these in turn earlier than CA1 (Lopez-Gallardo and Prada, 2001).

Administration of 10 mg/kg LTG resulted in a reduction in the number of damaged neurons in the CA1 region and tended to decrease damage in the CA3 and DG regions. Injection of 20 mg/kg LTG also decreased the percentage of damaged cells in the CA3 region.
and DG regions. Thus, we can conclude that the neuroprotective effect of LTG was dose-dependent.

There are no previous experimental data on the effects of LTG under conditions of perinatal HI. LTG has only been administered after ischaemic brain conditions in adult animals (Shuaib et al., 1995; Wiard et al., 1995). In studies of focal brain ischaemia in rats, after permanent middle cerebral artery occlusion, administration of 20 mg/kg LTG immediately after the insult reduces total and cortical infarct volume and improves neurological deficit (Smith and Meldrum, 1995). In a rat model of cardiac arrest-induced global cerebral ischaemia LTG reduced the damage to the hippocampal CA1 cells (Crumrine et al., 1997). It should also be noted that LTG protects striatal neurons against in-vitro ischaemia (Calabresi et al., 2003).

The hippocampal tissue levels of glutamate and aspartate were elevated immediately after the HI insult and remained enhanced even 7 d after the asphyctic insult suggesting a prolonged action. Although tissue values do not reveal the origin of the released neurotransmitters, they are, in regard to our study, a plain indicator that HI induced an imbalance in EAA turnover in the neonatal hippocampus. Furthermore, our study shows a correlation of damaged hippocampal neurons with elevated tissue values of glutamate and aspartate that supports the idea of a dominant role for EAAs in cell damage, induced under HI conditions in neonates. Further, since any kind of direct investigation of extracellular release of neurotransmitters in 7-d-old rats may lead to an unacceptable impairment of the newborn’s condition, we decided to adopt this method of evaluating the influence of LTG on levels of neurotransmitters in this set-up. Other studies have shown, both in vitro and in vivo, that elevation of EAAs plays a dominant role in the cell damage induced under HI conditions, i.e. excitotoxicity (Choi et al., 1987; du Plessis and Johnston, 1997; Fujisawa et al., 1993). A significant increase in the levels of glutamate has also been observed in adult humans immediately following an ischaemic stroke (Castillo et al., 1996), or acute brain ischaemia in adult rats (Kouvelas et al., 2006). The levels of the EAAs remained elevated even 7 d after the asphyctic insult suggesting a prolonged action.

In the present study, hippocampal tissue levels of GABA immediately after asphyxia were elevated. The degree of GABA elevation is in proportion to the elevation of the other neurotransmitters and may be caused by the same cellular events generated by asphyxia (Schwartz-Bloom and Sah, 2001). The release of preloaded labelled GABA and its structural analogue taurine is greatly enhanced in both the adult and developing hippocampus under cell-damaging conditions, including hypoxia, hypoglycaemia and ischaemia (Saransaari and Oja, 1998). In our study, 7 d after the HI insult, GABA levels returned to the control values. As with most neurotransmitters, GABA accumulates in the extracellular space during ischaemia but its level sharply returns to normal (within an hour of onset of reperfusion) presumably as GABA uptake resumes (Phillis et al., 1994; Schwartz-Bloom and Sah, 2001). There is also support that asphyxia leads to loss of GABAergic projection neurons and interneurons, especially in the striatum and the hippocampus of...
the rat (Van de Berg et al., 2003). A reduction in the number of the GABAergic neurons of these areas has been reported in several experimental animal models after perinatal asphyxia (Dell’Anna et al., 1996; Guan et al., 2000; Mallard et al., 1995). The release of massive amounts of GABA immediately after asphyxia may constitute an important protective mechanism against the excessive release of EAAs (Saransaari and Oja, 1998).

The levels of glutamine in hippocampal brain tissue were significantly decreased immediately after asphyxia. Our results confirm previous findings in the same model of asphyxia (Spandou et al., 1999). Glutamine synthase, which catalyses the conversion of glutamate to glutamine, is an ATP-requiring enzyme. HI leads to ATP depletion and thus decreased activity of the synthase resulting in decreased levels of glutamine and increased levels of glutamate. The increase in glutamine levels in the days following HI may be secondary to the conversion of the very high glutamate levels (Andine et al., 1991).

From the outcomes of the above experiments, it was noted that administration of LTG caused a dose-dependent decrease in the tissue levels of EAAs evoked under HI conditions in the neonatal hippocampus. LTG is believed to cause a reduction in glutamate release secondary to an inhibitory effect on type II Na$^+$-channels leading to an overall reduction in CNS excitability (Lees and Leach, 1993; Wang et al., 2001). Other studies have investigated the effect of acute and chronic LTG treatment on amino-acid release in the hippocampus of freely moving rats. A reduction of basal and veratridine-evoked extracellular release of EAAs was observed after treatment with LTG (Ahmad et al., 2004). LTG has also been used after focal brain ischaemia in adult rats showing neuroprotective effects (Smith and Meldrum, 1995). Furthermore, the injection of LTG immediately after an ischaemic insult, causes a reduction in the extracellular accumulation of glutamate in a model of global cerebral ischaemia in adult rabbits (Bacher and Zornow, 1997). Similar results were observed in in-vitro studies (Calabresi et al., 2000). The fact that administration of LTG abolished HI-induced hippocampal tissue levels of glutamate and aspartate indicates that the affected EAA release might be of vesicular origin since the mechanisms of action of LTG is thought to be both the stabilization of the presynaptic membrane through blockade of voltage-sensitive sodium channels, and the inhibition of high voltage-activated calcium currents interacting with the vesicular release of neurotransmitters (Lees and Leach, 1993; Wang et al., 2001; Grunze et al., 1998). To the best of our knowledge, there are no other targets of LTG.

Data on the effect of LTG on GABA release are controversial. In the present study, LTG did not influence GABA levels of hippocampal tissue. There is no literature on the effect of LTG on GABA levels after brain HI. An in-vivo study focused on the effect of LTG on the veratridine-evoked release of GABA in freely moving adult rats which found a decrease of evoked GABA release during chronic LTG treatment (Ahmad et al., 2004). A possible LTG-mediated decrease in spontaneous release of glutamate and enhanced GABA release was noted in the rat entorhinal cortex in vitro (Cunningham and Jones, 2000). In contrast LTG decreases GABA-mediated transmission under similar experimental conditions (Braga et al., 2002). Ex-vivo studies have shown acute LTG to have no effect on hippocampal tissue content of GABA in freely moving rats but GABA increased following chronic treatment with the drug (Hassel et al., 2001).

In conclusion the present study demonstrates that LTG has a neuroprotective effect on the neonatal rat hippocampus when administered immediately after the HI insult. Furthermore, injection of LTG decreased asphyxia-induced brain tissue levels of excitotoxicity causing the EAAs glutamate and aspartate but did not alter GABA and glutamine levels. These findings lend support to a crucial role for the reduction of EAAs being central to the mode of neuroprotective action of LTG. Our study suggests that LTG could be potentially used as a neuroprotective agent following perinatal asphyxia, but further behavioural and histological studies are required in order to evaluate its effects at later time-points in the adult animal’s life and define its action more precisely.

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Statement of Interest
None.

References


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