Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 485 (2004) 183–191
www.elsevier.com/locate/ejphar

Effect of late treatment with γ-hydroxybutyrate on the histological and behavioral consequences of transient brain ischemia in the rat

Alessandra Ottani[a,*], Anna Valeria Vergoni[a], Sabrina Saltini[a], Chiara Mioni[a], Daniela Giuliani[a], Marta Bartiromo[b], Davide Zaffe[c], Annibale R. Botticelli[d], Anna Ferrari[e], Alfio Bertolini[e], Susanna Genedani[a]

[a] *Section of Pharmacology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via G. Campi 287, Modena 41100, Italy*
[b] *Department of Medical Biotechnology, University of Napoli, Naples, Italy*
[c] *Department of Morphological Sciences and Forensic Medicine, University of Modena and Reggio Emilia, Largo del Pozzo 71, Modena 41100, Italy*
[d] *Department of Human Pathology, University of Pavia, Via Forlanini 14, Pavia 27100, Italy*
[e] *Section of Clinical Pharmacology and Toxicology, Department of Internal Medicine, University of Modena and Reggio Emilia, Largo del Pozzo 71, Modena 41100, Italy*

Received 6 November 2003; received in revised form 26 November 2003; accepted 28 November 2003

Abstract

It has been previously described that γ-hydroxybutyrate (GHB) provides significant protection against transient global cerebral ischemia in the rat (four vessel occlusion model), when given 30 min before or 10 min after artery occlusion. Here, we show that in the same rat model, significant protection can also be obtained when treatment is started 2 h after the ischemic episode. In saline-treated animals, 30 min of global ischemia followed by reperfusion caused a massive loss of neurons in the hippocampal CA1 subfield (examined 63 days after the ischemic episode), and an impairment of sensory-motor performance (tested on the 51st and 63rd days after ischemia) and of spatial learning and memory (evaluated starting 46 days after the ischemic episode). Treatment with GHB—300 mg/kg intraperitoneally (i.p.) 2 h after the ischemia–reperfusion episode, followed by 100 mg/kg i.p. twice daily for the following 10 days—afforded a highly significant protection, against both histological damage and sensory-motor and learning-memory impairments. These data further suggest the possible therapeutic effectiveness of GHB in brain ischemia, and indicate that the underlying mechanism of action involves non-immediate steps of the ischemia-induced cascade of events.

Keywords: Cerebral ischemia; GHB (γ-hydroxybutyrate); Immunohistochemistry; (Rat); Sensory-motor test; Spatial learning

1. Introduction

γ-Hydroxybutyrate (GHB) is a neurotransmitter in the mammalian brain, where it is mainly concentrated in the hippocampus and in the thalamus, principally in the synaptosomal compartment (Bernasconi et al., 1999; Maitre et al., 1983, 2002; Snead, 1991; Snead and Liu, 1984; Tunnicliff, 1992; Vayer et al., 1987). Specific mechanisms for its synthesis, release and uptake are present in neurons (Maitre et al., 1983; Vayer et al., 1987). High- and low-affinity specific binding sites are unevenly distributed in the brain, and most densely located in the rostral part of the brain hippocampus, fronto-parietal cortex, striatum, thalamus, olfactory tract, some dopaminergic nuclei (Hechler et al., 1987, 1992).

A protective effect of GHB and its lactone, γ-butyrolactone (GBL), in animal models of brain ischemia/hypoxia as well as in human conditions such as head injury-induced coma, has been repeatedly described (Bralet et al., 1979; Escuret et al., 1977; Lavyne et al., 1983; Vergoni et al., 2000; Wolfson et al., 1977). In particular, we have recently observed (Vergoni et al., 2000) that in a rat model of transient (30 min) global brain ischemia followed by incomplete reperfusion [four vessel occlusion model (Pulsinelli and Brierly, 1979)], the intraperitoneal (i.p.) injection of GHB (300 mg/kg, 30 min before or 10 min after artery occlusion, followed by 100 mg/kg i.p. twice daily for the following 10 days) affords a significant protection against

* Corresponding author. Tel.: +39-059-2055382; fax: +39-059-2055376.
E-mail address: alessandraottani@yahoo.it (A. Ottani).

0014-2999/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2003.11.072

both histological and behavioral outcomes (loss of neurons in the hippocampal CA1 subfield; impairment of sensory-motor performance, learning, and memory). However, it is impossible to start treatment 10 min after ischemia, and even more so 30 min before ischemia, in patients. So, our above-quoted previous rat data do not allow us to predict the possible therapeutic effect of GHB in humans with a brain ischemic insult. The present investigation was therefore designed with the aim to see whether GHB is still effective when the times of administration are similar to clinically practicable ones.

2. Materials and methods

2.1. Animals

Adult male rats of an SPF Wistar strain (Harlan Italy, Correzzana, Milano, Italy), weighing 220–240 g upon arrival, were used. They were housed four per cage (40 × 21 × 15 cm), with food in pellets (TRM, Harlan Teklab) and tap water available ad libitum, in a climatized colony room (temperature: 22 ± 1 °C; humidity: 60%), on a 12-h light/dark cycle (light on from 07:00 to 19:00 h). They were acclimatized to our housing conditions for at least 1 week before being used. Housing conditions and experimental procedures were strictly in accordance with the European Community ethical regulations on the care of animals for scientific research purposes.

2.2. Four vessel occlusion transient global cerebral ischemia procedure (Pulsinelli and Brierly, 1979)

On the first day, rats were anesthetized with ketamine plus xylazine (115 + 2 mg/kg i.p.; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milano, Italy, respectively). Both common carotid arteries were exposed through a ventral midline cervical incision and isolated. An atraumatic silk ligature was loosely placed around each carotid artery (carefully avoiding interrupting or reducing the blood flow) in order to allow easy and rapid access for occlusion on the following day; then the incision was sutured. A second dorsal midline incision from the occipital protuberance to the midcervical region was made. The paraspinal muscles were dissected and, with the aid of an operating microscope (25 × magnification factor, Carl Zeiss, Jena, Germany), the right and left alar foramina of the first cervical vertebra were exposed. A 0.5-mm electrocautery needle was inserted into each alar foramen and both vertebral arteries were permanently occluded by cauterization. While the rats were still under anesthesia, stainless-steel screw electrodes were inserted into the skull bone overlying the fronto-parietal cortex in order to perform electroencephalogram (EEG) recordings (Battaglia-Rangoni polygraph, Bologna, Italy). The rats were allowed to recover from surgery overnight. The next day, under brief halothane anesthesia (2% halothane in room air), the ventral neck suture was removed and the common carotid arteries were re-exposed. At this moment, halothane anesthesia was interrupted (and no more resumed) and both carotid arteries were occluded by clamping them closed with atraumatic clips. The clips were removed 30 min later, and resumption of blood flow was verified by direct visual inspection. During ischemia and the subsequent reperfusion, body temperature was maintained at 37.5 ± 0.5 °C by means of a rectal thermostat connected to a heating lamp until the rats recovered thermal homeostasis. Only those rats that lost their righting reflex immediately after carotid occlusion, were unresponsive to somatosensory stimulation, with pupils fully dilated, and had an isoelectric EEG throughout the ischemic period were included in the study. Rats that exhibited seizures were excluded. Four percent of rats died during the 30-min period of total ischemia.

Sham-operated controls were only subjected to anaesthesia, electrode implantation, and skin incision and sutures.

2.3. Spatial learning and memory

The Morris water maze test (Morris, 1984; Mcnamara and Skelton, 1993) was used with minor modifications (Vergoni et al., 2000). This test measures the ability of a rat to learn, remember and go to a place in space defined only by its position relative to distal extra-maze cues (Mcnamara and Skelton, 1993). Under our experimental conditions, the apparatus consisted of a circular white pool (1.60-m diameter) filled to a depth of 30 cm with water at 28 ± 1 °C, rendered opaque with milk powder. Rats were trained to find the platform (diameter 11 cm) which was made of clear perspex and submerged 1 cm below the water level. Four points on the pool rim (North, South, East, West) defined four 90° quadrants on the pool surface (NE, NW, SE, SW) and the platform occupied a position in the middle of a cardinal quadrant, 35 cm from the rim of the pool. The tank was virtually divided into four quadrants by two wires intersecting at right angles. Conspicuous cues (racking, wall plates, door, the observer himself) were placed in a fixed position around the pool. On the day before the start of training, each rat was given 120 s of adaptation to the pool (i.e., the rat was placed in the pool—without platform—and allowed to swim freely with no opportunity to escape). During training, a trial began when the rat, held facing the side wall, was immersed in the water. Latency to escape onto the hidden platform and time spent outside the target quadrant were recorded with a stopwatch. If the rat failed to locate the platform within 60 s, it was placed on it. The rat was left on the platform for 20 s and then removed. Each rat received four consecutive trials on each day, starting each time from a different cardinal point in random succession. In the first 5-day acquisition sessions, which started 46 days after the ischemic episode, the platform remained in its allocated position. In the second 3-day reversal learning, which started 60 days after ischemia, the position of the

platform was changed, while the extra-maze cues were maintained in the same position. Tests were performed between 10:00 and 13:00 h in a sound-proof room by an observer unaware of the treatments. The pool was drained and cleaned each day at the end of testing.

2.4. Sensory-motor orientation and coordinated limb use

To test sensory-motor orientation and coordinated limb use on each side of the body, rats were subjected to the test battery of Marshall and Teitelbaum (1974), modified by Bjorklund et al. (1980) and further slightly modified by ourselves, 51 and 63 days after the ischemic episode. In order to test orientation to sensory stimuli, the rat was placed on the surface of a bench. Head orientation toward (with or without biting of) the stimulus probe was recorded first on one side of the body and then on the other side for each of the following stimuli: (a) somesthesis—a pin prick was applied to six sites, involving combinations of dorsal and ventral placements at rostral, middle and caudal levels on the lateral surface of the body; (b) whisker touch—a toothpick was lightly brushed against the vibrissae, approaching from the lower rear of the animal so as to avoid the visual field; (c) snout probe—the toothpick was gently rubbed against the snout of the rat; (d) olfaction—a small cotton swab dipped in ammonia solution was gently approached in a lateral–medial direction toward the rat's nose.

Limb reflexes and coordinated limb use were assessed in the following tests: (a) forelimb placement—the rat was grasped around the abdomen and slowly lowered in a head-down orientation toward the surface of the bench. The accuracy and coordination of reflex placement of the forelimbs was noted; (b) forelimb suspension—the rat was grasped by a forepaw and suspended. Normal animals rapidly grasp the hand with the free paw and use this to pull themselves up onto the hand; latency to achieve successful pull-up was recorded, with a failure criterion of 10 s; (c) climbing grid—the rat was placed on a vertical wire grid; (d) mouth probe—the rat was held vertically around the body with head upwards, and a toothpick was inserted into the side of the mouth. Normal rats lick this stimulus; grasping of the probe with the ipsilateral forepaw, or attempts to bite the probe were recorded. The deficit in each orientation and limb-use test was rated on a three-point scale [0 (absent), 1 (weak), or 2 (strong)] and a total index score for each rat was obtained. The overall minimum and maximum scores for each rat were thus 0 and 24, respectively.

2.5. Histology

Six rats per group (randomly taken from each of the three groups: sham-operated; lesioned and treated with saline; lesioned and treated with GHB) were decapitated under ether anesthesia 63 days after the lesion. The brains were dissected out and immediately immersed in 10% buffered (pH 7.0) formalin solution for 24 to 48 h. After fixation, brain were sectioned to obtain slices containing the hippocampus (Toga et al., 1995). The slices were then embedded in paraffin blocks, and coronal 6-μm-thick sections were serially cut (step section technique), spanning the longitudinal axis of the hippocampus, and collected on glass slides coated with aminoakylsilane (Dako, Glostrup, Denmark). Histological sections were stained with hematoxylin–eosin (morphology) and Nissl (neurons). Immunohistochemical methods (all antibodies were from Zymed Laboratories, San Francisco, CA, USA) were applied to highlight the fibrillary acid protein of normal and reactive astrocytic cells [antibody: monoclonal mouse anti-glial fibrillary acid protein (GFAP), clone ZCG29 Isotype IgG1]. The slides were incubated overnight at 4 °C in a moist and darkened chamber. Slides were then incubated with 1:200 streptavidin biotinylated complex (Dako) for 60 min, and developed in diaminobenzidine.

Histomorphological analyses were performed using an Axiophot photomicroscope (Carl Zeiss). Morphometrical data were collected using an image analysis system by means of a digital camera (DC100; Leica Microsystems, Heerbrugg, Switzerland), applied to personal computer connected to a Leica Photomicroscope Labor Lux 20 (Jena, Germany). The area of injured tissue and cell density were evaluated using a Sony CCD color camera on a Leica microscope connected to a personal computer. Histometrical analyses (Anderson and Lowe, 1990) were performed at the magnification factor on TV screen of $\times 50$ (injured brain areas) and $\times 800$ (cell density). The area of injured tissue and the number of neurons and glial cells were evaluated on three serial slides, stained with hematoxylin–eosin (injured tissue), Nissl (neurons) and GFAP (glial cells), using an image system (SC Image DB, Casti, Venice, Italy) and automatically processed by means of an image analysis program (Microimage, Casti). The density of neurons and glial cells was estimated in a 100-μm-thick band overlapping the single cellular layer of the hippocampus (the pyramidal cell layer) of the injured areas.

2.6. Drugs and treatment

Rats subjected to transient global cerebral ischemia were randomly assigned to one of the following i.p. treatments: (1) saline, 2 ml/kg, 2 h after carotid artery occlusion, and then twice daily for the subsequent 10 days; (2) GHB, 300 mg/kg, 2 h after carotid artery occlusion, and then 100 mg/kg twice daily for the subsequent 10 days. Sham-operated rats were treated with saline, 2 ml/kg i.p. twice daily for 10 days. Ten rats per group were used. The doses of GHB were chosen on the basis of previous experiments performed by our group with the same strain of rats and using the same model of transient global brain ischemia followed by reperfusion (Vergoni et al., 2000). Sodium γ-hydroxybutyrate (GHB), a gift of Laboratorio Farmaceutico C.T., Sanremo, Italy, was dissolved in saline immediately before treatment and injected in a volume of 2 ml/kg i.p.

Table 1
Influence of sodium γ-hydroxybutyrate (GHB) on sensory-motor orientation and coordinated limb use, tested 51 and 63 days following a 30-min episode of global cerebral ischemia. GHB 300 = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 2 h after arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days

Group	Score at 51st day	Score at 63rd day
(1) Sham-operated	10	10
(2) Ischemia + saline i.p.	80[a]	76[a]
(3) Ischemia + GHB 300	42[a,b]	36[b]

Cumulative scores obtained from 10 rats per group.
[a] $P < 0.05$ compared with sham-operated group.
[b] $P < 0.05$ compared with ischemia + saline group (Friedman's test followed by Mann–Whitney *U*-test).

2.7. Statistical analysis

Data for spatial learning and memory were analyzed using analysis of variance (ANOVA) followed by Student–Newman–Keuls' test. Data for sensory-motor test were analyzed using Friedman's test followed by Mann–Whitney *U*-test. Data concerning the area, the neuronal density and the astrocyte density of the injured tissue were analyzed using Mann–Whitney *U*-test.

3. Results

3.1. Sensory-motor performance

Under our experimental conditions, a 30-min period of global brain ischemia followed by incomplete reperfusion caused an impairment in sensory-motor orientation and coordinated limb use, evaluated 51 days after the ischemic insult ($r\chi^2 = 16.2$, $P < 0.05$); the impairment was still significant and almost unchanged 12 days later ($r\chi^2 = 12.8$, $P < 0.05$) (Table 1). The administration of GHB, started 2 h after occlusion of the carotid arteries (300 mg/kg i.p) and continued for the following 10 days (100 mg/kg, twice daily i.p.), afforded a protection that was significant either 51 (sham-operated vs. ischemia + saline: $T = 55$, $P = 0.000$; sham-operated vs. ischemia + GHB: $T = 67$, $P = 0.003$; ischemia + saline vs. ischemia + GHB: $T = 67$, $P = 0.004$) or 63 days (sham-operated vs. ischemia + saline: $T = 55$, $P = 0.000$; sham-operated vs. ischemia + GHB: $T = 75$, $P = 0.007$; ischemia + saline vs. ischemia + GHB: $T = 71$, $P = 0.010$) after the ischemic episode (Table 1).

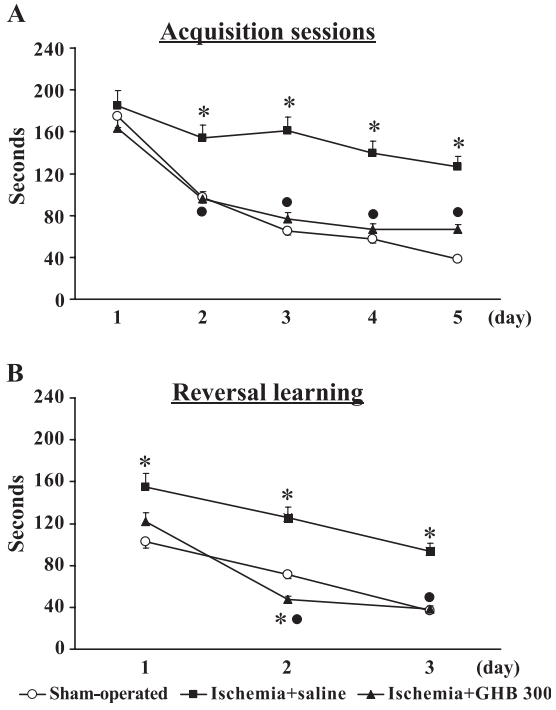

Fig. 1. Effect of sodium γ-hydroxybutyrate (GHB) on escape latency in the Morris water maze. GHB = 300 mg/kg i.p. 2 h after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. Acquisition sessions and reversal learning started 46 and 60 days, respectively, following a 30-min episode of global cerebral ischemia. Values are the means ± S.E.M. of the escape latency (s); 10 rats per group. *$P < 0.05$ compared with sham-operated group in the same session; •, $P < 0.05$ compared with ischemia + saline group in the same session (ANOVA followed by Student–Newman–Keuls' test).

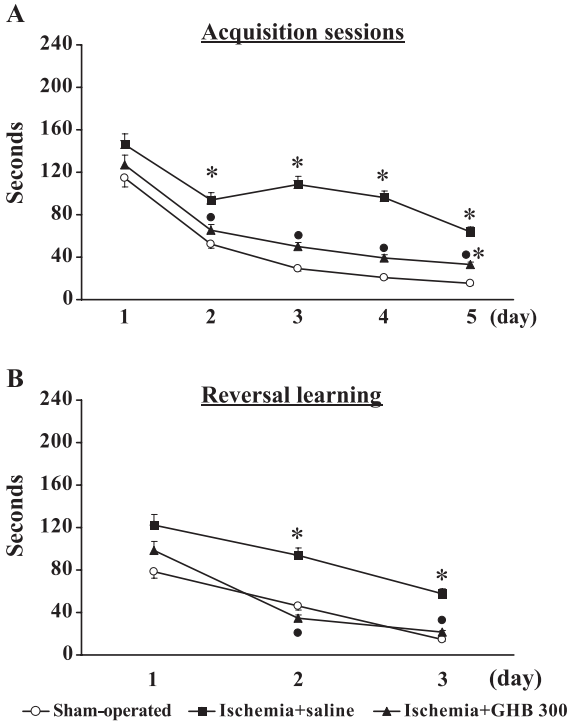

Fig. 2. Effect of sodium γ-hydroxybutyrate (GHB) on outside time in the Morris water maze. GHB = 300 mg/kg i.p. 2 h after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. Acquisition sessions and reversal learning started 46 and 60 days, respectively, following a 30-min episode of global cerebral ischemia. Values are the means ± S.E.M. of the escape latency (s); 10 rats per group. *$P < 0.05$ compared with sham-operated group in the same session; •, $P < 0.05$ compared with ischemia + saline group in the same session (ANOVA followed by Student–Newman–Keuls' test).

3.2. Spatial learning and memory

The 30-min episode of global brain ischemia followed by incomplete reperfusion produced a clear impairment of place learning and memory in the Morris water-maze test: the performance of these rats was in fact significantly worse than that of sham-lesioned ones. The performance of rats treated with GHB starting 2 h after the ischemia/reperfusion episode was significantly better than that of saline-treated rats already from the second day of training and throughout the whole 5-day acquisition session, and was almost identical to the performance of sham-operated rats [day 2:

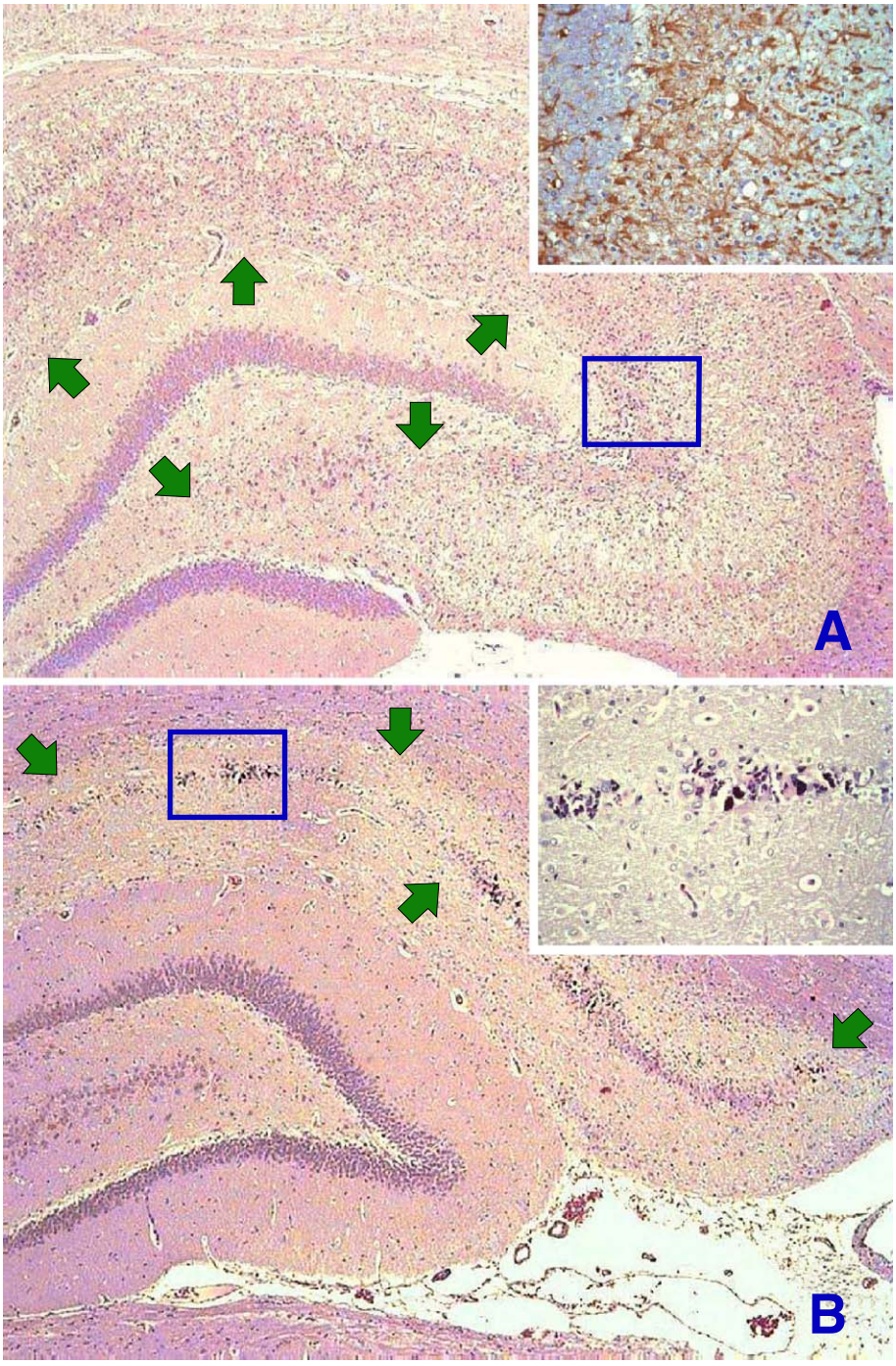

Fig. 3. Effect of sodium γ-hydroxybutyrate (GHB) on the hippocampus appearance 63 days after a 30-min episode of global cerebral ischemia. Ischemic rat hippocampus after (A) saline (2 ml/kg) or (B) GHB (300 mg/kg i.p. 2 h after arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days). The squared areas correspond to the fields of the high magnification insert on the right-top of each image. Note in A the large extent of the injured (due to gliosis) tissue (arrows) and the presence of reactive astrocytes (insert). Note in B the lesser extent of the injured area (arrows). Hematoxylin–eosin stain (main images and B insert), GFAP immunoreaction (A insert). Field with: main images = 2.5 mm; inserts = 400 μm.

$F(2,27)=8.35$, $P=0.001$; day 3: $F(2,27)=15.61$, $P=0.000$; day 4: $F(2,27)=8.62$, $P=0.001$; day 5: $F(2,27)=17.59$, $P=0.000$] (Fig. 1A). Similar results were obtained for reversal learning, lasting 3 days and started 10 days after the termination of the first 5-day acquisition session. Indeed, on the 2nd day of this second sequence, the performance of ischemia-lesioned rats treated with GHB was better than that of sham-lesioned rats [day 1: $F(2,27)=4.53$, $P=0.02$; day 2: $F(2,27)=33.63$, $P=0.000$; day 3: $F(2,27)=11.84$, $P=0.000$] (Fig. 1B). Data concerning the time spent outside the quadrant where the platform was located confirmed the latency-to-escape data (Fig. 2A and B).

3.3. Histology

No histological evidence of ischemic injury was seen in sham-operated rats. The stratum pyramydalis of hippocampus showed neurons without degeneration or necrosis and a feeble positivity to GFAP in sham-operated rats. In contrast, extensive hippocampal ischemia was detected in 100% of saline-treated rats, whereas a lower level of ischemia was detected in GHB-treated rats. Ischemic areas were found in the hippocampus proper (Ammon's horn).

In the damaged ischemic areas (saline-treated rats), neuronal cells inside the stratus pyramydalis of hippocampus have disappeared and were almost totally replaced by glial cell hyperplasia showing GFAP immunocytochemical positivity (Fig. 3A, insert). At the edges of these areas, the damaged tissue was mixed with pre-existent undamaged tissue with living neurons among necrotic ones (SNN—selective neuronal necrosis). These few living neurons did not show nuclear regression or nuclear displacement and an almost intact Nissl substance. In contrast, the necrotic neurons show pyknosis, nuclear dust, swollen perikaryon, cellular shrinkage and absence of Nissl substance. The GFAP immunohistochemical reaction (Fig. 3A, insert) showed that fibrous astrocytes formed the predominant cell type inside the ischemic area.

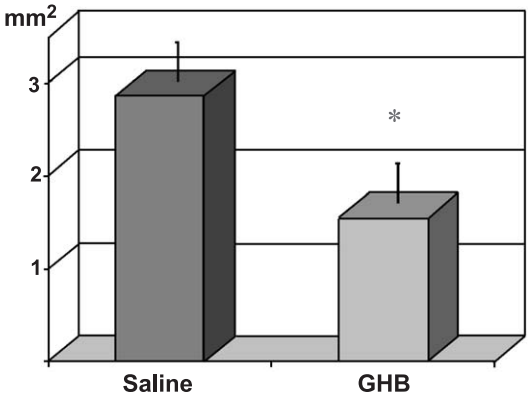

Fig. 4. Effect of sodium γ-hydroxybutyrate (GHB) on hippocampal ischemic area 63 days after a 30-min episode of global cerebral ischemia. GHB = 300 mg/kg i.p. 2 h after arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. Each bar represents the mean ± S.D. ischemic area (expressed as mm^2) in saline- ($n=6$) or GHB-treated rats ($n=6$). *$P<0.01$ (Mann–Whitney U-test).

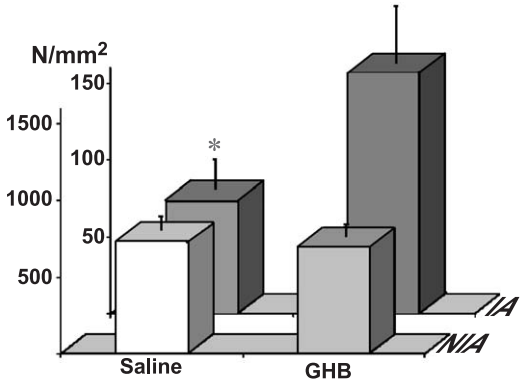

Fig. 5. Effect of sodium γ-hydroxybutyrate (GHB) on hippocampal neuronal density 63 days after a 30-min episode of global cerebral ischemia. GHB = 300 mg/kg i.p. 2 h after arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. Each bar represents the mean ± S.D. neuronal density (expressed as neurons/mm^2) of not-injured (NIA) and ischemic (IA) areas in saline-treated rats ($n=6$) and GHB-treated rats ($n=6$). *$P<0.01$, (Mann–Whitney U-test).

The ischemic area in GHB-treated rats had a moderate degree of damage, with the presence of living neurons among necrotic ones (SNN) (Fig. 3B, insert). The predominant glial element in these ischemic areas was found to be the protoplasmic astrocyte, as shown by the GFAP immunohistochemical reaction.

Histometric analyses of the injured tissue (Fig. 4) showed that the mean ± S.D. area of ischemic damage was 2.87 ± 0.44 mm^2 in lesioned and saline-treated rats, whereas it was 1.55 ± 0.43 mm^2 in lesioned and GHB-treated rats (Mann–Whitney U-test: $T=21.5$, $N1=6$, $N2=6$; $P<0.01$). The neuronal density in not-injured areas (Fig. 5) was $736.8 \pm 97.5/mm^2$ (m ± S.D.) in saline-treated rats and $700.8 \pm 78.3/mm^2$ (m ± S.D.) in GHB-treated rats (Mann–Whitney U-test: $T=43.5$, $N1=6$, $N2=6$; $P=0.47$). The neuronal density in injured

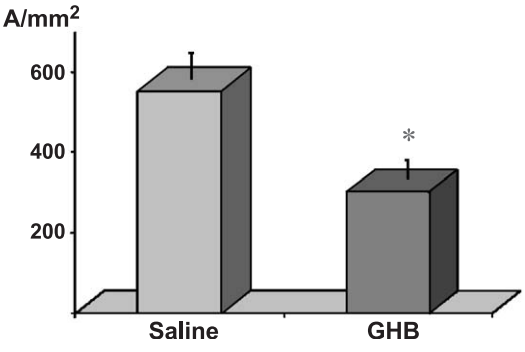

Fig. 6. Effect of sodium γ-hydroxybutyrate (GHB) on hippocampal astrocyte density 63 days after a 30-min episode of global cerebral ischemia. GHB = 300 mg/kg i.p. 2 h after arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. Each bar represents the mean ± S.D. astrocyte density (expressed as astrocytes/mm^2) of ischemic areas in saline- ($n=6$) or GHB-treated rats ($n=6$). *$P<0.01$ (Mann–Whitney U-test).

areas (Fig. 5) was $73.9 \pm 21.26/mm^2$ ($m \pm S.D.$) in saline-treated rats and $157.2 \pm 39.7/mm^2$ ($m \pm S.D.$) in GHB-treated rats (Mann–Whitney U-test: $T = 21.5$, $N1 = 6$, $N2 = 6$; $P < 0.01$). The astrocyte density of injured areas (Fig. 6) was $555 \pm 70.39/mm^2$ ($m \pm S.D.$) in saline-treated rats, and $304.4 \pm 50.63/mm^2$ ($m \pm S.D.$) in GHB-treated rats (Mann–Whitney U-test: $T = 21$, $N1 = 6$, $N2 = 6$; $P < 0.01$).

4. Discussion

The present behavioral data demonstrate that sensory-motor, learning and memory impairments and hippocampal damage produced in adult rats by a transient episode (30 min) of global brain ischemia followed by incomplete reperfusion are significantly reduced by the i.p. administration of GHB, started 2 h after the ischemic insult and continued for 10 consecutive days.

Qualitative and quantitative histomorphological results showed a significant effect of GHB on the extent and characteristics of the ischemic tissue. Morphometric analysis indicated that the ischemia-induced neuronal damage was significantly greater in the group of rats treated with saline. Both the morphologic and morphometric analyses indicated that the treatment with GHB in part preserved neurons and reduced the severity of ischemic damage.

The doses of GHB (first dose: 300 mg/kg; subsequent doses: 100 mg/kg every 12 h) are far from toxic ones (900–1200 mg/kg). These results confirm the previous data obtained with the same animal model in which GHB administration was started either 30 min before or 10 min after occlusion of the carotid arteries (Vergoni et al., 2000) and show that GHB is effective even if treatment is delayed by 2 h. This may give support to the idea (Vergoni et al., 2000) that GHB may be of therapeutic interest in the management of episode of brain ischemia.

It has already been shown that the lactone of GHB, γ-butyrolactone (GBL), is beneficial at low doses (60 mg/kg) in comatose head-injured patients (Escuret et al., 1977). In these subjects, GBL lowered the cerebral metabolism of oxygen without depressing cerebral perfusion pressure. In rats, GBL lowered cerebral glucose utilization more than did comparable doses of barbiturates or hypothermia, suggesting a potential beneficial effect in the treatment of evolving stroke (Wolfson et al., 1977). In mice kept under hypoxic conditions (5% oxygen), GHB and GBL significantly increased survival time, the maximum effect being obtained with both drugs at the dose of 300 mg/kg (Artru et al., 1980). In experiments performed with rats and our same model of transient global cerebral ischemia (Lavyne et al., 1983), it was found that the administration of GBL at the dose of 100 mg/kg i.p. every 2 h for 24 h, beginning at the time of vessel occlusion, prevented the development of regional cerebral hyperemia and the prolonged cerebral hypoperfusion that was experienced by the non-treated controls. The histological picture of the hippocampus was basically similar to that observed in our present study.

Our data suggest that the mechanism(s) of the protective effect of GHB against the brain damage produced by transient ischemia followed by reperfusion involve(s) non-immediate events of the ischemia/reperfusion-triggered cascade. The available literature indicates that GHB reduces cellular metabolism, thereby lowering oxygen demand (MacMillan, 1978). Moreover, GHB has oxygen radical scavenging activity (Dosmugambetova, 1983; Boyd et al., 1990). There is good evidence that oxygen-derived free radicals play a major role as mediators of damage to the cell membrane in the pathophysiology of injury caused by the reperfusion of ischemic tissue (Gardner et al., 1983; McCord, 1985; Granger et al., 1986; Weisiger, 1986; Horton and Borman, 1987; Von Ritter et al., 1988; Bitterman et al., 1988; Friedl et al., 1989; Lieners et al., 1989; Marzi et al., 1990; Redl et al., 1993). The ischemic insult triggers an inflammatory response (for review: Schlag et al., 1991) that includes the activation of transcription factors, such as nuclear-factor kappa-B (NF-kB) and the production of several factors of the inflammatory response, including inducible nitric oxide (NO) synthase, chemokines, cytokines [in particular tumor necrosis factor-α (TNF-α)], and oxygen radicals (Li and Karin, 1999).

At dose levels similar to those used in our present study, GHB binds $GABA_B$ receptors besides its own receptors (Emri et al., 1996; Maitre, 1997): activation of both GHB and $GABA_B$ receptors may decrease the release of excitatory amino acids (Behl et al., 1993; Bernasconi et al., 1999). Brain ischemia is associated with massive release of glutamate, massive activation of NMDA, AMPA and metabotropic receptors, and Ca^{2+} overload (Onodera et al., 1989).

By activating $GABA_B$ receptors and by producing an increase in potassium conductance, GHB hyperpolarizes hippocampal neurons (Xie and Smart, 1992): the effectiveness in stroke of drugs that increase GABAergic transmission is currently being investigated (Wahlgren and Martinsson, 1998), as well as that of K^+ channel openers (Basile et al., 1999).

Finally, it has long been known that GHB reduces the utilization of cerebral glucose and high energy phosphates (Wolfson et al., 1977) and increases the concentration of glycogen and glucose, while decreasing the concentration of pyruvate and lactate in brain tissue. This lowers brain oxygen demand and consumption (MacMillan, 1978).

In conclusion, our present results confirm that GHB provides significant protection against the consequences of transient global cerebral ischemia followed by reperfusion, in rats, and demonstrate that such protection can be obtained when treatment is started late (2 h) after the ischemia/reperfusion episode.

Acknowledgements

This work was supported in part by grants from Ministero dell'Istruzione dell'Università e della Ricerca (MIUR), Roma.

References

Anderson, J.M., Lowe, J., 1990. Histometry and image analysis. In: Bancroft, J.D., Stevens, A. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone, Edinburg, UK, pp. 597–618.

Artru, A., Steen, P., Michenfelder, J., 1980. Gamma-hydroxybutyrate: cerebral, metabolic, vascular and protective effects. J. Neurochem. 35, 1114–1119.

Basile, A.M., Nencini, P., Inzitari, D., 1999. Meccanismi molecolari dell'ischemia cerebrale: possibili interventi terapeutici. Ann. Ital. Med. Interna 14, 94–105.

Behl, C., Hovey, L., Krajewski, S., Schubert, D., Reed, J.C., 1993. BCL-2 prevents killing of neuronal cells by glutamate but not by amyloid beta protein. Biochem. Biophys. Res. Commun. 197, 949–956.

Bernasconi, R., Mathivet, P., Bischoff, S., Marescaux, C., 1999. Gamma-hydroxybutyric acid: an endogenous neuromodulator with abuse potential? Tips 20, 135–141.

Bitterman, H., Aoki, N., Lefer, A.M., 1988. Anti-shock effects of human superoxide dismutase in splanchnic artery occlusion (SAO) shock. Proc. Soc. Exp. Biol. Med. 188, 265–271.

Bjorklund, A., Dunnett, S.B., Stenevi, U., Lewis, M.E., Iversen, S.D., 1980. Reinnervation of the denervate striatum by substantia nigra transplants: functional consequences as revealed by pharmacological and sensorymotor testing. Brain Res. 199, 307–333.

Boyd, A.J., Sherman, I.A., Saibil, F.G., Mamelak, M., 1990. The protective effect of γ-hydroxybutyrate in regional intestinal ischemia in the hamster. Gastroenterology 99, 860–862.

Bralet, J., Beley, P., Bralet, A.M., Beliz, A., 1979. Influence of various agents on the development of brain edema in rat following microembolism. Stroke 10, 653–656.

Dosmugambetova, R.S., 1983. Prevention of stress-related disorders in the contractile function of non-ischemic areas of the heart during myocardial infarction using gamma-hydroxybutyric acid. Bull. Esp. Biol. Med. 96, 28–30.

Emri, Z., Antal, K., Crunelli, V., 1996. Gamma-hydroxybutyric acid decreases thalamic sensory excitatory postsynaptic potentials by an action on presynaptic $gaba_b$ receptors. Neurosci. Lett. 216, 121–124.

Escuret, E., Roquefeuil, B., Frerebean, P., Baldy-Moulinier, M., 1977. Effect of hyperventilation associated with administration of central nervous depressant in brain injuries. Acta Neurol. Scand. 56, 154–155.

Friedl, H.P., Till, G.O., Trentz, O., Ward, P.A., 1989. Roles of histamine, complement and xanthine oxidase in thermal injury of skin. Am. J. Pathol. 135, 203–217.

Gardner, T.J., Steward, J.R., Casale, A.S., Downey, J.M., Chambers, D.E., 1983. Reduction of myocardial ischemic injury with oxygen-derived free radical scavengers. Surgery 94, 423–427.

Granger, D.N., Hollwarth, M.E., Parks, D.A., 1986. Ischemia–reperfusion injury: role of oxygen-derived free radicals. Acta Physiol. Scand., Suppl. 548, 47–63.

Hechler, V., Weissmann, D., Mach, E., Pujol, J.F., Maitre, M., 1987. Regional distribution of high affinity γ-[^{3}h]hydroxybutyrate binding site as determined by quantitative autoradiography. J. Neurochem. 49, 1025–1032.

Hechler, V., Gobaille, S., Maitre, M., 1992. Selective distribution pattern γ-hydroxybutyrate receptors in the rat forebrain and midbrain as revealed by quantitative autoradiography. Brain Res. 572, 345–348.

Horton, J.W., Borman, K.R., 1987. Possible role of oxygen-derived, free radicals in cardiocirculatory shock. Surg. Gynecol. Obstet. 165, 293–300.

Lavyne, M., Hariri, R., Tankosic, T., Babiak, T., 1983. Effect of low dose γ-butyrolactone therapy on forebrain neuronal ischemia in unrestrained, awake rat. Neurosurgery 12, 430–434.

Li, N., Karin, M., 1999. Is NF-kappaB the sensor of oxidative stress? FASEB J. 13, 1137–1143.

Lieners, C., Redl, H., Molnar, H., Furst, W., Hallstrom, S., Schlag, G., 1989. Lipid peroxidation in a canine model of hypovolemic–traumatic shock. Prog. Clin. Biol. Res. 308, 345–350.

MacMillan, V., 1978. The effects of gamma-hydroxybutyrate and gamma-butyrolactone upon the energy metabolism of the normoxic and hypoxic rat brain. Brain Res. 146, 177–187.

Maitre, M., 1997. The gamma-hydroxybutyrate signalling system in brain: organization and functional implications. Prog. Neurobiol. 51, 337–361.

Maitre, M., Rumigny, J.F., Cash, C.D., Mandel, P., 1983. Subcellular distribution of γ-hydroxybutyrate binding sites in rat brain. Principal localization in the synaptosomal fraction. Biochem. Biophys. Res. Commun. 110, 262–265.

Maitre, M., Kummel, V., Andriamampandry, C., Gobaille, S., Aunis, D., 2002. The role of γ-hydroxybutyrate in brain function. In: Tunnicliff, G., Cash, C.D. (Eds.), Gamma-hydroxybutyrate: molecular, functional and clinical aspects. Taylor and Francis, London, pp. 236–247.

Marshall, J.F., Teitelbaum, P., 1974. Further analysis of sensory inattention following lateral hypothalamic damage in rats. J. Comp. Physiol. Psychol. 86, 375–395.

Marzi, I., Takei, Y., Knee, J., Menger, M., Gores, G.J., Buhren, V., Trents, O., Lemasters, J.J., 1990. Assessment of reperfusion injury by intravital fluorescence microscopy following liver transplantation in the rat. Transplant. Proc. 22, 2004–2005.

McCord, J.M., 1985. Oxygen-derived free radicals in postischemic tissue injury. N. Engl. J. Med. 312, 159–163.

Mcnamara, R.K., Skelton, R.W., 1993. The neuropharmacological and neurochemical basis of place learning in the morris water maze. Brains Res. Rev. 18, 33–49.

Morris, R.G.M., 1984. Development of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60.

Onodera, H., Araki, T., Kogure, K., 1989. Excitatory amino acid binding sites in the rat hippocampus after transient forebrain ischemia. J. Cereb. Blood Flow Metab. 9, 623–628.

Pulsinelli, W.A., Brierly, J.B., 1979. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10, 267–272.

Redl, H., Gasser, H., Schlag, G., Marzi, I., 1993. Involvement of oxygen radicals in shock related cell injury. Br. Med. Bull. 49, 556–565.

Schlag, G., Redl, H., Hallstrom, S., 1991. The cell in shock: the origin of multiple organ failure. Resuscitation 21, 137–180.

Snead, O.C., 1991. The gamma-hydroxybutyrate model of absence seizure: correlation of regional brain levels of gamma-hydroxybutyric acid and gamma-butyrolactone with spike and wave discharges. Neuropharmacology 30, 161–167.

Snead, O.C., Liu, C.C., 1984. Gamma-hydroxybutyric acid binding sites in rat and human brain synaptosomal membranes. Biochem. Pharmacol. 16, 2587–2590.

Toga, A.W., Santori, E.M., Hazani, R., Ambach, K., 1995. A 3d digital map of rat brain. Brain Res. Bull. 38, 77–85.

Tunnicliff, G., 1992. Significance of γ-hydroxybutyric acid in the brain. Gen. Pharmacol. 23, 1027–1034.

Vayer, P., Mandel, P., Maitre, M., 1987. Gamma-hydroxybutyrate, a possible neurotransmitter. Life Sci. 41, 1547–1557.

Vergoni, A.V., Ottani, A., Botticelli, A.R., Zaffe, D., Guano, L., Loche, A., Genedani, S., Gessa, G.L., Bertolini, A., 2000. Neuroprotective effect of gamma-hydroxybutyrate in transient global cerebral ischemia in the rat. Eur. J. Pharmacol. 397, 75–84.

Von Ritter, C., Hinder, R.A., Oosthuizen, M.M., Svensson, L.G., Hunter, S.J., Lambrecht, H., 1988. Gastric mucosal lesions induced by hemorrhagic shock in baboons. Role of oxygen-derived free radicals. Dig. Dis. Sci. 33, 857–864.

Wahlgren, N.G., Martinsson, L., 1998. New concepts for drug therapy after stroke. Can we enhance recovery? Cerebrovasc. Dis. 8, 33–38.
Weisiger, R.A., 1986. Oxygen radicals and ischemic tissue injury. Gastroenterology 90, 494–496.
Wolfson, L.I., Sakurada, O., Sokoloff, L., 1977. Effects of gamma butyrolactone on local cerebral glucose utilization in the rat. J. Neurochem. 29, 777–783.
Xie, X., Smart, T.G., 1992. γ-Hydroxybutyrate hyperpolarizes hippocampal neurons by activating $gaba_b$ receptors. Eur. J. Pharmacol. 212, 291–294.