ELSEVIER

European Journal of Pharmacology 397 (2000) 75–84

ejp

www.elsevier.nl/locate/ejphar

Neuroprotective effect of γ-hydroxybutyrate in transient global cerebral ischemia in the rat

Anna Valeria Vergoni [a], Alessandra Ottani [a], Annibale Renzo Botticelli [b], Davide Zaffe [c], Lorenza Guano [d], Antonella Loche [d], Susanna Genedani [a], Gian Luigi Gessa [e], Alfio Bertolini [a,*]

[a] *Section of Pharmacology, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Via G. Campi 287, 41100 Modena, Italy*
[b] *Department of Human Pathology, University of Pavia, Via Forlanini 14, 27100 Pavia, Italy*
[c] *Department of Morphological Sciences and Forensic Medicine, University of Modena and Reggio Emilia, Largo del Pozzo 71, 41100 Modena, Italy*
[d] *Laboratorio Farmaceutico C.T., Via Dante Alighieri 69–71, 18038 Sanremo, Italy*
[e] *Department of Neuroscience ''B.B. Brodie'', University of Cagliari, Via Porcell 4, 09100 Cagliari, Italy*

Received 20 January 2000; received in revised form 13 March 2000; accepted 21 March 2000

Abstract

The effect of γ-hydroxybutyrate on the histological and behavioral consequences of transient brain ischemia was studied in the four vessel occlusion rat model. In saline-treated animals, 30 min ischemia caused a massive loss of neurons in the hippocampal CA1 subfield (normal neurons: 14%, 5%, 23% and 30% on the 3rd, 10th, 15th and 65th day after ischemia, respectively). γ-Hydroxybutyrate — 300 mg/kg intraperitoneally (i.p.) 30 min before or 10 min after arteries occlusion, followed by 100 mg/kg i.p. twice daily for the following 10 days — afforded a highly significant protection (normal neurons on the 3rd, 10th, 15th and 65th day after ischemia: 88% and 91%, 80% and 80%, 91% and 90%, 72% and 71% in rats receiving the first dose before or after arteries occlusion, respectively). The ischemia-induced sensory–motor impairment was significantly attenuated in rats receiving the first dose of γ-hydroxybutyrate before arteries occlusion. Finally, the ischemia-induced impairment in spatial learning and memory, evaluated starting 27 days after the ischemic episode, was significantly attenuated by γ-hydroxybutyrate, either injected first at 30 min before or 10 min after arteries occlusion. Lower doses of γ-hydroxybutyrate had no significant effect. In conclusion, these results indicate that γ-hydroxybutyrate provides significant protection against both histological and behavioral consequences of transient global cerebral ischemia in rats.

Keywords: γ-Hydroxybutyrate; Cerebral ischemia; Hippocampus; Immunochemistry; Sensory–motor test; Spatial learning

1. Introduction

γ-Hydroxybutyrate and its lactone, γ-butyrolactone, are naturally occurring four-carbon compounds. γ-Hydroxybutyrate is unevenly distributed in the mammalian brain and particularly concentrated in the hippocampus and in the thalamus, located principally in the synaptosomal compartment (Maitre et al., 1983; Snead and Liu, 1984; Snead, 1991; Tunnicliff, 1992; Bernasconi et al., 1999). Specific mechanisms for synthesis, release and uptake have been described (Maitre et al., 1983; Vayer et al., 1987), and a role for γ-hydroxybutyrate in the neuronal transmission has therefore been suggested (Vayer et al., 1987); a hypothesis that, although not unanimously shared (Cash, 1994), is reinforced by the presence in both rat and human brain of specific binding sites for this compound (high affinity sites: K_{d1} = 30–580 nm; low-affinity sites: K_{d2} = 2.3–16 nm) (Snead and Liu, 1984; Maitre, 1997). These sites are heterogeneously distributed in brain tissue, and are densely located in the hippocampus and fronto-parietal cortex (Hechler et al., 1987).

In addition to its possible physiological role, γ-hydroxybutyrate has several pharmacological properties (Tunnicliff, 1992; Bernasconi et al., 1999). Systemic administration of γ-hydroxybutyrate to animals of different

* Corresponding author. Tel.: +39-59-428412; fax: +39-59-428428.
E-mail address: bertolini.alfio@unimo.it (A. Bertolini).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00246-6

species produces a variety of dose-dependent effects, including changes in behavioral state (Tunnicliff, 1992) and generation of behavioral and EEG activities that resemble human absence epilepsy (Winters and Spooner, 1965) (indeed, systemic injection of γ-hydroxybutyrate is an established pharmacological model for this type of epilepsy (Snead, 1991)). Moreover, γ-hydroxybutyrate has long been used in general anesthesia, in combination with other anesthetics, particularly during childbirth (Vickers, 1969; Kleinschmidt et al., 1997). Smaller doses are anxiolytic and myorelaxant and induce deep slow-wave sleep within a short time (Laborit, 1964; Schmidt-Mutter et al., 1998). For this reason, γ-hydroxybutyrate has been tested in sleep disorders: several open or double-blind placebo-controlled trials have demonstrated the therapeutic efficacy of γ-hydroxybutyrate in narcolepsy, without significant side-effects (Nishino and Mignot, 1997). Finally, γ-hydroxybutyrate has been shown to reduce ethanol consumption and suppress ethanol withdrawal symptoms both in laboratory animals and humans (Fadda et al., 1988, 1989; Gallimberti et al., 1989, 1992), probably by mimicking ethanol actions in the central nervous system and by substituting for ethanol's reinforcing effects (Fadda et al., 1989; Agabio et al., 1998).

Some years ago, several laboratories studied the effect of γ-hydroxybutyrate and γ-butyrolactone in conditions of brain ischemia and/or hypoxia, with somewhat conflicting results (Escuret et al., 1977; Bralet et al., 1979; Artru et al., 1980; MacMillan, 1980; Lavyne et al., 1983). Our present research was aimed at re-examining this peculiar aspect of the pharmacology of γ-hydroxybutyrate, by using a widely validated rat model of transient global cerebral ischemia, and by studying both the brain histological picture at different times after the ischemic episode and the degree of sensory–motor and learning deficits.

2. Materials and methods

2.1. Animals

Adult male rats of an SPF Wistar strain (Harlan Italy, Correzzana, Milano, Italy), weighing 230–260 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 freely available, in a climatized colony room (temperature: 22 ± 1°C; humidity: 60%), on a 12-h light/dark cycle (lights on at 0700 h, and off at 1400 h). Animals were acclimatized to our housing facilities for at least 1 week before use. Housing and experimental procedures were strictly in accordance with the European Community ethical regulations for the care of animals for scientific research.

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

On the first day, rats were anesthetized with ketamine plus xylazine (115 + 2 mg/kg intraperitoneally, i.p.; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milano, Italy, respectively). Both common carotid arteries were exposed through a ventral midline cervical incision and carefully isolated. An atraumatic silk ligature was loosely placed around each vessel without interrupting the blood flow, to allow easy and rapid access for occlusion on the following day, and 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, 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 animals were still under anesthesia, stainless-steel screw electrodes were inserted into the skull bone overlying the fronto-parietal cortex 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 common carotid arteries re-exposed. Halothane anesthesia was interrupted, and both carotid arteries occluded by clamping them closed with atraumatic clips. Clips were removed 30 min later, and restoration of blood flow was verified by direct visual inspection. During ischemia and reperfusion, body temperature was maintained at 37.5 ± 0.5°C with rectal thermostar connected to a heating lamp until the rats recovered thermal homeostasis. Only those rats losing their righting reflex immediately after carotid occlusion, unresponsive to somatosensory stimulation, with pupils fully dilated, and an isoelectric EEG throughout the ischemic period, were included in the study. Rats that exhibited seizures were excluded. Sham-operated controls were only subjected to anesthesia, electrode implantation, and skin incision and sutures.

2.3. Drug and treatments

Rats were randomly assigned to one of the following groups: (1) sham-operated and i.p. treated with saline, 2 ml/kg, 30 min before carotid occlusion, 10 min thereafter, and then twice daily for the subsequent 10 days; (2) subjected to global cerebral ischemia and i.p. treated with saline with the same schedule of group 1; (3) subjected to global cerebral ischemia and i.p. treated with γ-hydroxybutyrate, 100 mg/kg, 30 min before carotid occlusion and then 50 mg/kg twice daily for the subsequent 10 days; (4) subjected to global cerebral ischemia and i.p. treated with

γ-hydroxybutyrate, 100 mg/kg, 10 min after carotid occlusion and then 50 mg/kg twice daily for the subsequent 10 days; (5) subjected to global cerebral ischemia and i.p. treated with γ-hydroxybutyrate, 300 mg/kg, 30 min before carotid occlusion and then 100 mg/kg twice daily for the subsequent 10 days; and (6) subjected to global cerebral ischemia and i.p. treated with γ-hydroxybutyrate, 300 mg/kg, 10 min after carotid occlusion and then 100 mg/kg twice daily for the subsequent 10 days.

Sodium γ-hydroxybutyrate, a gift of Laboratorio Farmaceutico, Sanremo, Italy, was dissolved in saline immediately before treatment, and injected in a volume of 2 ml/kg.

2.4. Sensory–motor tests

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 Björklund et al. (1980) and further slightly modified by ourselves, 55 days after the episode of transient global cerebral ischemia. In order to test orientation to sensory stimuli, the rat was placed on the surface of a bench. Head orientation towards (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) somaesthesis — 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; and (d) olfaction — a small cotton swab dipped in ammonia solution was gently approached in a lateral–medial direction towards 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 towards 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 one 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; and (d) mouth probe — the rat was held vertically around the body with head upwards, and a toothpick inserted into the side of the mouth. Normal rats show a licking to 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.

2.5. Spatial learning and memory

The Morris water-maze test (Morris, 1984) was used. This test measures rat's ability to learn, remember and go to a place in space defined only by its position relative to distal extramaze cues (McNamara and Skelton, 1993). In 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 spatial location of a platform, made of clear perspex, hidden by arranging for its top surface, 11 cm in diameter, to be 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. Conspicuous cues (racking, wall plates, door, the observer himself) were placed in a fixed position around the pool. On the day before starting 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 for 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 was recorded with a stopwatch. If the rat failed to locate the platform within 60 s, it was placed on it. The rat remained on the platform for 30 s, then it was removed. Each rat received four consecutive trials on each day, starting each time from a different cardinal point in a random succession. In the first 4-day training sequence, which started 27 days after the episode of transient global cerebral ischemia, the platform remained in its allocated position. In the second 3-day training sequence — 7 days after the end of the first sequence — the position of the platform was changed, while the extramaze cues were maintained in the same position. Twenty days after the end of the second training sequence, rats were subjected to a final 3-day training, leaving the platform and the extramaze cues in the same position like during the second training sequence. Tests were performed between 1000 and 0300 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.

2.6. Histology

At 3, 10, 15 and 65 days after the episode of transient global cerebral ischemia, four rats per group were decapitated under ether anesthesia. The brains were rapidly dissected out and fixed in 4% buffered paraformaldehyde (pH 7.0) for 24 h. Coronal 7-μm-thick sections were serially cut (step section technique), spanning the longitudinal axis of the dorsal hippocampus. Sections were transferred onto coated slides for immunoreactions (Dako, Denmark),

stained with hematoxylin–eosin, Feulgen, neuron specific enolase (NSE) immunomethod, and glial fibrill antigen protein (GFAP) immunomethod. Both immunomethods used avidin–biotin complex (ABC) system. Histomorphological observation and microphotography were performed using an Axiophot photomicroscope (Zeiss, Germany). Area of injured tissue and number of cells were evaluated, at 50× magnification factor on the TV screen, using a Sony color camera on a Leica microscope connected with a personal computer. Data were collected using an image system (SC Image, Casti, Italy) and automatically processed by means of an image analysis program (Microimage, Casti, Italy). Number of normal and injured neurons, number of astrocytes and the area of their distribution were moreover estimated, at 515× magnification factor on the TV screen, using a CCTV camera (Panasonic, Japan) on a Universal microscope (Zeiss) connected by a coaxial cable to a Vidas Zeiss. Data were collected using an interactive semiautomatic program implemented on Vidas. The number of astrocytes was estimated only at the 15th and 65th day after ischemia.

Table 1
Influence of sodium γ-hydroxybutyrate on sensory–motor orientation and coordinated limb use, tested 55 days following a 30 min global cerebral ischemia episode. GHB 100 before = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 30 min before arterial occlusion followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 100 after = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 10 min after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 before = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 30 min before arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 after = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days
Cumulative scores obtained from 10 rats per group.

Group	Score
1. Sham-operated	10
2. Ischemia + saline i.p.	48[a]
3. Ischemia + GHB 100 before	38[a]
4. Ischemia + GHB 300 before	14[b]
5. Ischemia + GHB 100 after	41[a]
6. Ischemia + GHB 300 after	28

[a] $P < 0.05$ compared with sham-operated group.
[b] $P < 0.05$ compared with ischemia + saline group (Friedman's test followed by Mann–Withney *U*-test).

2.7. Data analysis

All data were collected by individuals unaware of the treatment conditions. Data for Morris water-maze test were analyzed using the analysis of variance (ANOVA) followed by Student–Newman–Keuls' test. Data for sensory–motor test were analyzed using Friedman's test followed by Mann–Withney *U*-test. Data concerning the number of normal and injured neurons, and the number of astrocytes, were analyzed using analysis of variance (ANOVA) followed by Student–Newman–Keuls' test.

3. Results

3.1. Sensory–motor performance

As shown in Table 1, a 30-min global cerebral ischemia produced a significant impairment in sensory–motor orientation and coordinated limb use, evaluated 55 days after the ischemic episode. Treatment with γ-hydroxybutyrate, at the lower regimen (a first dose of 100 mg/kg i.p. followed by 50 mg/kg twice daily for the subsequent 10 days) was ineffective either when started before or after the occlusion of common carotid arteries; whereas at the higher dose regimen (a first dose of 300 mg/kg i.p. followed by 100 mg/kg twice daily for the subsequent 10 days), γ-hydroxybutyrate, when started 30 min before ischemia (i.e., before the occlusion of both common carotid arteries), afforded a significant protection: the sensory–motor performance of γ-hydroxybutyrate-treated rats was in fact not significantly different from that of sham-operated ones. On the other hand, rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after brain ischemia were not significantly protected.

3.2. Spatial learning and memory

A 30-min period of global cerebral ischemia caused a significant impairment in place learning. These rats had a performance significantly worse than that of sham-operated ones during the last 2 days of the first training session, on the last day of the second session, and during the whole third (final) session (Table 2). Treatment with γ-hydroxybutyrate at the lower dose regimen had no significant effect (data not shown). On the other hand, rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before the ischemic insult had a performance worse than that of sham-operated ones during the last 2 days of the first training sequence and on the last day of the second sequence, but during the final sequence the performance was not different in the two groups. These rats performed better than ischemized saline-treated ones on the last day of the second training sequence, and during the whole final sequence (Table 2). Rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after the occlusion of both common carotid arteries had a performance significantly worse than that of sham-operated ones only on the first day of the final training session. These rats performed better than ischemized saline-treated ones during the last 2 days of the first training session, on the last day of the second session, and during the last 2 days of the final session (Table 2).

Table 2
Effect of sodium γ-hydroxybutyrate (GHB, 300 mg/kg i.p. 30 min before or 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days) on escape latency in the Morris water maze. The first session started 27 days following a 30-min global cerebral ischemia episode. GHB 300 before = first injection of GHB 300 mg/kg, 30 min before arterial occlusion; GHB 300 after = first injection of GHB 300 mg/kg, 10 min after arterial occlusion. Data concerning rats treated with GHB at the dose of 100 mg/kg i.p., either before or after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days, were never significantly different from those obtained in rats subjected to brain ischemia and treated with saline, and are not reported for the sake of clarity.
Values are the means ± S.E.M. of the escape latencies (s); 10 rats per group. 1st session: $F(5,54) = 5.53$, $P = 0.000$ and $F(5,54) = 4.44$, $P = 0.002$ for days 3 and 4, respectively; 2nd session: $F(5,54) = 8.93$, $P = 0.000$ for day 3; 3rd session: $F(5,54) = 19.43$, $P = 0.000$, $F(5,54) = 10.17$, $P = 0.000$ and $F(5,54) = 9.50$, $P = 0.000$ for days 1, 2 and 3, respectively.

Session	Day	Treatment			
		Sham-operated	Ischemia + saline i.p.	GHB 300 before	GHB 300 after
1st	1	178.2 ± 11.75	177.4 ± 8.98	181.0 ± 7.33	186.4 ± 20.83
	2	123.4 ± 19.2	167.2 ± 12.86	116.4 ± 11.6	140.6 ± 12.4
	3	82.2 ± 10.57[a]	119.6 ± 2.3[b]	110.0 ± 6.75[b]	90.8 ± 2.78[a]
	4	62.0 ± 7.85[a]	107.6 ± 3.15[b]	102.8 ± 18.53[b]	72.0 ± 2.5[a]
(7 days gap)					
2nd	1	117.0 ± 10.62	124.8 ± 13.8	112.4 ± 11.42	140.6 ± 17.51
	2	69.4 ± 6.29	76.6 ± 9.19	71.2 ± 2.45	93 ± 6.78
	3	49 ± 4.34[a]	78.2 ± 1.73[b]	60.4 ± 6.98[a,b]	47.8 ± 3.05[a]
(20 days gap)					
3rd	1	47.8 ± 4.17[a]	97.8 ± 3.51[b]	56.6 ± 1.44[a]	85.4 ± 7.21[b]
	2	39 ± 4.24[a]	70.6 ± 5.55[b]	51 ± 4.15[a]	35.8 ± 5[a]
	3	35 ± 1.45[a]	45.4 ± 2[b]	26 ± 1.91[a,b]	27 ± 2.13[a,b]

[a] $P < 0.05$ compared with ischemia + saline group in the same session (ANOVA followed by Student–Newman–Keuls' test).
[b] $P < 0.05$ compared with sham-operated group in the same session.

3.3. Histology

The results are shown in Figs. 1–4, and are expressed in terms of the mean number of cells/reticle (250 μm side). A 30-min global cerebral ischemia produced a marked damage in the hippocampal CA1 subfield. Three days after the ischemic insult the number of apparently normal pyramidal cells was dramatically reduced. Eighty-

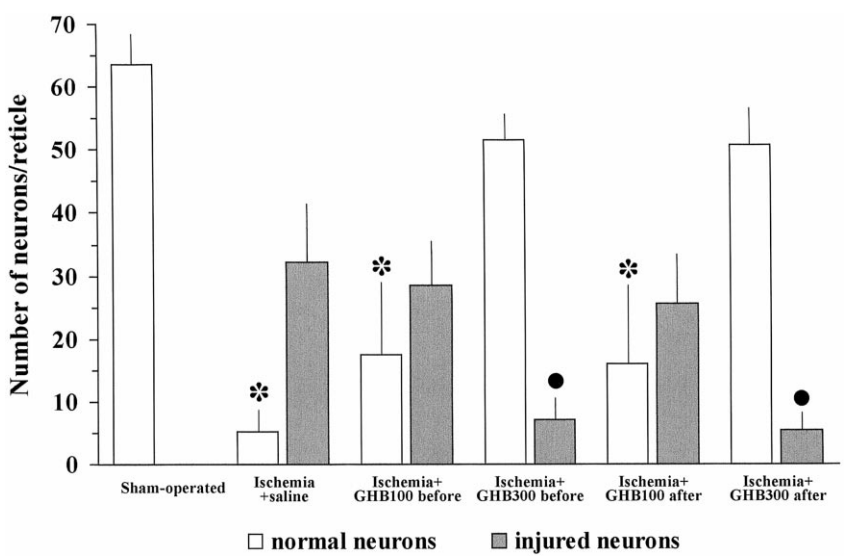

Fig. 1. Effect of sodium γ-hydroxybutyrate on the histological picture of the hippocampal CA1 subfield, 3 days after a 30-min global cerebral ischemia episode. Each value is the mean ± S.E.M. of two reticles/two hemispheres/two sections/four rats per group. GHB 100 before = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 30 min before arterial occlusion followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 100 after = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 10 min after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 before = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 30 min before arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 after = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. * $P < 0.05$ compared with sham-operated group [$F(5,18) = 9.25$; $P = 0.000$]; (●) $P < 0.05$ compared with ischemia group [$F(5,18) = 4.96$; $P = 0.005$] (ANOVA followed by Student–Newman–Keuls' test).

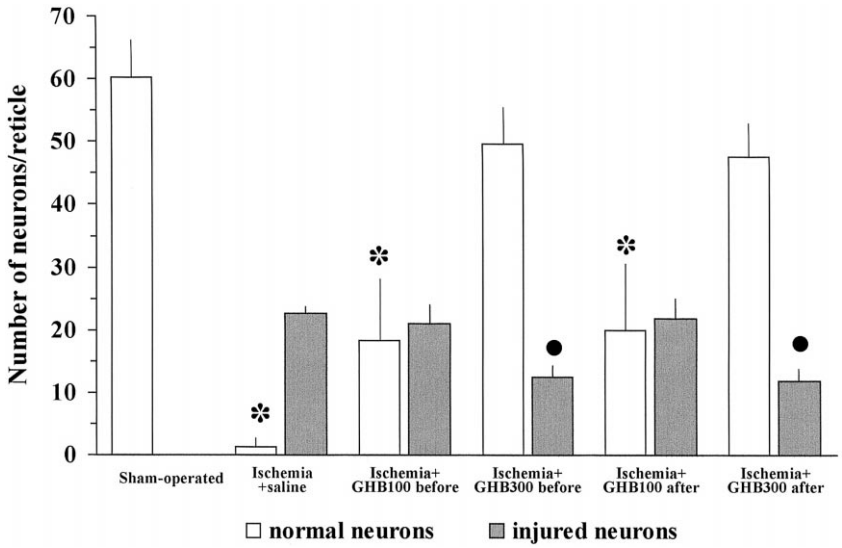

Fig. 2. Effect of sodium γ-hydroxybutyrate on the histological picture of the hippocampal CA1 subfield, 10 days after a 30-min global cerebral ischemia episode. Each value is the mean ± S.E.M. of two reticles/two hemispheres/two sections/four rats per group. GHB 100 before = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 30 min before arterial occlusion followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 100 after = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 10 min after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 before = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 30 min before arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 after = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. $^{*}P < 0.05$ compared with sham-operated group [$F(5,18) = 8.63$; $P = 0.000$]; (●) $P < 0.05$ compared with ischemia group [$F(5,18) = 15.69$; $P = 0.000$] (ANOVA followed by Student–Newman–Keuls' test).

six percent of neurons had a shrunken appearance, with pycnotic nucleus and without nucleolus; the perykaryon was homogeneous, amorphous, without Nissl bodies. Treatment with γ-hydroxybutyrate at the higher dose regimen (either started 30 min before or 10 min after the occlusion of carotid arteries) afforded an impressive protection, the percentage of apparently normal pyramidal cells being 88% in rats where treatment was started 30 min

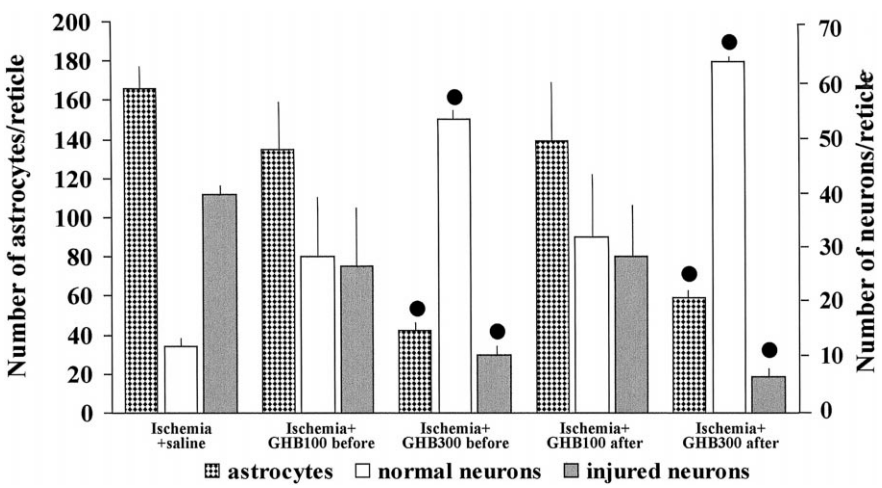

Fig. 3. Effect of sodium γ-hydroxybutyrate on the histological picture of the hippocampal CA1 subfield, 15 days after a 30-min global cerebral ischemia episode. Each value is the mean ± S.E.M. of two reticles/two hemispheres/two sections/four rats per group. GHB 100 before = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 30 min before arterial occlusion followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 100 after = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 10 min after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 before = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 30 min before arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 after = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. (●) $P < 0.05$ compared with ischemia group [$F(4,15) = 7.28$, $F(4,15) = 5.40$ and $F(4,15) = 4.51$ for astrocytes, normal neurons and injured neurons, respectively; $P = 0.002$, $P = 0.007$ and $P = 0.014$, respectively] (ANOVA followed by Student–Newman–Keuls' test).

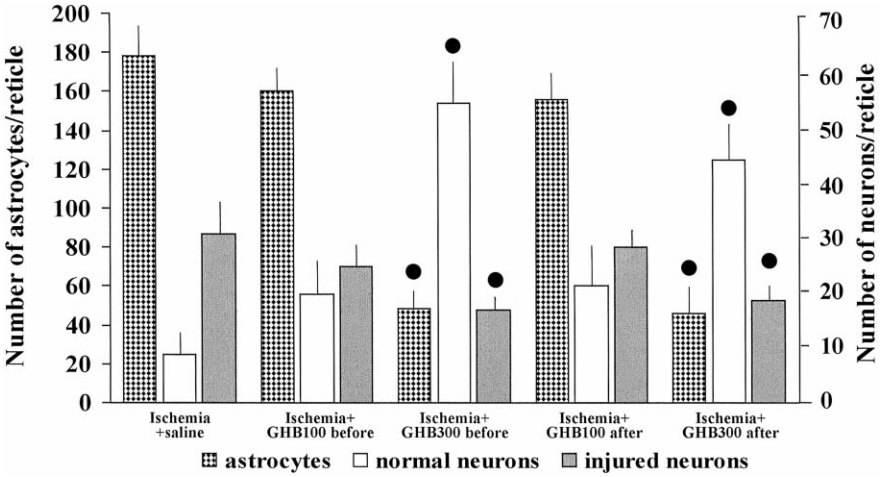

Fig. 4. Effect of sodium γ-hydroxybutyrate on the histological picture of the hippocampal CA1 subfield, 65 days after a 30 min global cerebral ischemia episode. Each value is the mean ± S.E.M. of two reticles/two hemispheres/two sections/four rats per group. GHB 100 before = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 30 min before arterial occlusion followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 100 after = sodium γ-hydroxybutyrate, 100 mg/kg i.p. 10 min after arterial occlusion, followed by 50 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 before = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 30 min before arterial occlusion followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. GHB 300 after = sodium γ-hydroxybutyrate, 300 mg/kg i.p. 10 min after arterial occlusion, followed by 100 mg/kg i.p. twice daily for the subsequent 10 days. (●) $P < 0.05$ compared with ischemia group [$F(4,15) = 44.83$, $P = 0.000$; $F(4,15) = 7.56$, $P = 0.002$ and $F(4,15) = 4.08$ $P = 0.02$ for astrocytes, normal neurons and injured neurons, respectively] (ANOVA followed by Student–Newman–Keuls' test).

before occlusion of carotid arteries, and 91% in rats where treatment was started 10 min after carotid occlusion (Fig. 1). The picture was similar in rats examined 10 days following cerebral ischemia: the percentage of apparently normal neurons was 5% in saline-treated ischemized rats, 80% in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before carotid occlusion, 80% in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after carotid occlusion (Fig. 2). In rats examined 15 days after the ischemic insult, the histological picture of the hippocampal CA1 subfield in saline-treated rats was slightly different, the percentage of normal neurones being 23%. However, the protective effect of γ-hydroxybutyrate was again quite evident, the percentage of normal neurons being 91% in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before carotid occlusion, and 90% in rats where treatment was started 10 min after carotid occlusion. As far as astrocytes are concerned, their number was 165.94 ± 13.10/reticle in saline-treated ischemized rats, 42.11 ± 5.41/reticle in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before carotid occlusion, and 58.73 ± 4.48/reticle in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after carotid occlusion (Fig. 3). In rats examined 65 days after the ischemic insult, the percentage of apparently normal neurons was 30% in saline-treated ischemized rats, 72% in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before carotid occlusion, 71% in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after carotid occlusion. The number of astrocytes was 178 ± 12.7/reticle in saline-treated ischemized rats, 48.70 ± 6.82/reticle in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 30 min before carotid occlusion, and 46.12 ± 8.20/reticle in rats treated with γ-hydroxybutyrate at the higher dose regimen starting 10 min after carotid occlusion (Fig. 4). In no case γ-hydroxybutyrate afforded a significant protection at the lower dose regimen, either starting treatment before or after occlusion (Figs. 1–4).

4. Discussion

The present data show that γ-hydroxybutyrate provides significant protection against both histological and behavioral consequences of a transient (30 min) global cerebral ischemia in rats. In our experimental conditions, the permanent occlusion of both vertebral and common carotid arteries for 30 min (four vessel occlusion model; Pulsinelli and Brierly, 1979) produced an extensive neuronal damage in the hippocampal CA1 subfield. The percentage of apparently normal neurons ranged from 5% to 30%, depending on the time lapse between ischemic insult and histological examination (3, 10, 15 or 65 days), the most severe picture being observed 10 days after ischemia. In late ischemia (15 and 65 days after the ischemic episode), injured areas showed also a substantial increase in the number of protoplasmic astrocytes, detected by GFAP immunoreaction.

γ-Hydroxybutyrate afforded a significant protection. At the i.p. dose of 300 mg/kg, followed by 100 mg/kg i.p.

twice daily up to the 10th day after ischemia, γ-hydroxybutyrate caused a highly significant reduction of hippocampal neuronal loss, the percentage of apparently normal neurons ranging from 71% to 91%. The neuroprotective effect of γ-hydroxybutyrate was roughly of the same degree either when the first dose was administered 30 min before ischemia or 10 min after ischemia. That is, also the post-ischemic administration of γ-hydroxybutyrate had a significant neuroprotective effect. γ-Hydroxybutyrate caused also a significant reduction of the astrogliosis reaction observed in late ischemia.

The ischemia-induced histological damage was concurrently associated with long-lasting significant deficits in sensory–motor performance and learning ability, still evident 2 months after the ischemic insult. Treatment with γ-hydroxybutyrate, at the above doses, started 30 min before the four vessel occlusion, significantly prevented also these behavioral and motor outcomes.

A lower dose regimen (first dose 100 mg/kg i.p., followed by 50 mg/kg i.p., twice daily up to the 10th day after ischemia) had no significant effect. Higher doses were not used because previous studies had shown that in animal conditions of brain hypoxia γ-hydroxybutyrate and γ-butyrolactone are maximally effective in the range of 200–300 mg/kg (Artru et al., 1980).

Previous studies concerning the effect of γ-hydroxybutyrate and γ-butyrolactone in conditions of cerebral ischemia had produced rather conflicting results.

In a mouse model of cerebral hypoxia, γ-hydroxybutyrate and γ-butyrolactone afforded a moderate protection (Artru et al., 1980). In the anesthetized dog [halothane 1% and nitrous oxide 60–70%, in oxygen], γ-hydroxybutyrate and γ-butyrolactone induced a severe depression in cerebral metabolic rate for oxygen (product of cerebral blood flow and cerebral arterial–venous blood oxygen content difference), associated with an almost two-thirds reduction in cardiac output at the largest doses (600–1100 mg/kg) (Artru et al., 1980). In a model of cerebral hypoxia–oligoemia (produced in anesthetized rats by clamping the right common carotid artery, and by reducing the oxygen concentration in the breathed gas mixture to give arterial PO_2 of about 30 mm Hg), γ-hydroxybutyrate retarded the restitution of energy phosphates and the oxidation of the accumulated lactate, whereas γ-butyrolactone led to a delayed metabolic deterioration (MacMillan, 1980). On the other hand, it was found that γ-butyrolactone lowers cerebral glucose utilization in rats more than comparable doses of barbiturates or that hypothermia, so to suggest a potential benefit in the treatment of evolving stroke (Wolfson et al., 1977). Indeed, a low dose γ-butyrolactone therapy (60 mg/kg) came out to be more effective than barbiturates in lowering the cerebral metabolic rate of oxygen in comatose head-injured patients without depressing cerebral perfusion pressure, a life-threatening side effect of barbiturate therapy (Escuret et al., 1977); and it was described that γ-butyrolactone inhibited the development of brain edema 12 h after an embolic stroke in the rat (Bralet et al., 1979). Finally, it was found that in a rat model of transient global cerebral ischemia, γ-butyrolactone prevented the development of regional cerebral hyperemia, and later, the prolonged cerebral hypoperfusion that was experienced by the non-treated controls; moreover, the γ-butyrolactone-treated rats had significantly reduced neuronal tissue loss in striatum and hippocampus than controls (Lavyne et al., 1983).

Our present results show that γ-hydroxybutyrate not only greatly reduces the ischemia-induced hippocampal damage, but also prevents the ensuing sensory–motor and cognitive deficits. Our histological data are basically in agreement with those obtained by Lavyne et al. (1983), who used γ-butyrolactone (100 mg/kg i.p. every 2 h for 24 h, beginning at the time of vessel occlusion) in our same model of transient forebrain ischemia, in the same animal species. It is not possible, on the other hand, to compare our results with those obtained in other previous animal studies, because the experimental conditions were quite different. So, Artru et al. (1980) evaluated the effect of γ-hydroxybutyrate and γ-butyrolactone — i.p. injected at doses of 33, 75, 150, 300 600, 900 and 1200 mg/kg — on the mean survival time of mice in a hypoxic atmosphere (5% oxygen). However, even in such rather rough condition, survival time came out to be significantly increased in treated animals, the maximum effect being obtained with both drugs at the dose of 300 mg/kg. Increasing the dose produced a lesser effect. The animal study of MacMillan (1980) concerned the effect of γ-hydroxybutyrate and γ-butyrolactone on brain energy metabolism in rats subjected to clamping of a common carotid artery and ventilated with a hypoxic gas mixture: the overall results indicated that γ-hydroxybutyrate and γ-butyrolactone were without apparent beneficial effects.

As far as the possible mechanisms of action are concerned, the following features of the pharmacodynamics of γ-hydroxybutyrate may be in question.

(a) γ-Hydroxybutyrate causes reduction of cerebral glucose and high energy phosphate utilization rates (Wolfson et al., 1977) and increases glycogen and glucose and decreases pyruvate and lactate contents of brain tissue (Godin et al., 1968; MacMillan, 1978). This lowers brain oxygen demand and consumption (MacMillan, 1978). Similar effects — albeit to a lesser degree (Wolfson et al., 1977) — are shared by barbiturates (Steen and Michenfelder, 1978), which also protect the brain in a variety of animal models of hypoxia and ischemia. On the other hand, other anesthetics, which also reduce cerebral oxygen consumption, do not provide any apparent protection (Yatsu et al., 1972; Smith et al., 1974).

(b) It has been suggested that γ-hydroxybutyrate may protect the brain against ischemic injury not only by reducing cellular metabolism, thereby lowering oxygen demand, but also by scavenging oxygen radical species (Dosmugambetova, 1983; Boyd et al., 1990).

(c) At dose levels similar to those used in our present study, γ-hydroxybutyrate reduces the firing rate of neurones and provokes hypothermia (Kaufman et al., 1990; Godbout et al., 1995).

(d) At these same dose levels, γ-hydroxybutyrate binds $GABA_B$ receptors besides its own receptors (Emri et al., 1996; Maitre, 1997): activation of both γ-hydroxybutyrate and $GABA_B$ receptors may produce a diminution of excitatory aminoacids release (Behl et al., 1993; Bernasconi et al., 1999).

(e) γ-Hydroxybutyrate hyperpolarizes hippocampal neurons by activating $GABA_B$ receptors and by producing an increase in potassium conductance (Xie and Smart, 1992). The effectiveness in stroke of drugs that increase GABAergic transmission is at present under clinical investigation (Wahlgren et al., 1998), as well as that of potassium channel openers (Basile et al., 1999).

In conclusion, the present data confirm and extend previous results indicating a favourable influence of γ-hydroxybutyrate on the morphological and behavioral consequences of brain ischemia. The fact that, moreover, γ-hydroxybutyrate does not depress cerebral perfusion pressure (Escuret et al., 1977) may add to the possible therapeutic interest of this drug.

Acknowledgements

This work was supported in part by grants from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Roma.

References

Agabio, R., Colombo, G., Loche, A., Lobina, C., Pani, M.L., Reali, R., Gessa, G.L., 1998. γ-Hydroxybutyric acid reducing effect on ethanol intake: evidence in favour of a substitution mechanism. Alcohol Alcohol. 33, 1–10.

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. Int. 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.

Björklund, A., Dunnett, S.B., Stenevi, U., Lewis, M.E., Iversen, S.D., 1980. Reinnervation of the denervate striatum by subtantia nigra transplants: functional conequences as revealed by pharmacological and sensorimotor 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.

Cash, C.D., 1994. Gamma-hydroxybutyrate: an overview of the pros and cons for its being a neuro-transmitter and/or a useful therapeutic agent. Neurosci. Biobehav. Rev. 18, 291–304.

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. Exp. 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.

Fadda, F., Mosca, E., Colombo, G., Gessa, G.L., 1988. Gamma-hydroxybutyric acid suppresses ethanol consumption in alcohol-preferring rats. Psychopharmacology 96, 107.

Fadda, F., Colombo, G., Mosca, E., Gessa, G.L., 1989. Suppression by gamma-hydroxybutyric acid of ethanol withdrawal syndrome in rats. Alcohol Alcohol. 24, 447–451.

Gallimberti, L., Canton, G., Gentile, N., Ferri, M., Cibin, M., Ferrara, S.D., Fadda, F., Gessa, G.L., 1989. Gamma-hydroxybutyric acid for treatment of alcohol withdrawal syndrome. Lancet 30, 787–789.

Gallimberti, L., Ferri, M., Ferrara, S.D., Fadda, F., Gessa, G.L., 1992. Gamma-hydroxybutyric acid in the treatment of alcohol dependence: a double-blind study. Alcohol.: Clin. Exp. Res. 16, 673–676.

Godbout, R., Jelenic, P., Labrie, C., Schmitt, M., Bourguignon, J.J., 1995. Effect of gamma-hydroxybutyrate and its antagonist NCS-382 on spontaneous cell firing in the prefrontal cortex of the rat. Brain Res. 673, 157–160.

Godin, Y., Mark, J., Mandel, P., 1968. The effects of 4-hydroxybutyric acid on the biosynthesis of amino acid in the central nervous system. J. Neurochem. 15, 1085–1091.

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

Kaufman, E.E., Porrino, L.J., Nelson, T., 1990. Pyretic action of low doses of gamma-hydroxybutyrate in rats. Biochem. Pharmacol. 40, 2637–2640.

Kleinschmidt, S., Grundmann, U., Janneck, U., Kreienmeyer, J., Kulosa, R., Larsen, R., 1997. Total intravenous anaesthesia using propofol, gamma-hydroxybutyrate or midazolam in combination with sufentanil for patients undergoing coronary artery bypass surgery. Eur. J. Anaesthesiol. 14, 590–599.

Laborit, H., 1964. Sodium 4-hydroxybutyrate. Int. J. Neuropharmacol. 3, 433–452.

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.

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.

MacMillan, V., 1980. Effects of gamma-hydroxybutyrate and gamma butyrolactone on cerebral energy metabolism during exposure and recovery from hypoxemia–oligoemia. Stroke 11, 271–277.

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. Comm. 110, 262–265.

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

McNamara, R.K., Skelton, R.W., 1993. The neuropharmacological and neurochemical basis of place learning in the Morris water maze. Brain 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.

Nishino, S., Mignot, E., 1997. Pharmacological aspects of human and canine narcolepsy. Prog. Neurobiol. 52, 27–78.

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

Schmidt-Mutter, C., Pain, L., Sandner, G., Gobaille, S., Maitre, M., 1998. The anxiolytic effect of gamma-hydroxybutyrate in the elevated plus maze is reversed by the benzodiazepine receptor antagonist, flumazenil. Eur. J. Pharmacol. 342, 21–27.

Smith, A.L., Hoff, J.T., Nielsen, S.L., Larson, C.P., 1974. Barbiturate protection in acute focal cerebral ischemia. Stroke 5, 1–7.

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.

Steen, P.A., Michenfelder, M.D., 1978. Cerebral protection with barbiturates. Relation to anesthetic effect. Stroke 9, 140–142.

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.

Vickers, M.D., 1969. Gamma-hydroxybutyric acid. Int. Anesthesiol. Clin. 7, 75–89.

Clomethiazole Acute Stroke Study Collaborative Group, Wahlgren, N.G., 1998. The Clomethiazole Acute Stroke Study (CLASS): safety results in 1356 patients with acute hemispheric stroke. Neurology 50, 212.

Winters, W.D., Spooner, C.E., 1965. Various seizure activities following γ-hydroxybutyrate. Int. J. Pharmacol. 4, 197–200.

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.

Yatsu, F.M., Diamond, I., Graziano, C., Lindquist, P., 1972. Experimental brain ischemia: protection from irreversible damage with a rapid-acting barbiturate (methohexital). Stroke 3, 726–732.