Gamma-hydroxybutyrate accelerates functional recovery after focal cerebral ischemia

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Abstract

BACKGROUND AND PURPOSE: Gamma-Hydroxybutyrate (GHB), a natural metabolite of gamma-aminobutyric acid and a drug used in humans to promote slow-wave sleep and treat narcolepsy, has been suggested to protect against ischemic stroke at high doses. This study aimed to assess recovery-promoting effects of GHB at a low dose similar to that used in patients. METHODS: Adult mice, subjected to 30 min of intraluminal middle cerebral artery occlusion, were intraperitoneally treated with GHB (100 mg/kg, twice/day, 8 h apart) or saline for 10 days. Motor recovery was evaluated by the grip strength test. The brain lesion was assessed by cresyl violet and NeuN staining 5 weeks after stroke. Expression of neuroplasticity-related genes (GAP43, c-jun, neurocan and ephrin B1) was analyzed by Taqman real-time PCR. RESULTS: GHB-treated mice regained their body weight faster and recovered grip strength (3 weeks after stroke) more quickly than saline-treated mice. This was noteworthy as GHB did not influence ischemia-induced brain injury, as revealed by cresyl violet and neuronal staining. The Taqman PCR assay revealed a decreased expression of c-jun and neurocan in the ischemic striatum of GHB-treated mice in comparison to saline-treated mice. CONCLUSION: GHB at a low dose accelerates neurological recovery following ischemic stroke. Further studies are necessary to determine the potential relationship between GHB, neuroplasticity, sleep and stroke recovery.
Gamma-Hydroxybutyrate Accelerates Functional Recovery after Focal Cerebral Ischemia

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Introduction

γ-Hydroxybutyrate (GHB), a natural metabolite of γ-aminobutyric acid, is a neurotransmitter and neuromodulator by action on both the GHB receptor and γ-aminobutyric acid type B receptor in the CNS. Exogenously administered GHB that freely crosses the blood-brain barrier induces diverse neuropharmacological effects including sedation and promotion of deep sleep. Clinically, GHB is an important drug in the treatment of such symptoms as cataplexy, excessive daytime sleepiness and sleep disturbance associated with narcolepsy.

GHB has been found to have protective action against ischemia/brain damage in rat models. However, its beneficial effects were observed in studies in which higher doses (≥300 mg/kg) than those used in patients (60–130 mg/kg) were applied.

Ischemic insults trigger considerable changes in the expression of neuroplasticity-related genes, many of which are involved in the pathological process of ischemic stroke. GHB has been shown to have protective action against ischemia/brain damage in rat models. However, its beneficial effects were observed in studies in which higher doses (≥300 mg/kg) than those used in patients (60–130 mg/kg) were applied.

Conclusion

GHB at a low dose accelerates neurological recovery following ischemic stroke. Further studies are necessary to determine the potential relationship between GHB, neuroplasticity, sleep and stroke recovery.
with distinct temporal patterns that correspond with initiation, maintenance and termination phases of post-stroke axonal sprouting [12]. These molecular events are important, at least in part, for axonal sprouting, tissue repair and reorganization and are currently under extensive investigation in search for drug targets to develop new therapies to promote stroke recovery. So far there is no information whether GHB influences the expression of plasticity-related genes.

In the present study, in a mouse model of focal cerebral ischemia [13], we aimed to evaluate the effects of GHB at a low dose similar to that used in patients on neurological recovery from brain injury and the expression of several plasticity-related genes, including growth-promoting genes GAP43, c-jun, the growth-inhibiting gene neurocan and the developmentally associated growth inhibitor ephrin B1, that exhibit a unique early/sustained expression pattern in response to ischemia [12].

**Methods**

**Animals and Induction of Focal Striatal Ischemia**

Male C57BL/6J mice, weighing 25.4 ± 3 g at the time of surgery, were used in this study. They were housed (4–5 animals/cage) in standard polycarbonate cages (42.5 × 26.6 × 18.5 cm) and kept under a 12-hour light/dark cycle (light on at 9 a.m.). Food and water were provided ad libitum. All experiments were conducted with governmental approval according to local guidelines for the care and use of laboratory animals.

Focal cerebral ischemia was induced by intraluminal occlusion of the middle cerebral artery (MCA) as described before [13]. Briefly, animals were anesthetized with 1% halothane (30% O2, remainder N2O). The left common carotid artery was exposed briefly, animals were anesthetized with 1% halothane (30% O2, remainder N2O). The left common carotid artery was exposed up to the bifurcation at which the MCA goes anterior to the carotid artery and advanced through the internal carotid artery up to the bifurcation at which the MCA goes off. MCA blood flow was thereby interrupted. Ischemia was monitored by laser Doppler flowmetry via a flexible 0.5-mm fiberoptic probe (Perimed, Stockholm, Sweden) that was attached to the intact skull overlying the MCA territory. Thirty minutes after MCA occlusion, the monofilament was retracted, and reperfusion was established. Sham control animals were only subjected to the veno-occlusion, the monofilament was retracted, and reperfusion was monitored by laser Doppler flowmetry via a flexible 0.5-mm fiberoptic probe.

**Analysis of Ischemic Brain Injury**

Coronal 16-μm cryostat sections were collected from brains at the level of the striatum. Sections were fixed with 0.1 M phosphate-buffered saline containing 4% paraformaldehyde for 20 min at room temperature. Cresyl violet staining was performed to outline the damaged area. Adjacent sections were used for immunofluorescence staining against the neuronal marker NeuN (Chemicon, Temecula, Calif., USA) and the astrocyte marker glial fibrillary acidic protein (GFAP; Dako, Glostrup, Denmark). After incubation with the antibody to NeuN (a mouse antibody, 1:200) or to GFAP (a rabbit antibody, 1:5,000) at 4°C overnight, sections were incubated with Cy3-conjugated secondary antibody (1:300; Jackson Immunoresearch, West Grove, Pa., USA). Stained sections were analyzed with a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) by counting the density of NeuN-positive neurons in the striatum in a total of 12 regions of interest, each measuring 62,500 μm² (500 μm apart). Cell counting was conducted by 2 individuals. Neuronal survival in the ischemic striatum was finally expressed as percentage by dividing the values obtained by cell densities determined in the nonischemic striatum [15]. The NIH image J software was used to analyze injury size.

**Tagman® Gene Expression Assay**

From a 2-mm brain slice obtained from the brain after collecting cryosections, 4 small blocks, containing the striatum and cortex, were carefully dissected both ipsilateral and contralateral to the stroke. Blocks were pooled together for each treatment group. Total RNA was extracted by the Trizol® method (Life Technologies, Rockville, Md., USA) and was treated with RQ1 DNase (Promega, Madison, Wis.). The mRNA samples were reverse transcribed and analyzed using the Taqman® Gene Expression Assay protocol [16].

**Drug Treatment**

At the onset of reperfusion, either 50 μl of saline or GHB (100 mg/kg; Lipomed AG, Arlesheim, Switzerland) were intraperitoneally applied, followed by twice daily (8 h apart) administrations starting at light onset for 10 days. The dose of 100 mg/kg was chosen based on a pilot experiment in which the effects of different doses (50–200 mg/kg) on behavior were observed in normal mice. No abnormal behavior was induced by the dose of 100 mg/kg. The following groups were assessed: (1) ischemic surgery followed by GHB treatment (n = 7); (2) ischemic surgery followed by saline treatment as vehicle control (n = 6); (3) sham operation followed by saline treatment (n = 4), and (4) sham operation followed by GHB treatment (n = 6). Five weeks after surgery, the animals were decapitated under halothane anesthesia, and brains were removed immediately and stored at −80°C. Since GHB appears to have a very narrow dose range in mice, i.e. >50 and <150 mg/kg [14], dosages other than 100 mg/kg were not tested in this study. In addition, two groups of mice treated (the same protocol as above) for 3 days either with saline (n = 6) or GHB (n = 6) after ischemic surgery were used to assess acute GHB effects on neuronal survival. In this experiment mice were decapitated 3 days after stroke.

**Evaluation of Behavioral Deficits and Motor Recovery**

Prior to surgery and at weekly time points after MCA occlusion, the animal’s body weight was assessed. In order to evaluate the animal’s motor recovery, the grip strength test was performed prior to surgery and at 1, 3, 5 weeks after MCA occlusion, during the light phase (5 h after light onset). The test was conducted on the spring balance (Kern 281–401, Kern & Sohn GmbH, Balingen-Frommern, Germany) attached to a triangular metal wire as the grip bar. Five measurements were averaged at each session (a) for the contralateral paretic limb and (b) for both limbs.

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mega, Madison, Wisc., USA) to digest genomic DNA. Oligo(dT)$_{15}$-primed first-strand cDNA synthesis was performed with AMV reverse transcriptase (Promega). The 5’-FAM$^\text{TM}$ label probes used in the Taqman real-time quantitative RT-PCR assay for GAPDH (endogenous control), GAP43, c-JUN, ephrin B1 and neurocan were purchased from Applied Biosystems (Forster City, Calif., USA). Reactions were performed in triplicate on a 7900HT fast real-time PCR system (Applied Biosystems). The relative level of mRNA expression in a given brain area was calculated as follows: mRNA = $2^{-\Delta\Delta C_T}$ (GHB or saline – ΔC$_T$ sham), where ΔC$_T$ = (C$_T$ target – C$_T$ GAPDH).

**Statistics**

All data were presented as means ± SD. Repeated-measures ANOVA and one-way ANOVA (SPSS, 12.01 for Windows) were used where appropriate, followed by post hoc (Tukey HSD) comparison. The 2-sided independent t test was used to estimate group differences and the corresponding 95% confidence intervals (95% CI). The significance level was set at $p \leq 0.05$.

**Results**

**Effects of GHB on Behavioral Recovery**

Focal cerebral ischemia led to a reduction of the body weight in both saline- and GHB-treated mice (fig. 1a). During recovery, GHB-treated mice showed much quicker regain of body weight to the presurgery level (at 2 weeks) than saline-treated mice (at 4 weeks). Repeated-measures ANOVA showed a significant effect of week × treatment ($F_{8.4, 53.4} = 4.7$, $p < 0.001$). There was a significant difference between the ischemia + GHB and ischemia + saline group for the second week (95% CI 0.16–2.55, $p = 0.048$) and the fifth week (95% CI 0.09–4.39, $p = 0.043$).

The grip strength test (fig. 1b) revealed a remarkable decrease in motor force of the paretic forelimb after focal cerebral ischemia in saline- and GHB-treated mice. A
decrease was also observed when both forelimbs were simultaneously tested. Over the recovery period, GHB accelerated restitution of grip strength in the paretic forelimb. Two-way repeated-measures ANOVA showed a significant effect of week × treatment for the paretic forelimb ($F_{9, 57} = 3.1, p = 0.004$) and for both forelimbs ($F_{9, 54} = 2.68, p = 0.012$). A significant difference was detected between the ischemia + GHB and ischemia + saline group in the paretic limb at 3 weeks by independent Student t tests (95% CI 5.1–22.9, $p = 0.005$).

**Effects of GHB on Ischemia-Induced Brain Injury**

As described before [13], cresyl violet staining showed that a 30-min MCA-induced localized ischemic lesion was confined to the striatum (fig. 2a). The lesion size in the GHB group was $0.77 ± 0.21 \text{ mm}^2$ and in the saline group $0.65 ± 0.28 \text{ mm}^2$, with a slightly unfavorable effect for the GHB group (95% CI −0.39 to 0.17, $p = 0.41$). Neuronal survival in the injured striatum, calculated by NeuN-positive cells (fig. 2b), was $48.7 ± 9.1\%$ in the saline versus $41.0 ± 5.9\%$ in the GHB group 5 weeks after ischemia, and $46 ± 27\%$ versus $42 ± 29\%$ in the GHB group 3 days after ischemia. The difference of the reactive GFAP area (fig. 2c) in the injury site between the two groups ($0.99 ± 0.48 \text{ mm}^2$ in the saline vs. $1.24 ± 0.35 \text{ mm}^2$ in the GHB group) appeared also small (95% CI: −0.76 to 0.26, $p = 0.3$). Taken together, these results suggest that after ischemic stroke the GHB treatment at the dose of 100 mg/kg has little effect on the brain damage in mice.

**Effects of GHB on Expression of Plasticity-Related Genes**

To investigate whether the brain plasticity was responsible, at least in part, for the beneficial effects of
GHB, a quantitative real-time PCR assay was performed for expression of several ischemia-induced genes [12]. The results (fig. 3) revealed that, at the mRNA level, expression of c-jun, ephrin B1 and neurocan in all regions was increased in both ischemia groups treated with either saline or GHB (fig. 3). When compared with the saline group, GHB-treated mice exhibited reduced expression for c-jun (13.5 ± 1.8 for saline vs. 8.9 ± 0.4 for GHB, 95% CI 1.9–7.3, p = 0.005) and neurocan (6.7 ± 0.8 for saline vs. 4.1 ± 0.2 for GHB, 95% CI 1.3–3.7, p = 0.001) in the ischemic striatum. The neurocan mRNA level in the ischemic cortex was slightly reduced by GHB (4.7 ± 0.1 for saline vs. 4.1 ± 0.08 for GHB, 95% CI 0.6–0.07, p = 0.001), whereas ephrin B1 was slightly increased (3.2 ± 0.3 for saline vs. 3.8 ± 0.05 for GHB, 95% CI –0.6 to 0.2, p = 0.035).

**Discussion**

In summary, GHB treatment at a small dose similar to that used in patients, beginning at reperfusion after ischemic stroke, (1) accelerates regain of body weight and recovery of the forelimb muscle strength (fig. 1), (2) has no
appreciated neuroprotective effects (fig. 2b, c) and (3) alters expression of several neuroplasticity-related genes (fig. 3).

The finding that GHB accelerates recovery of the muscle strength after stroke in the mouse is consistent with previous reports from rat models of ischemia/brain damage that GHB improves sensorimotor activities and learning memory performance [6–8]. The GHB-promoted rapid regain of body weight observed in this study may contribute to the motor strength recovery of both forelimbs (fig. 1b). However, the GHB-induced significantly quicker recovery in the paretic limb (compared to saline at 3 weeks) appears a specific effect on the neural tissue, since the muscle strength in the sham + GHB group remained unchanged during this period (fig. 1b).

To our surprise, GHB at the dose used in this study had little effects on the brain damage evaluated by cresyl violet staining, NeuN-positive cell counting and GFAP immunohistochemistry. For example, Lavine et al. [5] used a protocol in which 12 injections of 100 mg/kg of GHB were given at 2-hour intervals within 24 h. Vergoni et al. [6] and Ottani et al. [7] used 300 mg/kg for the first injection after the stroke surgery. These doses are much higher than those used in clinical practice (60–130 mg/kg, daily) and may induce a coma-like state in mice [14].

Interestingly, GHB changes expression profiles of several neuroplasticity-associated genes in the injured striatum and the nearby region (fig. 3). GHB tends to suppress the stroke-evoked increase in expression of these genes, and c-jun and neurocan genes in the ischemic striatum are the most affected (reduction by 2- to 4-fold; fig. 3). As a component of transcription factor AP-1, c-jun is highly inducible in response to neuronal injury [16] and has a dual function in both neuron death and survival after stroke [17]. Neurocan is a family member of chondroitin sulfate proteoglycans and an inhibitory extracellular matrix molecule. The increase in expression of chondroitin sulfate proteoglycans is believed to play a crucial part in hindering axon outgrowth [18]. It is not clear at present how GHB-induced changes in expression of neuroplasticity-related genes may influence neuronal tissue repair at the cellular level.

GHB is a reliable sleep stimulant [2] which at present is used to treat narcolepsy-related sleep-wake disorders. Sleep is thought to facilitate neuroplasticity in normal subjects [19, 20] and may play a role in mediating functional recovery after stroke [21]. Further studies are necessary to clarify the relationship between sleep and stroke recovery, and the potential role of GHB in promoting stroke recovery.

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