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The effect of 3-hydroxybutyrate methyl ester on learning and memory in mice

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ABSTRACT

Learning and memory requires energy-demanding cellular processes and can be enhanced when the brain is supplemented with metabolic substrates. In this study, we found that neuroglial cell metabolic activity was significantly elevated when cultured in the presence of polyhydroxybutyrate (PHB) degradation product 3-hydroxybutyrate (3-HB) and derivatives. We demonstrated that the receptor for 3-HB, namely, protein upregulated in macrophages by IFN-γ (PUMA-G), was expressed in brain and upregulated in mice treated with 3-hydroxybutyrate methyl ester (3-HBME). We also affirmed increased expression of connexin 36 protein and phosphorylated ERK2 (extracellular signal-regulated kinase 2) in brain tissues following 3-HBME treatment, although these differences were not statistically significant. Mice treated with 3-HBME performed significantly ($p < 0.05$) better in the Morris water maze than either the negative controls (no treatment) or positive controls (acetyl-L-carnitine treatment). Moreover, we found that 3-HBME enhanced gap junctional intercellular communication between neurons. Thus, 3-HB and derivatives enhance learning and memory, possibly through a signaling pathway requiring PUMA-G that increases protein synthesis and gap junctional intercellular communication.

1. Introduction

Memory formation after a learning experience correlates with increases in synaptic transmission and morphological alterations at the synapse, both of which add to the energetic workload of the neuron. Distinct areas of the hippocampus are metabolically active at different times during spatial learning tasks, supporting the involvement of a type of metabolic plasticity involving neuron–glia coupling [1,2]. Astrocytes couple synaptic activity to glucose consumption through molecular mechanisms that involve the sequential intervention of astrocyte-specific glutamate transporters and the sodium–potassium ATPase, activation of glycolysis in astrocytes, and monocarboxylate transporter-mediated exchange of lactate from astrocytes to neurons [3–5].

The monocarboxylate transporter also transports ketone bodies including 3-HB. Ketone bodies are normally produced by the liver from fatty acids and released into the vasculature as an energy source for extra hepatic tissues, especially during starvation or disease [6]. 3-HB is the most common degradation product of microbial polyhydroxybutyrate (PHB) that has been investigated for tissue engineering application [7]. Evidence has been accumulated for a therapeutic role of ketone bodies in neuronal disorders. For example, 3-HB conferred partial protection to hippocampal neurons against beta-amyloid 1–42 toxicity, and preserved neuronal integrity and stability during glucose deprivation [8,9]. Alzheimer's disease and many other multifactorial cognitive or neurological disorders are rapidly growing public health concerns with potentially devastating effects [10,11]. Although numerous studies have described either the etiology of these diseases or compounds that protect neurons, an effective therapy is yet to be developed [12,13].

We previously reported that 3-HB enabled cultured cells to reach high confluencies by preventing cell death [14]. We speculated that 3-HB was converted to acetyl-CoA, which would support a greater mitochondrial membrane potential ($\Delta\psi_m$) and in turn prevent cell death. Subsequently, 3-HB was proposed as the endogenous ligand for PUMA-G [15], a G-protein-coupled receptor expressed in adipose tissue. This supports the alternative explanation that 3-HB protects cells through a signaling pathway activated by this receptor, although PUMA-G was reported to have a limited tissue distribution that did not include the brain [16]. Additionally, our previous observation that 3-HB was most effective at high confluencies hints

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that functional gap junctions promote cell survival, so the possibility that gap junctional intercellular communication is upregulated under these conditions must be considered. Interestingly, neuronal gap junctions incorporating connexin 36 play a role in learning and memory [17]. Thus, the possibility that 3-HB may foster learning and memory must also be considered.

Many researches focus on the applications of polyhydroxyalkanoates (PHA) as implant biomaterials, and found that PHA has good biocompatibility and biodegradability [18–20]. In vivo, PHB degradation products 3-HB or derivatives may enter the brain through the blood–brain barrier and then produce some effects. This study sought to investigate the effect of 3-HB and derivatives on neuroglial cell metabolic activity and gap junctional intercellular communication of hippocampal neurons, to evaluate the hippocampal expression of PUMA-G and proteins related to memory following treatment with 3-HB, and to determine whether 3-hydroxybutyrate methyl ester (3-HBME) improves learning and memory in the normal mouse.

2. Materials and methods

2.1. Materials

BALB/c mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Shanghai, China). Polyhydroxybutyrate (PHB) was kindly donated from Microbiology Laboratory, Tsinghua University (Beijing, China). 3-HB, B27 additives, 6-CFDA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (USA). Penicillin–streptomycin, trypsinase were purchased from Guangzhou Whiga Technology Co. Ltd. (China). Fetal Bovine Serum (FBS) (HyClone, New Zealand), low glucose Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F12 and Neruobasal medium powder from Invitrogen Corporation (USA). Protein extraction kit and Bradford protein assay kit were purchased from Beijing SBS Genetech (China). Primary antibody Rabbit anti-NSE, Rabbit anti-GFAP, second antibody goat anti-Rabbit Cy3-IgG, HRP goat anti-rabbit IgG and DAB chromogenic detection kit were purchased from Wuhan Boster Biological Technology Co. Ltd. (China). The blocking solution was purchased from Beyotime Institute of Biotechnology (China) and used as it was. Primary antibody anti-connexin 36 rabbit polyclonal antibody, anti-pERK2 rabbit polyclonal antibody and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody were from Santa Cruz Biotechnology, Inc. (USA). Real time RT-PCR primers and the fluorogenic probe were purchased from Shanghai Xinghan Biotechnology Co. Ltd. (China). All other chemicals with analytical purity were purchased from Guangdong Guanghua Chemicals Co. Ltd. (China).

2.2. Neuroglial and neuron cell isolation and in vitro cultivation

BABL/c mice (0–3 days old) were sacrificed, the brain removed, and rinsed with dissection buffer to remove blood. Minced pieces of cortical gray matter were digested with 0.25% trypsinase and centrifuged for 5 min at 300 g at 4 °C. The pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were seeded into 50 ml polystyrene tissue culture flasks and allowed to adhere for 24 h. The cells were then incubated in a CO_2 incubator (5% CO_2, 95% air) (Thermo electron corporation, USA) at 37 °C and the medium was changed every four days. Glial fibrillary acidic protein staining was used to identify neuroglial cells. After the cells reached confluency, 2 ml of DMEM containing 0.25% trypsinase was added to the culture flask to create a cell suspension. The effect of 3-HB and derivatives (3-hydroxybutyrate methyl ester, 3-HBME, came from the methanolysis of polyhydroxybutyrate, and 3-hydroxybutyrate ethyl ester, 3-HBEE, came from the ethanolysis of polyhydroxybutyrate) on cell metabolic activity was determined for cultures seeded with 5×10^3 cells per well into 96 well plastic plates.

Dissociated hippocampal neurons were prepared from postnatal 0–3 day BABL/c mice by digestion of hippocampal tissue with 0.125% (w/v) trypsin for 10 min. DMEM/F12 medium containing 10% FBS was added, and three mild trituration and centrifugation steps were performed prior to resuspension in DMEM/F12 medium containing 10% FBS. The cells were seeded at 5×10^6 cells per well into glass bottom dishes coated with poly-L-lysine and incubated for 12 h to allow cells to adhere. Cytarabine was then added to a final concentration of 2.5 μg/ml to prevent glial cell proliferation, and after 24 h cells were cultured in neurobasal medium with 2% B27 and 1% Gln, with half the medium changed every 3 days. Immunostaining with neuron-specific enolase was used to identify neuronal cells.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The metabolic activity of neuroglial cells was evaluated by a MTT assay [21]. MTT was prepared as a 5 mg/ml stock solution in phosphate-buffered saline (0.1 M NaCl, 0.01 M Na_2PO_4, pH 7.2). To each well, 0.02 ml of MTT was added, and plates were incubated at 37 °C. After 4 h, the medium was removed, 0.1 ml dimethyl sulfoxide was added to each well, and the plates were gently rotated for 10 min to dissolve formazan. Six samples were prepared for each time point. The absorbance of samples at 570 nm was measured using an ELISA microplate reader (Bio-Rad model 550, USA). Control experiments indicated that 3-HB and derivatives did not stimulate cellular formazan production.

2.4. Fluorescence redistribution after photobleaching (FRAP) analysis of intercellular communication

Imaging and FRAP were performed using a Zeiss LSM 510 meta laser scanning confocal microscope (Germany) equipped with an argon laser source at 488 nm. Prior to microscopy, neurons were treated with 3-HBME, cells were rinsed with Hank's Buffered Salt Solution (HBSS), and loaded with 10 μg/ml 6-carboxyfluorescein diacetate (6-CFDA) at 37 °C in the presence of 5% CO_2 for 10 min. Cells were then rinsed with HBSS and observed in fresh culture medium without FBS. The CFDA fluorescence in a single cell soma was photobleached using a 488 nm laser, and fluorescence recovery in the photobleached compartment was then monitored for 620 s. For every photobleaching treatment, the entire soma of a single neuron was bleached and the fluorescence recovery was measured. All data represent the average ± SEM fluorescence recovery of six cells and are representative of three different FRAP experiments.

2.5. Morris water maze

Upon arrival, mice were allowed seven days to recover from transport before treatment. Mice were kept in standard rearing cages (30 × 20 × 14 cm) within an air-conditioned room (24 ± 1 °C) with a 12:12 h light:dark cycle. Food and water were supplied ad libitum. Sixty-two-month-old BALB/c mice were divided into five groups, each containing six males and six females: Negative control (treated with deionized water), 30 mg/kg/d acetyl-L-carnitine, 20 mg/kg/d 3-HBME, 30 mg/kg/d 3-HBME, and 40 mg/kg/d 3-HBME, each dissolved using deionized water as the solvent. Acetyl-L-carnitine improves rodents' performance in the Morris water maze and served as a positive control [22,23]. Mice were treated once daily through intragastric gavage for one month prior to the Morris water maze experiment.

The Morris water maze (Shanghai Jiliang Software Technology Co. Ltd., China) consisted of a circular pool (diameter 120 cm; height 60 cm) with black bottom and wall and water heated to 24 ± 1 °C. A black circular platform (diameter 6 cm; height 30 cm) was submerged 1 cm beneath the surface of the water. Because the color of the water tank including the pool bottom and the pool wall as well as the platform were black, the position of the platform was sufficiently obscured from the mice when the platform was submerged 1 cm beneath the surface of the water. On each of the walls of the four quadrants, a distinct colored paper was pasted as a visual positional cue. Four 45 W daylight lamps were positioned on the floor in the four corners of the arena and aimed at the ceiling to indirectly illuminate the surface of the water. A closed-circuit television camera was mounted onto the ceiling directly above the centre of the pool to monitor subject-swimming parameters.

2.5.1. Hidden platform test

All testing began at 09:30. During four daily acquisition sessions, each mouse was placed in the water facing the wall of the tank at one of the four designated starting points and allowed to swim and find the hidden platform located in the SW quadrant of the maze. During each trial, a mouse was given 60 s to find the hidden platform. If still in the water after 60 s, a mouse was gently guided to and placed on the platform. 10 s after climbing onto the platform, the mouse was placed on the next starting point. Swim paths and other data were recorded using a video camera connected to a tracking analysis system. The time the mouse required to reach the platform (escape latency), the total distance swam for each trial, and the swimming path in the pool was recorded.

2.5.2. Probe test

On the sixth day, the platform was removed from the pool and mice were challenged to a single search trial for 60 s (probe test). Four starting positions in the hidden platform were used for all mice. For the probe test, three parameters were measured, including the number of crossings of the exact place where the platform had been located, the swimming distance in the quadrant of the former platform position, and the swimming path in the pool.

2.5.3. Retention test

The retention test was conducted two days after the probe test and consisted of three trials from unique starting positions. After mounting the platform, mice were immediately placed in the holding cage for 40 s until the next trial.

2.5.4. Thigmotaxis

Thigmotactic swimming is defined as the behavior that mice display when swimming close to the walls of the water maze. The maze was divided into three circles of equal area, and the time spent in the outer ring of the pool was designated as "thigmotactic swimming" [24]. Thigmotaxis was reported as the percent of the total swimming time.