

Glutamine synthetase activity and glutamate uptake in hippocampus and frontal cortex in portal hypertensive rats

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Abstract

AIM: To study glutamine synthetase (GS) activity and glutamate uptake in the hippocampus and frontal cortex (FC) from rats with prehepatic portal vein hypertension.

METHODS: Male Wistar rats were divided into sham-operated group and a portal hypertension (PH) group with a regulated stricture of the portal vein. Animals were sacrificed by decapitation 14 d after portal vein stricture. GS activity was determined in the hippocampus and FC. Specific uptake of radiolabeled L-glutamate was studied using synaptosome-enriched fractions that were freshly prepared from both brain areas.

RESULTS: We observed that the activity of GS increased in the hippocampus of PH rats, as compared to control animals, and decreased in the FC. A significant decrease

in glutamate uptake was found in both brain areas, and was more marked in the hippocampus. The decrease in glutamate uptake might have been caused by a deficient transport function, significantly and persistent increase in this excitatory neurotransmitter activity.

CONCLUSION: The presence of moderate ammonia blood levels may add to the toxicity of excitotoxic glutamate in the brain, which causes alterations in brain function. Portal vein stricture that causes portal hypertension modifies the normal function in some brain regions.

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Key words: Portal hypertension; Glutamine synthetase; Glutamate uptake; Frontal cerebral cortex; Hippocampus; Rat

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INTRODUCTION

Two major complications appear in severe liver failure: hepatic encephalopathy (HE) and portal hypertension (PH), but the mechanism involved in the production of brain damage is still unclear.

PH is found in patients with cirrhosis, and in portal vein thrombosis, it is characterized by an increase in splanchnic blood flow and pressure, caused by abdominal blood flow resistance, secondary to important liver parenchyma alterations (fibrosis or cirrhosis). Normally, the liver splanchnic blood must reach, through the liver, the hepatic veins and finally the vena cava^[1]. As a result

of increased splanchnic blood flow, collateral vein shunts appear and abdominal circulation avoids the damaged liver parenchyma to reach the systemic circulation^[2].

HE in acute and chronic liver disease is a complex syndrome, associated frequently with hyperammonemia. Increases in blood brain barrier (BBB) permeability present in PH, allow ammonia ions and other neurotoxic substances to penetrate brain tissue^[3-6]. Hyperammonemia is caused by a defect in the liver parenchyma that forms urea from intestinal nitrogenous substances, and vein shunts from the splanchnic circulation carry it into the general circulation^[7].

According to Erceg *et al*^[8], chronic hyperammonemia, with or without liver failure, impairs the glutamate-nitric oxide-cGMP pathway in the brain and reduces extracellular cGMP in the brain. This function is associated with a decreased ability of rats to learn a Y maze conditional discrimination task. It has been suggested that a decrease in extracellular cGMP is involved in impaired learning ability and intellectual function.

Ammonia is a well-known toxic substance for the central nervous system (CNS), especially when levels exceed the antitoxic capacity of the brain cells. Glutamate plays an important role in cellular metabolism, and contributes to normal excitatory neurotransmission in the brain. When this function is not accomplished effectively, and either ammonia or glutamate are not sufficiently detoxified, their concentrations increase pathologically, neuron and astrocyte functions deteriorate, and damage and even cell death can result^[9]. It has been shown clearly that acute ammonia toxicity and liver failure lead to excitotoxicity as a result of activation of N-methyl-D-aspartate (NMDA) receptors in the brain^[10], and that blocking these receptors can lead to ammonia-induced death^[11]. In contrast, chronic hyperammonemia leads to down-regulation of signal transduction pathways associated with these receptors, which contributes to cognitive impairment^[8].

The glutamine/glutamate cycle participates in cell metabolism, and has important relevance in normal and pathological functions. When this cycle does not function adequately, CNS functional damage can appear, and even cellular death can be produced^[12]. To accomplish the transformation to ammonia and glutamate into glutamine, the brain depends on the activity of the enzyme glutamine synthetase (GS) in astrocytes. This is associated with correct function of the glutamate transporters, to provide an adequate uptake, release and metabolism of ammonia and glutamate^[13]. Therefore, the importance of correct function of the glutamine/glutamate cycle during this detoxifying step in brain is clear.

The aim of the present study was to analyze the participation of GS activity and glutamate uptake in the hippocampus and cerebral frontal cortex (FC), using a prehepatic PH rat model, with the intention to mimic the two major complications that appear in chronic liver failure. By using this model, it may be possible to understand more clearly the defense mechanism of the brain against the two toxic substances, ammonia and the excitatory neurotransmitter glutamate.

MATERIALS AND METHODS

Animals and surgical procedures

Male Wistar rats with an average weight of 240 g were used. The animals were placed in individual cages, with free access to food (standard laboratory rat chow) and water, and a 12-h light cycle: 8.00-20.00 h. Special care for perfect air renewal was taken. PH was obtained by calibrated stenosis of the portal vein, according to the method of Chojkier and Groszmann^[14]. Rats were lightly anesthetized with ether and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3.0 silk sutures was placed around the vein, and snugly tied to a 20-gauge blunt-end needle placed alongside the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Fourteen days after portal vein ligation, animals exhibit an increase in portal pressure. Sham-operated rats underwent the same experimental procedure, except that the portal vein was isolated but not stenosed. Animals were placed in individual cages and allowed to recover from surgery. Animals were sacrificed by decapitation at 14 d after portal vein stricture.

Experiments were carried out in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication N° 80-23/96) and local regulations. All efforts were made to minimize suffering of animals and to reduce the number of animals used.

Portal pressure measurement

Fourteen days after the corresponding operation, the rats were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL), and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico), coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA, USA).

GS activity

GS activity was assessed as described by Rowe *et al*^[15], with some minor modifications. Fourteen days after portal vein stricture, rats were sacrificed by decapitation and the FC and hippocampus were incubated in 500 μ L HEPES-Tris buffer, which contained 140 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L HEPES, 10 mmol/L glucose (adjusted to pH 7.4 with Tris base) for 30 min at 37°C. After incubation, each brain region was homogenized in 200 μ L 10 mmol/L potassium phosphate, pH 7.2. Reaction mixtures contained 150 μ L brain region homogenate and 150 μ L stock solution (100 mmol/L imidazole-HCl buffer, 40 mmol/L MgCl₂, 50 mmol/L β -mercaptoethanol, 20 mmol/L ATP, 100 mmol/L glutamate and 200 mmol/L hydroxylamine, adjusted to pH 7.2). Tubes were incubated at 37°C for 15 min.

The reaction was stopped by adding 0.6 mL ferric chloride reagent (0.37 mol/L FeCl_3 , 0.67 mol/L HCl and 0.20 mol/L trichloroacetic acid). Samples were placed for 5 min on ice. Precipitated proteins were removed by centrifugation at 10000 g , and the absorbance of the supernatants was read at 535 nm against a reagent blank. Under these conditions, 1 μmol γ -glutamylhydroxamic acid gave an absorbance of 0.340. GS specific activity was expressed as μmol γ -glutamylhydroxamate per hour per milligram of protein.

Preparation of tissue samples for glutamate uptake

As described for the GS activity assay, at 14 d after portal vein stricture, animals were killed by decapitation. The brain was removed from the cranial cavity, and the FC and hippocampus were dissected onto a Petri dish at 0°C, according to the method of Glowinski and Iversen^[16], and homogenized with a glass-PTFE homogenizer in 15 volumes of 0.32 mol/L sucrose. The homogenates were centrifuged at 800 g for 10 min, the pellet was discarded, and the supernatant was centrifuged at 20000 g for 20 min. The pellet (P2 = crude synaptosomal fraction) was suspended with a glass-PTFE homogenizer in fresh 0.32 mol/L sucrose, and again centrifuged at 20000 g for 20 min. The procedure was repeated three times, and the resulting pellet was suspended and used in uptake experiments within 5 h after preparation.

Glutamate uptake procedure

Uptake experiments were carried out using fresh synaptosomal fractions that originated from 20 mg of tissue of FC and hippocampus (wet weight) per 1 mL incubation medium. This consisted of 125.0 mmol/L NaCl, 3.5 mmol/L KCl, 1.5 mmol/L CaCl_2 , 1.2 mmol/L MgSO_4 , 1.25 mmol/L KH_2PO_4 , 25 mmol/L NaHCO_3 , 10 mmol/L HEPES and 10 mmol/L D-glucose, pH adjusted to 7.4. The tissue was first incubated for 5 min at 30°C, as described by Takarada *et al.*^[17], followed by the addition of 10 nmol/L radiolabeled substrate of [^3H] L-glutamate, and subsequent incubation for 5–30 min (time course study). The incubation was terminated by vacuum-filtration through Whatman glass fiber-filters (type D) and rapid washing, three times, with isotonic saline solution (at 2–4°C). The radioactivity on the filters was measured using liquid scintillation counting. Parallel experiments were always performed without incubation as time zero, to obtain radioactivity not specifically taken up into brain preparation for the radiolabeled substrate used in this experiment.

Protein content was estimated by the technique of Lowry *et al.*^[18] using bovine serum albumin as a standard.

Drugs, chemicals and radiolabeled compounds

[^3H] L-Glutamate (specific activity: 52.0 Ci/mmol) was from Perkin Elmer NEN Life Science Inc. (Boston, MA, USA). Plasma ammonia concentration was determined by the Ammoniac Enzymatic UV kit (Biomerieux, France). All other chemicals and reagents were analytical grade and obtained through regular commercial sources.

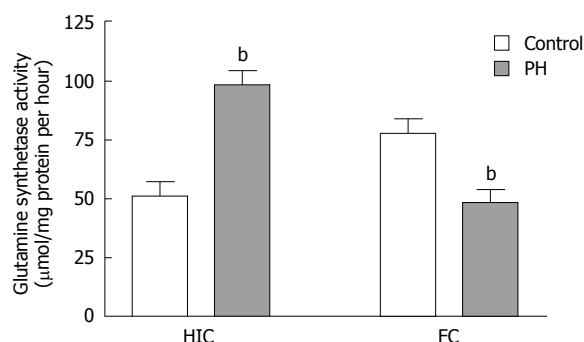


Figure 1 GS activity in the hippocampus and FC. Values are the mean \pm SE in eight experiments. ^b $P < 0.01$ compared with respective control group. HIC: Hippocampus.

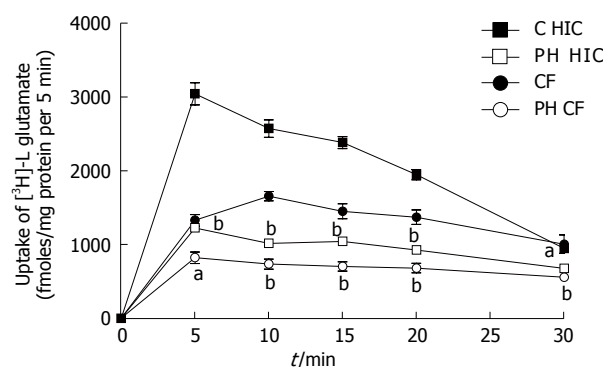


Figure 2 Time course of [^3H] L-glutamate uptake in FC and hippocampus. Synaptosomes were incubated with [^3H] L-glutamate at 10 nmol/L substrate concentration, for > 30 min, in the presence of 125 mmol/L NaCl at 30°C. Values are the mean \pm SE in six or seven separate experiments done in triplicate. ^a $P < 0.05$; ^b $P < 0.01$ compared with respective control group. C: Control group; PH: Portal hypertension group.

Statistical analysis

The uptake was estimated with Graph Pad Prism 3.1 software (San Diego, CA, USA). Data of uptake activity were presented as the mean \pm SE, and were analyzed by one-way analysis of variance, followed by Tukey test^[19]. Student's t test was used to assess differences between paired groups. $P < 0.05$ was considered as significant.

RESULTS

In these experiments using prehepatic portal hypertensive animals, with almost normal liver function (2–2.5 times normal values), we found a significant increase in hippocampal GS activity, as compared to sham-operated animals ($P < 0.001$) (Figure 1). On the contrary, in FC, a significant decrease in the enzyme activity was documented ($P < 0.001$), in comparison with control rat brains (Figure 1).

The uptake of [^3H] glutamate occurred in a temperature-dependent manner and increased with incubation time up to 5 min, with a plateau thereafter at 30 min. Glutamate uptake in the hippocampus and FC showed that, at 30°C, [^3H] L-glutamate was taken up into synaptosomes in a time-dependent manner. Its level reached a plateau after 20 min in the presence of Na^+ ions (Figure 2), and remained linear with time for about 2–3 min in the hippocampus and FC, in PH and

sham-operated rats. The uptake of [^3H] L-glutamate was temperature- and Na^+ -dependent in both regions studied. Time course experiments verified that the uptake of this excitatory amino acid was essentially linear up to 2-3 min at the respective incubation temperature (Figure 2).

The portal hypertensive rat, with almost normal liver function (only moderately increased aspartate aminotransferase and alanine aminotransferase activity and blood ammonia), was shown to have morphological and functional alterations of GS and glutamate uptake in astrocytes and neurons.

There was a significant decrease in glutamate uptake in the portal hypertensive group, as compared to the sham-operated rats, in the hippocampus ($P < 0.001$) and FC ($P < 0.05$) (Figure 2). We found a more pronounced decrease in glutamate uptake in the hippocampus. [^3H] L-Glutamate uptake was concentration-dependent at 30°C and was dramatically suppressed at 2°C (data not shown). At 30 min, we observed a decline in [^3H] L-glutamate uptake in the two regions, which might have been caused by the metabolic changes in the glutamine/glutamate cycle.

Liver homogenates obtained from both groups of animals showed no biochemical alterations and no histological damage (data not shown).

Portal pressure was 8.5 ± 0.5 mmHg in the sham-operated group and 12.5 ± 0.8 mmHg in the PH group ($P < 0.01$).

Plasma ammonium level was 23 ± 9 $\mu\text{mol/L}$ in the control group and 79 ± 15 $\mu\text{mol/L}$ in the PH group, which corresponded to an increase of 243% ($P < 0.01$).

DISCUSSION

PH is responsible for severe circulatory derangements in the splanchnic and systemic circulation, and brain damage results from HE, as a consequence of acute and chronic liver failure. In particular, HE is responsible for severe and often lethal outcomes in these diseases. The HE can be characterized by a wide spectrum of neuropsychiatric abnormalities.

In the present study of prehepatic portal hypertensive rats, we demonstrated different activities of GS and glutamate uptake in the hippocampus and FC. We observed that the activity of GS increased in the hippocampus of PH rats compared to control animals, while there was a decrease in the FC. There was a moderate increase in ammonia concentration.

Using fresh synaptosomal fractions from the FC and hippocampus, we found that glutamate uptake was decreased significantly in PH rats, with a more marked decrease in the hippocampus. In the presence of 125 mmol/L NaCl, uptake of this radiolabeled amino acid occurred in a temperature-dependent manner, and increased with incubation time up to 2-3 min, with a plateau thereafter at 30 min. These results suggest that there are biochemical differences between the brain regions, possibly caused by the toxic metabolic action of ammonia, glutamate, and perhaps glutamine, on the rat brain.

The morphological alterations in the liver parenchyma in chronic disease and in portal vein thrombosis create

collateral veins that shunt splanchnic blood flow to the systemic circulation, in an attempt to overcome the increased pressure of portal vein flow. This phenomenon modifies the normal physiology of several organs, including the CNS, and transports intestinal toxins directly to the brain.

In previous experiments with this rat model, alterations in the CNS have been documented, including BBB permeability modifications^[5,20]. The impact of PH on the BBB has been demonstrated in rats, in which, 40 d after portal vein stricture, the BBB recovers its function associated to normalize portal pressure and the impermeability to dyes^[20].

Glutamate is a major excitatory neurotransmitter, and any alteration in the glutaminergic pathway must modify brain function. Normally, glutamate is synthesized in brain tissue from glucose.

Butterworth *et al*^[21], studied the brain in liver failure, glutamate, some other amino acids and neurotransmitters, such as serotonin and dopamine, that are involved in the development of HE. Glutamate uptake and transport are important steps in protecting CNS cells from glutamate excitotoxicity^[22,23].

As the removal of ammonia in the brain is linked to the metabolism of glutamate in astrocytes, damage to these cells has been described in PH animals, which involves glutamate uptake and clearance^[24].

Rapid clearance of neurotransmitters released from synapses, especially glutamate, acts to limit its signaling and prevents its harmful over-stimulation^[25]. It has been well established for several decades that hyperammonemia leads to reduced glutamate uptake, which is caused by a reduced amount and function of glutamate transporters^[26-28]. Moreover, it has been shown recently that reduced glutamate transporters and increased extracellular glutamate are responsible for hypokinesia in rats with HE and hyperammonemia^[29].

GS plays a central role in the metabolic regulation of the excitatory neurotransmitter glutamate and in the detoxification of ammonia. GS is located mainly in astrocytes. It has been suggested that glutamate can regulate GS brain distribution^[30]. This enzyme is responsible for the protection of neurons against excess ammonia and glutamate, by metabolizing both substances into glutamine. Approximately 85% of ammonia is converted to glutamine^[31,32]. In addition, glutamate from neurons can be reconverted into glutamine^[33]. Astrocytes play a key role in the pathogenesis of ammonia-induced neurotoxicity and HE. Schliess *et al*^[34] have found that ammonia induces protein tyrosine nitration in cultured rat astrocytes, which is sensitive to the NMDA receptor antagonist MK-801. Actually, the production of reactive nitrogen intermediates and protein tyrosine nitration may alter astrocyte function and contribute to ammonia neurotoxicity^[34].

Acute intoxication with large doses of ammonia leads to CNS cell damage. The main mechanism for ammonia elimination in the brain is its reaction with glutamate to form glutamine. This reaction is catalyzed by GS and consumes ATP^[35]. It has been observed that GS activity and

glutamine content in the brain are modulated by NMDA receptors and nitric oxide.

There are two main types of hyperammonemia: (1) chronic moderate hyperammonemia, as occurs in liver cirrhosis, which leads to altered cerebral function, and is responsible for the neurological alterations in different hyperammonemic states, and also for some of the neurological alterations in liver disease and HE; and (2) acute intoxication with large doses of ammonia, which may lead to rapid death of animals or patients. This situation can occur in acute liver failure.

Direct toxic effects of glutamine on CNS cells have been demonstrated in isolated mitochondria, which shows that elevated accumulation of glutamine has injurious effects on these cells^[36].

Isolated rat cerebral mitochondria treated with high glutamine concentrations (5 mmol/L), as present in acute hyperammonemic rats, show swelling and mitochondrial permeability transition (MPT)^[37]. Murthy *et al.*^[38] have shown that ammonia alone does not induce MPT, but that its metabolism to glutamine is necessary to produce this alteration. Mitochondrial swelling, as a result of the presence of high levels of glutamine and ammonia, is known to stimulate the uptake of glutamine^[39].

The addition of glutamine to cultured astrocytes induces MPT development and the formation of free radicals^[40]. Hence, glutamine synthesis, from ammonia and glutamate in astrocytes, represents an important process in brain ammonia detoxification, but also can produce negative effects.

The increased activity of GS in the FC, as seen in this PH rat model, may represent a response to moderate increases in ammonia. However, in the hippocampus, we observed decreased activity of GS, which may correspond to an increase in glutamate, caused by decreased uptake and a prolonged toxic effect.

The astrocyte is the brain cell that is directly involved in HE. It participates in chronic porto-systemic encephalopathy, with ultrastructural alterations in the brain. Astrocyte mitochondria are included in this pathophysiological mechanism in the hippocampus^[41] and alterations in its respiratory chain, and show ultrastructural damage. By using proton magnetic resonance, Häussinger^[42] has shown that brains from HE patients have an increase in glutamine/glutamate signaling. Structural and functional alterations in cultured astrocytes, when their exposure to different ammonia concentrations is modified, suggest the possibility that HE may constitute a primary gliopathy^[43].

The mechanisms that produce brain damage in chronic encephalopathy may include the following. (1) Increased ammonia in the brain, which is associated with an increase in glutamate, caused by its reduced uptake, which leads to altered metabolic pathways and functional and morphological damage to the mitochondria in the hippocampus and FC; and (2) The hippocampus possesses a fundamental function in behavioral patterns on memory and spatial mechanisms among other functions. Therefore, it is possible that hippocampus suffers more readily from toxicity than the FC, which also

participates in this process, but with minor involvement.

It is possible that the results obtained in the hippocampus in PH rats are indicative of its plasticity, which provokes, during toxic insult, changes in the expression of its enzymes, which maintain the functional equilibrium during this pathological situation. In the mammalian brain, this region is fundamental for the encoding of recent and past experience, including spatial and non-spatial information^[44]. The FC has an efficient adaptative mechanism, not described in the hippocampus; its structure perhaps suffers more damage when toxic substances, such as ammonia, glutamate and glutamine are not detoxified efficiently. It may be that, in patients with cirrhosis, these mechanisms can participate in memory processes and behavioral alterations. It might be that the extracellular glutamate that arises from impairment of the glutamate/glutamine cycle in PH participates in the pathogenesis of HE. Furthermore, in PH, increases in ammonia and glutamate and/or glutamine may participate in oxidative stress that is induced by a glutamate-mediated pathway.

Warren and Schenker^[44] have demonstrated that inhibition of GS by methionine sulfoximine decreases the death rate in rats intoxicated with ammonia. They found fewer metabolic alterations in HE, including brain edema, less astrocyte swelling, and a decrease in ammonia-induced reactive oxygen species. These experiments demonstrate the negative role that glutamine can play in the pathophysiology of HE^[39,45-47]. In liver failure, brain edema, intracranial hypertension, neurotransmitter derangements and neurological symptoms represent the cerebral repercussions of distant liver parenchymal toxicity.

Our results from a PH rat model showed that two different brain regions had different responses in terms of GS activity and glutamate uptake. The BBB in these animals showed an increase in its permeability, apparently as a result of increased portal pressure, because when pressure returned to normal, the BBB recovered its permeability^[20]. Also, an increase in astrocyte number, associated with an increase in endothelial cells (angiogenesis), has been documented previously^[48].

Finally, it is clear that a partial stricture of the portal vein is capable of modifying functions in two brain regions, the hippocampus and FC. The different changes observed are difficult to explain. Perhaps, further experiments with PH models, using blockers and antagonist of glutamate uptake are needed to explain these results.

COMMENTS

Background

To analyze the participation of glutamine synthetase (GS) activity and glutamate uptake in the hippocampus and cerebral frontal cortex (FC), using a rat model of prehepatic portal hypertension (PH), with the intention of mimicking the two major complications that appear in chronic liver failure: hepatic encephalopathy (HE) and PH.

Research frontiers

In this field, the research hotspots are how to use prehepatic PH rats and how to study the different activities of GS and glutamate uptake in the hippocampus and FC.

Innovations and breakthroughs

This study determinate the uptake of [³H] L-glutamate by synaptosomes of rat cerebral cortex. That provides accurate and reproducible data and can be employed to measure kinetic parameters, and this study determinate GS activity to maintain a stable glutamate/glutamine ratio in the brain.

Applications

Using the PH model, it is possible to understand more clearly the mechanism of toxicity and defense of the brain against two toxic substances: ammonia and the excitatory neurotransmitter glutamate.

Terminology

The extracellular actions of glutamate are limited by glutamate-specific Na⁺-dependent transporters that remove glutamate, mostly by taking it up into astrocytes. Astrocytes convert glutamate into glutamine and pass it back to neurons, where it can be converted into glutamate and used to replenish the neurotransmitter stores in synaptic vesicles.

Peer review

The authors have used a rat model of PH and have analyzed the activity of GS and the uptake of glutamate by synaptosomes in the hippocampus and FC 14 d after surgery. The paper seems to be important for understanding HE. The whole paper is relatively easy to read and understand.

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