Elucidation of neuroprotective role of endogenous GABA and energy metabolites in middle cerebral artery occluded model in rats

M Ramanathan1*, C Saravana Babu2, A Justin1 & S Shanthakumari3

1Department of Pharmacology, PSG College of Pharmacy, Peelamedu, Coimbatore 641 004, India
2Center for Toxicology & Developmental Research, Sri Ramachandra University, Chennai 600 116, India
3Department of Pathology, PSG Institute of Medical Sciences & Research, Peelamedu, Coimbatore 641 004, India

Received 28 January 2012; revised 28 March 2012

The excitatory amino acids (EAA) like glutamate, aspartate and inhibitory neurotransmitter GABA (gamma amino butyric acid) play an important role in the pathophysiology of cerebral ischemia. The objective of the present study is to elucidate the role of endogenous GABA against EAA release in different regions during ischemia. The transient focal ischemia was induced in rats by using middle cerebral artery occlusion model (MCAo). The results indicate gradual elevation of brain glutamate, aspartate and GABA level at different brain regions and attained peak level at 72 h of ischemic reperfusion (IR). At 168 h of IR the EAA levels declined to base line but GABA level was found to be still elevated. The biochemical analysis shows the depleted brain ATP, Na+K+ATPase content and triphasic response of glutathione activity. It can be concluded that time dependent variation in the EAA and GABA release, endogenous GABA can be neuroprotective and earlier restoration of energy deprivation is essential to prevent further neurodegeneration. To have efficient treatment in ischemic condition, multiple approaches like energy supply, antagonism of EAA, controlling calcium function are essential.

Keywords: ATP, Aspartate, Cerebral ischemia, Glutamate, Glutathione, Neuroprotection

Excitatory amino acids (EAA) glutamate and aspartate play an important role in neurodegeneration apart from their central neurotransmitter role. The elevated concentrations of these EAA were reported in ischemic conditions1. The release of EAA from the brain mediates through transduction pathways involving phospholipase A and C with protein kinase C2. The release of EAA can also be regulated with energy deprivation3 and excessive calcium entry4. Treatment with calcium channel blockers, NMDA antagonist and controlling the AMPA function can be neuroprotective5,6 in middle cerebral artery occlusion (MCAo) model. The GABAergic (gamma amino butyric acid) neurotransmission may also found to be a useful target to have neuroprotective activity in ischemic status7,8. In vitro studies have indicated increase in the sensitivity of GABAergic neurons in oxygen and glucose deprived conditions. Further, an increase in the intracellular chloride ions was observed in the hippocampal neuron in the similar condition9. GABA agonists found to modulate depolarization-evoked glutamate release in the hippocampus by inhibiting Ca2+ entry into neurons, an effect mediated by presynaptic GABA_A receptors10. Stimulation of GABA receptors inhibited the striatal glutamate release in focal cerebral ischemia model in rat11. These studies denote that drugs targeting GABAergic transmission can exert neuroprotective effects in ischemic condition via the activation of both GABA_A and GABA_B receptors12. In transient cerebral ischemic conditions normal GABA level was returned within 1 h after reperfusion13. There is no evidence to indicate the interrelationship of GABA with EAA release and energy imbalance. It is not clear that at the time of EAA release in vivo, what happens to the endogenous GABA activity? If GABA is released endogenously, whether it will protect the neuronal cells? To understand this, the level of EAA in different brain regions and their correlation is important in the later phase of ischemic damage. Hence it has been hypothesized that GABA release in

*Correspondent author
Telephone: +91 422 4345841
Fax: +91 422 2594400
E-mail: muthiah.im@gmail.com
excess can act as a defense mechanism in the brain to
antagonize the EAA activity in ischemic state.
The present study is designed to determine the time
dependent changes (up to 168 h) and correlate the
level of EAA along with GABA in different brain
regions. To understand the importance of energy
restoration and its role in neurodegeneration,
energy metabolites biochemical markers such as ATP,
LDH and pyruvate have been measured.
The neurodegenerative observations are supported
with histopathological studies.

Materials and Methods

Chemicals and reagents—GABA, L- glutamic
acid, L- aspartic acid were purchased from Sigma,
US; adenosine triphosphate (ATP) and nicotinamide
adenine dinucleotide (NAD) were purchased from
SISCO Research Laboratories, Mumbai, India.
Hematoxylin and eosin were procured from
Hi-Media, India. 4-0 nylon monofilament Ethicon
was procured locally. All other chemicals, reagents
and solvents unless specified were of analytical grade.

Animals—Male Sprague Dawley rats (290–340 g)
used in the study were housed in individual
polypropylene cages in a well ventilated room
(air cycle: 15/hr) under an ambient temperature of
23±2 °C and 40–65% RH, with a 12:12 h light/dark
artificial photoperiod. They were provided with food
(Nutrilab Rodent, Tetragon Chemie Pvt Ltd, India)
and purified water ad libitum. All the animals were
acclimatized at least for 7 days to the laboratory
conditions prior to experimentation. Guidelines of
“Guide for the Care and Use of Laboratory Animals”
(Institute of Laboratory Animal Resources, National
Academic Press 1996; NIH publication number
#85-23, revised 1996) were strictly followed
throughout the study. Institutional Animal Ethical
Committee (IAEC), Sri Ramachandra University,
Chennai, India approved the study

Surgical procedure—Focal cerebral ischemia
was induced by middle cerebral artery occlusion
with minor modifications. Rats were anesthetized with
chloral hydrate (350 mg/kg, ip) and the right common
carotid artery was exposed at the level of external
and internal carotid artery bifurcation. 4-0 nylon
monofilament was used and its tip was made round
headed by exposing it to flame. The Filament was
coated with 0.01% poly-L-Lysine and inserted into
the external carotid artery and advanced to the
internal carotid artery for a length of about 20–21 mm
until a slight resistance was felt. On achieving
occlusion, the filament was held in place with ligature
and the external incision was sutured temporarily.
After 2 h of ischemia the rats were anesthetized,
suture was opened, the filament was pulled out and
reperfusion in internal carotid artery was ensured
visually. Throughout the surgical procedure, body
temperature was measured by inserting a
thermometric probe into the rectum of rat and it was
maintained at 37±0.5 °C using thermostatically
controlled heating blanket. Animals were then kept in
a cage with a heating lamp, which maintained the
cage temperature between 29±1 °C for another 1 h to
counteract any possible hypothermic effect. In the
sham-operated (SO) group, external carotid artery was
surgically prepared for insertion of filament, but the
filament was not inserted.

Experimental design

<table>
<thead>
<tr>
<th>Anesthesia and surgery</th>
<th>2 h Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>0 min</td>
<td>30 min</td>
</tr>
<tr>
<td>No-ischemia (sham)</td>
<td>I</td>
<td>II</td>
</tr>
</tbody>
</table>

Neurochemical analysis

Glutamate, aspartate and GABA—Tissues were
homogenized in 0.1 N HCl in 80% ethanol (for every
10 mg tissue/200 μL) and were transferred to
polypropylene tubes and centrifuged at 4500 rpm for
20 min at 25 °C. The supernatant was then transferred
into micro centrifuge tubes and used immediately for
glutamate estimation by HPTLC (CAMAG — version
1.3.4, USA) chromatographic condition: silica gel
GF254 as stationary phase; n-butanol: glacial acetic
acid: water (65:15:25;v/v) as mobile phase;
applicator: Linomat V; scanner: CAMAG TLC
scanner III; developing chamber: twin trough glass
chamber (20×10); developing mode: ascending mode
(multiple development); detection reagent: 0.2%
ninhydrin in acetone; scanning wavelength: 486 nm;
experimental condition: temp: 25±2 °C: 55–65% RH.
Standard solutions of L-glutamic acid (20–200
ng/spot), aspartic acid (10–80 ng/spot) and GABA
(5-80 ng/spot) were prepared for plotting the
calibration curve.

Biochemical analysis

ATP content—Aliquots of the homogenates were
sonicated immediately in ice cold perchloric acid
(0.1 N) to inactivate ATPases. After centrifugation
(14,000 g, 4 °C, 5 min), supernatants containing
ATP were neutralized with 1N NaOH and stored
at –80 °C until analysis. ATP level in supernatants
were quantified using a reverse-phase HPLC (Perkin Elmer). RP-HPLC determination was performed on a reversed-phase Hypersil C18 (4.6 mm×250 mm, 5 μ) column (Elite, Dalian, China) attached to two LC-10ATvp pumps (Shimadzu, Kyoto, Japan), equipped with UV–Vis detector. The mobile phase was 100 mM K2HPO4–KH2PO4 buffer solution (pH 6.0), the flow rate 1.2 mL/min, the column temperature 25 °C and the detection wavelength 254 nm. A reference solution of ATP was prepared according to dissolving standards (Sigma, St. Louis, MO, USA).

Glutathione—Brain tissue homogenate (0.25 mL) was added to equal volume of ice cold 5% TCA. The precipitate was removed by centrifugation at 4000 rpm for 10 min. To 1 mL aliquot of supernatant, 0.25 mL of 0.2 M phosphate buffer (pH 8.0) and 0.5 mL of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) were added and mixed well. The absorbance was read at 412 nm using spectrophotometer (Perkin Elmer, λ 25, USA). Values were expressed in nano moles/g tissue.

Lactate dehydrogenase (LDH)—Plasma LDH was measured according to the kit manufacturer’s instructions (LDH reagent kit - Aspen Laboratories) by using auto analyser (Merck).

Pyruvate—To 500 μL of the plasma, 1.5 mL of 5% tricarboxylic acid was added and centrifuged at 2500 rpm for 10 min. To 500 μL supernatant, 1 mL distilled water and 500 μL of 2,4-dinitro phenyl hydroxide (prepared in 2 N HCl) were added, mixed well and allowed to stand at room temperature for 5 min. To the above mixture, 2.5 mL of 1.5 N NaOH was added. The intensity of the reddish pink colour was measured at 540 nm OD using spectrophotometer (Perkin Elmer, λ 25, USA). The standard calibration curve was plotted using pyruvate in the concentration range of 4–20 μg.

Na’K’ATPase—Na’K’ATPase was assayed by taking 250 μL of Tris HCl (184 mM; pH 7.5) buffer followed by the addition of 50 μL of 600 mM NaCl, 50 μL of 50 mM KCl, along with 50 μL of 1 mM Na.EDTA, and 50 μL of 80 mM ATP. The reaction mixture was pre-incubated at 37 °C for 10 min. Then 25 μL of 10% homogenate was added to the test alone and further incubated at 37 °C for 1 h. The reaction was immediately arrested by the addition of 10% TCA. The control reaction was correspondingly performed by adding 25 μL of 10% homogenate only after arresting the reaction. The resultant precipitate was removed by centrifugation at 3500 rpm for 10 min. From the 50 μL of the supernatant, the liberated inorganic phosphorus was measured as mentioned in GS estimation method19.

Protein estimation—The protein content in the brain homogenate was estimated by Lowry’s method20.

Histopathological study—After 72 h of ischemic reperfusion (IR), euthanasia on the experimental animals were done. The brain was dissected out quickly, fixed in 10% formalin and 5 μm thick sections were taken. The sections were processed and stained in hematoxylin and eosin21. The stained sections were observed under a binocular light microscope and photographed and compared with SO groups.

Statistical analysis—Data were expressed as mean±SE. Mean difference between the neurochemicals and biochemical parameters were analysed by one way ANOVA followed by Dunnet’s multiple comparison tests. For graphical representation pyruvate value was multiplied by 1000 and ATP value was multiplied by 100. Statistical analysis was performed using GraphPad Prism, 4.03 (San Diego, US). P <0.05 was fixed as the statistical significance criterion.

Results

Time dependent effect on brain glutamate, aspartate and GABA levels after ischemic reperfusion in different brain regions—

Cortex: A steady increase in the cortical glutamate level with peak concentration at 72 h (P < 0.001) was observed after IR. Thereafter a gradual decline in the glutamate level was observed up to 168 h. However at this time interval the glutamate concentration did not reach the base line value. The aspartate level was found to be elevated at 30 min and there after gradual increase in aspartate level was observed and the peak level was reached at 72 h (P < 0.001). GABA level was found to be elevated at 6 hr (P < 0.001) and reached the peak concentration at 72 h (P < 0.001) and then started decreasing till the time of study. However, significantly higher GABA level was observed when compared to the SO rats at 168 h (Table 1).

Striatum: In striatal region, glutamate (P < 0.001) and GABA (P < 0.01) levels were found to be high at 6 hr after ischemic reperfusion in comparison to SO rats. The GABA level even at 168 h (P < 0.001) was found to be significantly high and exhibited a slow
reversal of GABA in this region. Aspartate release was found to be higher at 30 min and gradual increase in aspartate concentration was observed and attained peak concentration at 72 h ($P < 0.001$). Like glutamate, aspartate level was also found to decline after 72 h, however both these amino acids failed to reach the basal value at 168 h (Table 2).

Hippocampus: The excitatory amino acids content and GABA level in hippocampal region were found to be similar to that of cortex and striatum. In hippocampus concentration of all the amino acids significantly increased after reperfusion following ischemia and the peak concentration reached at 72 h. In comparison to cortex and striatum hippocampus aspartate concentration at 72 h was found to be significantly less and the GABA level was found to be maintained up to 168 h ($P < 0.001$) (Table 3).

**Time dependent effect of energy metabolites after IR—**

Glutathione: Occlusion of middle cerebral artery followed by reperfusion resulted in a triphasic response with respect to brain glutathione level. After 30 min the glutathione level was found to be decreased followed by increase up to 6 h ($P < 0.01$) and then depletion upto 168 h of study ($P < 0.01$). Reversal of glutathione level was not observed during the study period (Fig. 1).

---

**Table 1**—Time dependent effect on glutamate, aspartate and GABA levels after IR in cortex  
[Values are mean±SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Sham 0</th>
<th>1/2</th>
<th>2</th>
<th>6</th>
<th>24</th>
<th>72</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>0.57±0.10</td>
<td>0.72±0.08</td>
<td>0.74±0.09</td>
<td>1.48±0.20</td>
<td>2.62±0.30**</td>
<td>4.76±0.50**</td>
<td>7.30±1.25**</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.13±0.06</td>
<td>0.53±0.04</td>
<td>0.96±0.10</td>
<td>1.24±0.19</td>
<td>1.81±0.27***</td>
<td>3.14±0.28***</td>
<td>5.98±0.31***</td>
</tr>
<tr>
<td>GABA</td>
<td>0.10±0.01</td>
<td>0.43±0.04</td>
<td>0.56±0.08</td>
<td>0.94±0.19</td>
<td>1.92±0.22**</td>
<td>3.18±0.32**</td>
<td>4.68±0.51**</td>
</tr>
</tbody>
</table>

*P* values: * < 0.01; ** < 0.001 in comparison to sham operated group

**Table 2**—Time dependent effect on glutamate, aspartate and GABA levels after IR in striatum  
[Values are mean±SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Sham 0</th>
<th>1/2</th>
<th>2</th>
<th>6</th>
<th>24</th>
<th>72</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>0.39±0.05</td>
<td>0.70±0.05</td>
<td>1.22±0.15</td>
<td>1.64±0.22</td>
<td>2.70±0.36***</td>
<td>5.48±0.41***</td>
<td>8.72±1.50***</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.11±0.01</td>
<td>0.69±0.09</td>
<td>1.08±0.12</td>
<td>1.58±0.19*</td>
<td>2.40±0.36***</td>
<td>4.34±0.23***</td>
<td>7.48±0.65***</td>
</tr>
<tr>
<td>GABA</td>
<td>0.19±0.03</td>
<td>0.39±0.05</td>
<td>0.68±0.07</td>
<td>1.56±0.29</td>
<td>2.11±0.32**</td>
<td>3.94±0.41***</td>
<td>6.18±0.68***</td>
</tr>
</tbody>
</table>

*P* values: * < 0.05; ** < 0.01; < 0.001 in comparison to sham operated group

**Table 3**—Time dependent effect on glutamate, aspartate and GABA levels after IR in hippocampus  
[Values are mean±SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Sham 0</th>
<th>1/2</th>
<th>2</th>
<th>6</th>
<th>24</th>
<th>72</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>0.65±0.70</td>
<td>0.84±0.06</td>
<td>0.92±0.11</td>
<td>1.81±0.31</td>
<td>2.70±0.22**</td>
<td>4.88±0.63***</td>
<td>8.09±1.34***</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.18±0.03</td>
<td>0.87±0.09</td>
<td>1.27±0.15</td>
<td>1.73±0.15*</td>
<td>1.90±0.31**</td>
<td>2.95±0.30***</td>
<td>3.69±0.21***</td>
</tr>
<tr>
<td>GABA</td>
<td>0.12±0.02</td>
<td>0.37±0.04</td>
<td>0.77±0.18</td>
<td>1.23±0.15</td>
<td>2.09±0.31**</td>
<td>2.85±0.40***</td>
<td>5.69±0.51***</td>
</tr>
</tbody>
</table>

*P* values: * < 0.05; ** < 0.01; < 0.001 in comparison to sham operated group
LDH: Plasma LDH level was found to be elevated even at zero hr followed by steady increase in LDH content in ischemic brain up to 2 h \( (P < 0.01) \) in comparison to SO rats. From 2 h the LDH concentration was found to decrease and it reached the basal value at 72 h and further decline was noted at 168 hr (Fig. 2).

Pyruvate: The plasma pyruvate level during ischemic reperfusion did not alter up to 168 h of observation in comparison to SO rats (Fig. 2).

ATP: In comparison to SO rats a gradual decline in the brain ATP content at different time intervals was observed. The ATP level did not recover up to 168 h \( (P < 0.01) \) and the energy imbalance was maintained throughout the observation period (Fig. 2).

Na⁺K⁺ATPase activity: In the present study, Na⁺K⁺ATPase activity decreased significantly \( (P < 0.01) \) in the cortex (67%), striatum (70.8%) and hippocampus (75.9%) of the IR group rats when compared to the SO rats. The decrease in the enzyme activity was found to be more prominent in the striatal region (Fig. 3).

Histopathological studies—The cerebral cortex of SO rats appears normal without any lesions or shrinkage of neurons. At 72 h, the IR group shows focal degenerative changes in the cerebral cortex with a few nucleated neurons (Fig. 4a, b).

![Fig. 2—Time dependent effect of brain ATP (µmoles/g tissue) content, Plasma LDH (U/mL) and pyruvate (mmoles/L) after IR [Values are mean±SE from 6 rats in each group] \( P: \* < 0.05; \** < 0.01 \) in comparison to sham operated group](image)

![Fig. 3—Brain Na⁺K⁺ ATPase activity after IR [Values are mean±SE from 6 rats in each group] \( P: \* < 0.01 \) in comparison to sham operated group](image)

![Fig. 4—Representative photographs showing (a) cerebral cortex of SO rats (b) ischemic cortex after 72 h of IR.](image)
Discussion

The present study represents the time dependent variation in brain glutamate, aspartate and GABA levels and energy deprivation which are characterized by decline of ATP, Na$^+$K$^+$ATPase content, reduced glutathione activity and increased LDH level after IR. These observations indicate that energy imbalance associated neurotransmitter changes can have crucial role in pathology of stroke. Memantine and nimodipine attenuated the altered glutamate, increased GS activity, oxidative stress in brain along with neurological and behavioural alterations after ischemic damage. In general, during cerebral ischemic condition decreased cerebral blood flow leads to restriction in the supplies of primary energy substrates like glucose and oxygen. The ability of neurons to obtain energy other than primary energy substrates contributes importantly to cellular survival. Reports indicate that 15-47% of excessive glutamate available in extracellular fluid can undergo TCA cycle for energy supply apart from its excitotoxicity function. The glutathione level was elevated at 2 h of IR to neutralize the free radical generation, but in 72 h of IR complete depletion of glutathione was observed or it may be due to non availability of glutamate for the synthesis of glutathione at astrocytes. The glutamate transport depends on glial bound Na$^+$K$^+$ATPase (EAA transporter) activity; this enzyme is very susceptible to free radicals. Na$^+$K$^+$ATPase is a ubiquitous cell membrane enzyme responsible for establishment of ion gradients necessary for cell function, including maintenance of the resting membrane potential of neurons. Excess ROS released during ischemia would have decreased the Na$^+$K$^+$ATPase enzyme activity there by accumulation of glutamate in extracellular fluid resulting in decreased synthesis of glutathione which was reflected in depletion of glutathione level at 72 h of IR.

The extracellular accumulation of GABA in different brain regions during ischemia may be attributed to open the voltage dependent Ca$^{2+}$ channels of presynaptic terminals of GABAergic neurons due to decreased ATP induced depolarization. This calcium overload in GABAergic neurons triggers the release of GABA from the synaptic vesicles. In recent years, the inhibitory neurotransmitter system GABA has been the focus in pharmacological research for neuroprotection in conditions like stroke because of its effective and protective role in ischemia-induced neuronal death and its functions in opposition to that of glutamate transmission. The present experimental findings connotes that the gradual elevation of GABA level did not reach the base line in the vulnerable areas like cortex, striatum and hippocampus region even at 168 h of IR. Hence it can be considered that GABA release in excess can act as a defense mechanism in the neuronal cells to antagonize the EAA activity in ischemic status. But importantly this phenomenon could not give full protection to the neuronal cells.

Changhan et al. suggest that both GABA$\alpha$ and GABA$\beta$ receptors play an important role in regulating extracellular concentration of glutamate in rat striatum during ischemia although the activity of GABA$\beta$ receptors seems to be more predominant. Released GABA, may potentially counteract the effects of excitatory neurotransmitters by hyperpolarizing neuron membrane potential and inhibiting glutamate transmission during ischemia. Further, pretreatment with exogenous GABA compounds suppressed the ischemia induced glutamate and aspartate release which supports the present finding. In the present study without any external stimulus excessive endogenous GABA content in cortex, striatum and hippocampus region of brain has been demonstrated. It would have acted as a defense mechanism during ischemia by antagonizes EAA induced neurodegeneration by regulating EAA release through enhanced GABA receptor activity. Though GABA act as neuroprotective, neurodegeneration was observed which suggests GABA stimulation alone may not be sufficient and role of other parameters like cerebral blood flow, energy supply, and Ca$^{2+}$ overload may contribute the neurodegeneration. The study can be concluded that time dependent variation in the EAA and GABA release, and endogenous GABA can be neuroprotective and earlier restoration of energy deprivation is essential to prevent further neurodegeneration. To have efficient treatment in ischemic condition, multiple approaches like energy supply, antagonism of EAA, controlling calcium function are essential.

References


