J Physiol Biochem (2014) 70:901–913
DOI 10.1007/s13105-014-0358-8

ORIGINAL PAPER

# Alleviation of glutamate mediated neuronal insult by piroxicam in rodent model of focal cerebral ischemia: a possible mechanism of GABA agonism

**Pallab Bhattacharya · Anand Kumar Pandey · Sudip Paul · Ranjana Patnaik**

Received: 12 July 2013 / Accepted: 17 September 2014 / Published online: 8 October 2014


**Abstract** Neurotransmitter imbalance is an inevitable outcome in cerebral ischemia that leads to neuronal death. In the present study, we evaluated the effects of piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), on extracellular brain glutamate and γ-aminobutyric acid (GABA) release, survival time, and neuronal cell death. Transient focal cerebral ischemia in male Charles Foster rat led to neuronal infarction and compromised intrinsic antioxidant status. Thirty-minute preadministration of piroxicam (10 mg/kg b.w.) showed a significant ($P<0.01$) reduction in cerebral infarct volume and potentiation of the intrinsic antioxidant status. High-performance liquid chromatography of brain cortex and striatum revealed changes in extracellular concentrations of neurotransmitters which were found to be 0.519±0.44 pmole/mg (GABA); 1.18±0.2 pmole/mg (glutamate), and 0.63±0.21 pmole/mg (serotonin), respectively. Hydroxyl radical (·OH) adduct of salicylate in the frontal cortex and striatum of control, untreated, and treated groups was found to be 0.261±0.06, 0.68±0.52, and 0.401±0.68 pmole/mg, respectively. After stroke, the extracellular level of glutamate in rat brain increases continuously as compared to that of control group. However, piroxicam administration in stroke rat significantly reduced ($P<0.05$) elevated extracellular cerebral glutamate. This indicates that piroxicam attenuates extracellular glutamate release and also reduces neuronal cell death due to reduction in oxidative stress in cerebral ischemia. Our results also indicate a consequent increase of extracellular GABA in brain regions administered with piroxicam, which hints that piroxicam alleviates glutamate excitotoxicity possibly by GABA agonism.

**Keywords** Cerebral ischemia · GABA · Glutamate · Antioxidant · Piroxicam · Neuroprotection

**Abbreviation**

| | |
|---|---|
| GABA | γ-Aminobutyric acid |
| Glu | Glutamate |
| 2,3-DHBA | 2,3-Dihydroxy benzoic acid |
| 2,5-DHBA | 2,5-Dihydroxy benzoic acid |
| NSAID | Nonsteroidal anti-inflammatory drug |
| SA | Salicylic acid |

## Introduction

Cerebral ischemia is a devastating condition which affects many people each year and is one of the leading

Pallab Bhattacharya and Anand Kumar Pandey have equal contribution

P. Bhattacharya (✉)
Department of Neurology, Leonard M. Miller School of Medicine, Miami, FL 33136, USA
e-mail: pbhattacharya@med.miami.edu

P. Bhattacharya · A. K. Pandey · S. Paul · R. Patnaik (✉)
School of Biomedical Engineering, Indian Institute of Technology, Banaras Hindu University, Varanasi 221005 UP, India
e-mail: drranjanapatnaik@gmail.com

S. Paul
Department of Biomedical Engineering, North Eastern Hill University (NEHU), Shillong, India

causes of mortality and disability around the world [17, 28, 34, 48]. The narrow time window for the therapeutic intervention is one of the causes of its progressive pathophysiology and temporal progression of the ischemic insult. The insult initiates with an occluded blood vessel which deprives the neurons of glucose and oxygen, resulting in cell death in the immediate infarct core. Further, disruptions of ionic homeostasis, release of intracellular potassium, accumulation of intracellular calcium and sodium increased extracellular levels of glutamate, and production of cytotoxic free radicals leads to cellular injury, necrosis, and apoptosis in areas adjacent to the initial insult [17, 34]. Reperfusion can revert metabolic derangement in the surrounding penumbral tissue and prevent progression, but at the same time, it brings oxidative insult which acts as a curse [17, 23, 34]. Neuroinflammation is also one of the major consequences after cerebral ischemia. There are several resident cell populations within brain tissue that are able to secrete proinflammatory mediators after an ischemic insult [52, 42, 54, 21, 55, 26, 34]. These factors add layers of complexity, in both adducing their pathophysiological roles in stroke and in the goal of developing new therapeutics for stroke therapy. Nonsteroidal anti-inflammatory drugs (NSAIDs) emerge as one of the candidate molecule to redress neuroinflammation and brain swelling as reported in past studies [8, 24].

Stroke leads to release of excessive glutamate and overstimulation of glutamate receptors which results into neuronal excitotoxicity [18, 17, 8] by triggering postsynaptic depolarization and influx of positive charges into the neurons. As a result of this, there is every possibility for the death of neurons [3, 16, 38, 46]. But, the intrinsic neuroprotective mechanism of the brain can also not be overlooked. The most possible internal neuroprotective mechanisms might be due to simultaneous release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) to counteract glutamate excitotoxicity by hyperpolarizing neuron membrane potential and inhibiting glutamatergic transmission [13, 20, 31, 32]. Drug acting on regulatory mechanism at the point of glutamate and GABA release in the cerebral regions sensitive to ischemic insult can owe to its neuroprotective efficacy that may be the deciding factor for the fate of the surviving neurons.

GABA receptors have a regulatory role on glutamate release in the insulted brain regions [33, 36, 41, 44, 49]. The striatum is not only known to be vulnerable to ischemic insult, but also populated with both GABA and glutamatergic neurons. Previous studies have already reported that i.p. administration of exogenous GABA decreases the glutamate release in cortex following cerebral ischemia [41]. This provides a clue regarding the positive role of GABA agonist to have neuroprotective effect. These results were also authenticated by validating the role of GABA by administration of GABAa and GABAb antagonists which failed to obliterate the glutamate level as reported by Ouyang et al. [41].

Owing to the past results regarding neuroprotective efficacy of piroxicam in cerebral ischemia [4–8, 53], in the present study, we have tried to observe the role of piroxicam in modulating the exogenous GABA which might bring changes in the extracellular release of glutamate [50]. This study was undertaken on the basis of past studies reported by Coyne et al., where it was reported that NSAIDs have a GABA agonism property, but no influence on glutamate or glycine [14]. The main aim to consider striatum with cortex was that according to the past reports striatal neurons are known to have both GABAa and GABAb receptors and activation of which may increase potentiation-evoked glutamate and dopamine release in brain regions including cortex [37, 49, 50]. However, the mechanism of GABAa and GABAb regulation of glutamate release is yet to be explored with lucidity. In the present study, we have evaluated the neuroprotective effects of piroxicam on the extracellular levels of glutamate and GABA in two vulnerable brain regions, the striatum and the cortex and also its role in reducing oxidative stress [35].

## Experimental procedure

### Chemicals

Piroxicam was purchased from Sigma (USA). GABA, glutamate, 2,3-dihydroxy benzoic acid (2,3-DHBA), 2,5-dihydroxy benzoic acid (2,5-DHBA), salicylic acid, ethylenediaminetetraacetic acid disodium salt (EDTA), *O*-pthaldialdehyde, and heptane sulfonic acid were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Acetonitrile and methanol were purchased from Merck (USA). All other chemicals were purchased from Merck and Hi-Media if not otherwise specified.

### Animals and drug treatment

Male Charles Foster rats (6 weeks, 270±10 g) that were inbred at the Central Animal House of Banaras Hindu

University (Registration No. 542/AB/CPCSEA) were used for the experiments. Animals were kept under standard laboratory conditions. Rats were fasted overnight and maintained at 12-h day/night cycle.

## Dose optimization of piroxicam and experimental design

Piroxicam was dissolved in normal saline and administered i.p. 30 min prior to middle cerebral artery (MCA) occlusion. Pretreatment of untreated animal model with different concentrations of piroxicam revealed that 10-mg/kg body weight i.p. was the optimum dose as revealed by cerebral infarct volume and neurological score [8]. The diagrammatic representation of the experimental design has been mentioned in Fig. 1. Randomization and exclusion criteria were taken into consideration to assign groups and their findings.

Total no. of animals used in this study were 111. Fourteen time points were selected to optimize the base line for both glutamate and GABA in vehicle and drug-treated vehicle (in triplicate). For TTC staining, untreated and treated rats were again considered in triplicate. To measure the level of oxidative stress and effect of piroxicam on the level antioxidants following ischemic stroke, rats were considered in triplicate. Those rats($n$=8) were excluded from the study which either did not show significant reduction in cerebral blood flow by 70 % or they died during surgery or after surgery before completing the experimental time frame.

Detailed stratification of animals in all end points are as follows: control=6, untreated=6, and treated=84(HPLC) +7 (total=103). For glutamate estimation in triplicate (14 time points)=42. For GABA estimation in triplicate (14 time points)=42. TTC staining (control)=(3–1=2); untreated=(3–1=2); treated=(3–0)=3 [$n$=9] [2 excluded from study]. OH and DHBA estimation (control)=(3–1=2); untreated=(3–1=2); treated=(3–1=2) [$n$=9] [3 excluded from study]. Antioxidant estimation (control)=(3–1=2); untreated=(3–1=2); treated=(3–1=2) [$n$=9] [3 excluded from study]. Total=42+42+7+6+6=103; excluded=8.

## Induction of focal cerebral ischemia in rat

Focal cerebral ischemia was induced by MCA occlusion (MCAo) with modified intraluminal technique [30]. Rats were anesthetized by the administration of a combination of ketamine (75 mg/kg b.w.) and xylazine (10 mg/kg b.w.) and then transferred to the surgical table with a heating pad (INCO, India) to maintain a constant body temperature of 37± 0.5 °C. Rat was placed in a supine position with forelimbs fixed on the table by tape, and the fur on the ventral neck was shaved, and the skin was cleaned by 0.5 % butadiene and 75 % alcohol. Eye cream was applied to protect corneal drying. Midline incision in the neck on ventral side was done to expose the left common carotid artery (CCA). A 5.0-cm length 3-0 silicon-coated nylon monofilament suture was introduced into the carotid artery (CA) lumen through a small nick and gently pushed from internal carotid (ICA) lumen to block the origin point of MCA. Approximately 18–22-mm length of nylon filament was inserted to reach the MCA blockade site from the bifurcation point. The external CA (ECA) stump was clamped around the intraluminal nylon suture to prevent bleeding. Reperfusion was done by gently removing the filament after 1 h of ischemia. Animals were allowed to recover from anesthesia and, on regaining the righting reflex, were transferred to polypropylene cages in the animal room with temperature maintained at 26±2.5 °C with food and water supply in ad libitum. In control animals, all the procedures were carried on except the insertion of nylon filament. Doppler monitoring (AD, USA) showed that all rats subjected to MCAO and cortical cerebral blood flow were reduced by at least 70 % of preischemic values within 5 min of advancing the filament and induction of MCAO (data not shown). Rats not exhibiting significant reduction in cerebral blood flow by 70 % were excluded from the study.

## Neurological score

Neurological scores were derived on five-point scale with 10 grading scores: A score of 0 indicated no neurological deficit; a score of 1 means failure to extend opposite forepaw fully, a score of 2 was assigned when contralateral circling was seen. While the rat which was not able to grip the wire meshes and fell on the contralateral side of brain damage, a score of 3 was assigned. Further, when the rats were unable to walk spontaneously and had a depressed level of consciousness, they were given

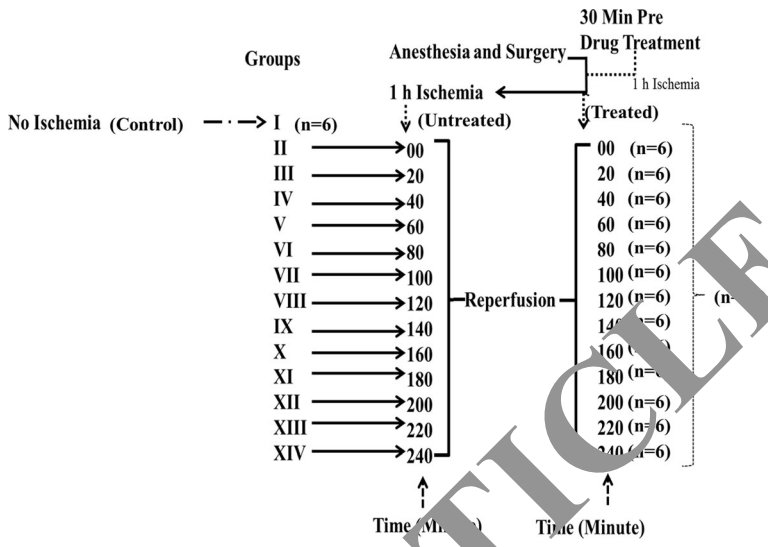


**Fig. 1** Experimental design

a score of 4. The neurobehavioral scores obtained after testing on each scale were averaged to denote the degree of neurological deficit [30].

Physiological parameters

Rectal temperature of the rats were measured at an ambient temperature of 21.5±1 °C with a lubricated digital thermometer probe (Panlab) inserted 3 cm into the rectum, the rat being lightly restrained by holding in the hand. Temperature was recorded before any drug treatment and thereafter every 60 min up to 8 h. Probes were reinserted from time to time until the temperature stabilized. Arterial blood parameters (pH, $PaCO_2$, $PaO_2$) and MAP (Diagno, India) were monitored in all the animals starting at 30 min preischemia and continuously throughout the experiment until 120 min of postischemia in those rats which underwent MCAO and drug treatment, but no significant differences between the experimental groups were observed as shown in Table 1.

Measurement of infarct volume

Rat brains were perfused with normal saline. The brains were immediately transferred to −20 °C after removal of the cerebellum. The frozen brains were sliced into uniform coronal sections of 2-mm thickness for TTC staining. The brain slices were incubated in TTC (1 %) followed by 10 % formalin overnight. Viable brain tissues became brick red after TTC staining whereas infarcted portion of brain tissue remains unstained (appeared white). The infarcted areas were captured and quantified using image analysis software (NIH Image J) with optical setup [30, 46]. The area of infarction was measured by subtracting the area of the lesioned hemisphere from that of the nonlesioned contralateral hemisphere. The volume of infarction was calculated by integration of the lesion areas [47].

Measurement of ·OH by high-performance liquid chromatography-electrochemical detector (HPLC-ECD)

We used the salicylate-trapping method to detect extracellular ·OH by measurement of the stable adducts 2,3- and 2,5-dihydroxybenzoic acid (DHBA) in the brain dialysates [29]. Salicylic acid (SA) 100 mg/kg i.p. can trap the ·OH generated in vivo, and the hydroxylation products 2,3-DHBA and 2,5-DHBA indicate the quantity of ·OH generated in the brain which ensures the oxidative stress produced following cerebral ischemia [51].

Estimation of antioxidant status

Superoxide dismutase (SOD) activity was assayed using an indirect inhibition assay, in which xanthine and xanthine oxidase serve as a superoxide generator, and nitro blue tetrazolium (NBT) is used as a superoxide indicator. The assay mixture consisted of 960 μl of 50-mM sodium carbonate buffer (pH 10.2) containing 0.1 mM

**Table 1** General physiological parameters observed during experiment

| Preischemia | Ischemia | Postischemia | 30 min | 60 min | 120 min |
|---|---|---|---|---|---|
| maBP mmHg | 125±2.0 | 118±3.0 | 124±4.0 | 127±3.0 | 115±2.0 |
| $PaCO_2$ mmHg | 38±0.5 | 39.6±0.5 | 38.5±1.53 | 38.3±1.32 | 37.8±0.32 |
| pH | 7.39±0.004 | 7.375±0.013 | 7.392±0.012 | 7.384±0.018 | 7.4±0.002 |
| $PaO_2$ mmHg | 159±13 | 161±9.0 | 151±6.0 | 148±10.0 | 154±12.0 |

xanthine, 0.025 mM NBT, and 0.1 mM EDTA, 20 μl of xanthine oxidase, and 20 μl of the supernatant of brain tissue homogenate. Changes in absorbance were observed spectrophotometrically at 560 nm. The activity was expressed as units/min/mg protein. Catalase activity was assayed by measuring the rate of decomposition of hydrogen peroxide at 240 nm. The assay mixture consisted of 50 μl of 1 M Tris-HCI buffer (pH 8.0) containing 5 mM EDTA, 900 μl of 10 mM $H_2O_2$, 30 μl of MQ water, and 20 μl of the supernatant of brain tissue homogenate. The rate of decomposition of hydrogen was observed spectrophotometrically at 240 nm. The enzyme activity was expressed as nmol of hydrogen peroxide decomposed/min/mg protein [39, 1]. Glutathione (GSH) levels in the brain tissue homogenate were determined fluorimetrically by the method of Jollow [25]. The glutathione peroxidase activities were determined in tissue using glutathione peroxidase assay kits as per manufacturer instructions.

## Estimation of glutamate and GABA in discrete brain areas by HPLC-ECD

Rats were euthanized by decapitation at different time points as most relevant to significant neurochemical changes. Extracted brains were immediately dissected into cortex and striatum from the hemisphere. Dissected regions were stored at −80 °C until the time of analyses.

*O*-pthaldialdehyde (OPA) is used as derivatizing agent. OPA of 22 mg is dissolved in 0.5 ml of sodium sulphite (1 M) to which 0.5 ml of absolute alcohol and 9.0 ml of sodium tetraborate buffer (0.1 M) are added, adjusted to pH 10.4 with 5 M NaOH. Sodium tetraborate buffer (0.1 M): Add 20.12 g granular anhydrous sodium tetraborate or 38.14 g sodium tetraborate decahydrate (borax) to 1,000 ml deionized water. Stir to dissolve. The pH is adjusted to 10.4 with 5 M NaOH. Derivatizing agent of 20 μl is mixed with 1 ml of amino acid standards. After 10-min incubation, 20 μl of the samples is injected into the HPLC (Waters, USA). For the biogenic amines, prepare 40-mM stock solutions. This is diluted hundred times, and of this solution, 10 μL (4 pmole) is injected into the HPLC as standard. For HPLC analysis of glutamate and GABA, the brain tissue (5 to 8 % of cortex and striatum) homogenate was made in 0.1 M $HClO_4$ containing 0.01 % EDTA. After sonication, samples are kept on ice for 20 min for protein precipitation. Samples are then centrifuged at 17,500×*g* for 10 min at 4 °C. Twenty microliters of the supernatant is mixed with 0.4 μl of the derivatizing agent and incubated at RT for 10 min. Ten microliters is injected into the HPLC. For the amino acids, 0.001 M solution is used as stock solutions. From this, 10 μl is injected into the HPLC as standard.

Calibration curve was obtained by running standards. Chromatograms were compared to that of standard. Amount of neurotransmitter was estimated as per method reported by Shankaranarayana et al. [9]

## Trypan blue exclusion test for cell viability

This test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not possess this ability. Hence, following cerebral ischemia, the dead cells would have altered membrane permeability, thereby facilitating the entry of this dye into the cell and staining the cytoplasm blue, and the live cells would have a clear cytoplasm.

This test was performed by adding trypan blue into control normoxic neuronal plates containing NB medium or ischemia-exposed neuron plates containing Locke's buffer. Following 3–5-min incubation with trypan blue, the cells in the culture plates were fixed with 4 % buffered formaldehyde and counted under a normal light microscope. In each field, the dead and total numbers of cells were counted, and their ratio provided an estimate of percentage cell death.

Statistical analysis

Data are represented as mean±SD. Statistical significance was analyzed using one-way ANOVA followed by Tukey's post hoc test. A $P$ value of <0.05 was considered significant.

## Results

Effect of piroxicam on cerebral infarction

It was found that ischemic infarction covered the cerebral cortex in vehicle rats. A significant reduction in infraction area was found in cerebral cortex in treated rats. Quantitatively, the infarction volume and neurological deficit score in the treated rats were found significantly lesser than that of untreated rats. The mean of infarct volume was found 192.24±12.3 $mm^3$ in untreated rats whereas 42.98±3.3 $mm^3$ in treated rats (Fig. 2a) while improved neurological deficit was also observed in treated group (Fig. 2b) (optimization of pretreatment and posttreatment regime data not shown). Representative images of neuronal survival and cell death of cortical neurons control, untreated, and treated following focal cerebral ischemia are shown in Fig. 3. Dead neurons were permeable to trypan blue, staining the cytoplasm whereas the live cells have a clear cytoplasm.

Effect of piroxicam on in vivo ·OH formation

Salicylate hydroxylation was shown as an in vivo marker of the ·OH formation by Chiueh [12]. In our studies, it was observed that SA given 30 min following cerebral ischemia, and animals sacrificed 2 h after SA administration, could produce a considerable amount of 2,3-DHBA and 2,5-DHBA, the most reliable indicator of ·OH adduct as assayed by HPLC with electrochemical detection [22].

We observed that treated group had a reduced level of these biomarkers as compared to that of untreated as shown in Table 2. This clearly justifies that piroxicam potentiates the intrinsic antioxidative mechanism to bring about neuroprotection.

Effect of piroxicam on antioxidant level

The changes in antioxidant levels during focal ischemia are shown in Table 3. There was a significant decrease in GSH levels ($P$<0.05) in the cortex and striatum in untreated group; however, in the presence of piroxicam, higher levels of GSH were observed. Further, there was a significant decrease in GPx ($P$<0.05) activities untreated, while treatment with piroxicam restored $GP_X$ levels toward the control values. The present results showed that piroxicam pretreatment increased the activities of SOD and catalase. Acute treatment of piroxicam caused a significant enhancement in the specific activity of SOD and catalase in both cortex and striatum.

Effect of piroxicam on the level of glutamate and GABA

Figure 4 shows the effect of piroxicam administration on monoamines and amino acid neurotransmitter levels in two brain regions, frontal cortex and striatum in untreated rat groups. The data demonstrate that glutamate and GABA levels in frontal cortex and striatum increased significantly in treated rat groups.

We have found that the level of glutamate and GABA increases instantly after the stroke and glutamate reach maximum at 80 min whereas GABA at 120 min. Our finding resembles with the finding of Ouyang et al. [40]. To our observation, we found that piroxicam administration significantly ($P$<0.05) decreased glutamate by 34 %. The peak value of glutamate measured at 80 min of treated group was 54 % (256±62.3 μM vs 484.3±166.6 μM) to that of the untreated or in other words piroxicam reduced 48 % of glutamate release shown in Fig. 5.

On the other hand, however, GABA concentration during ischemia was also remarkably increased. GABA may protect neurons not only by directly hyperpolarizing neurons but also by exerting an inhibitory influence on glutamate-mediated neuronal activity, as suggested by this and many other studies [13]. Although the increase in extracellular GABA (12.1-fold) in the cortex and striata (10.09 fold) from baseline to ischemia induced by MCAO was much greater than that of glutamate (4.9-fold), the peak level of GABA concentration (2.02±0.39 μM) after ischemia was only one third to that of glutamate (6.52±1.9 μM). Therefore, it was not unreasonable to use piroxicam to examine the effect of GABA on regulation of glutamate release as shown in Figs. 6 and 7.

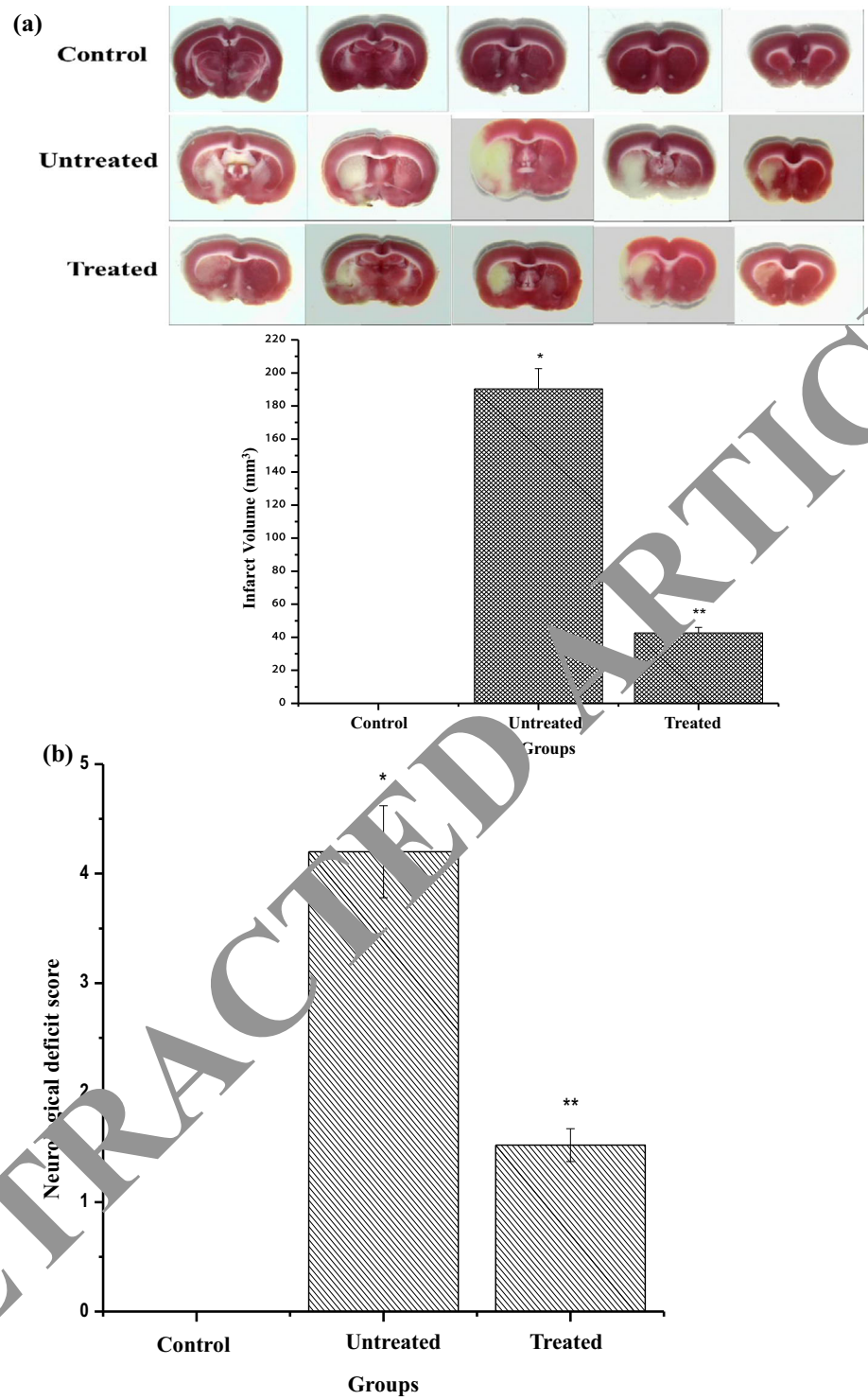




**Fig. 2** 2,3,5-Triphenyltetrazolium chloride (TTC) staining for detection of brain infarct and functional assessments by neurological deficit scoring. **a** TTC-stained coronal brain sections of control, untreated, and treated rats (**b**) neurological deficit score

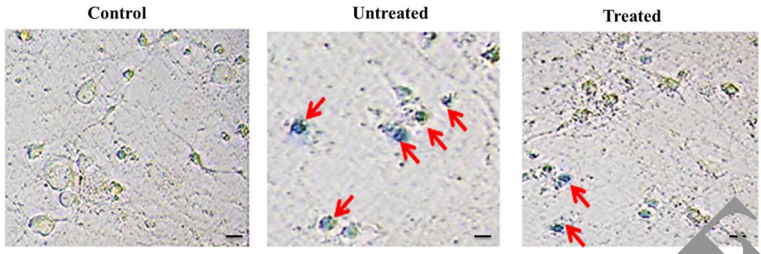


**Fig. 3** Representative images of neuronal cell death in control, untreated, and treated rats (piroxicam) following focal cerebral ischemia. *Scale bar*= 5 μm

Effect of piroxicam on the ratio of Glu to GABA

The ratio of Glu to GABA reflects the balance between the level of cerebral excitatory amino acids and inhibitory amino acids in the brain, which is stable in normal situation. The result of our finding showed that the ratio of Glu to GABA in cerebral striatum was 2.92 before ischemia. After ischemia, the ration of Glu to GABA rose and reached to the maximal level at 4.95 after 100 min ischemia, which is a significant increase as compared to that of the ratio before ischemia. The ratio gradually decreased afterward and reached toward the minimum at 240 min after ischemia, which was an increase as compared to that of the ratio before ischemia. The ration of Glu to GABA in drug-treated group was remarkably reduced. The results provided the evidence that piroxicam could inhibit Glu level more than GABA under the circumstance of cerebral ischemia (Fig. 4).

## Discussion

The glutamate/GABA study was undertaken on the basis of past studies by Costa et al. [13]. Reports suggested that NSAIDs have a GABA agonism property but has no influence on glutamate [14]. The values of glutamate and GABA prior to MCAo were 1.39±0.34 and 0.18±0.12 μM, respectively. Following MCAo, the peak values were 6.52±1.9 μM for glutamate at 80 min and 2.02±0.39 μM for GABA at 120 min. Although the time needed for a rise in the peak level of glutamate was much longer than the time reported by some studies [45], our results were similar to those of some other studies [10]. This discrepancy may be due to ischemia by different methodology or different insult zone.

As reported previously, neither GABAa nor GABAb receptor antagonists were used to block GABAa or GABAb influence on glutamate level; hence, it was resolved that only GABAa agonist may bring changes, if administered [41]. Further, studies reported that exogenous GABA significantly decreased glutamate release; hence, on this basis, we tried to find a relation between NSAID and its role as GABA agonist. To our observation, we found that piroxicam administration decreased 34 % glutamate. The peak value of glutamate measured at 80 min of piroxicam administration was 54 % (261.52 ±62.3 vs 484.3±166.6) to that of the untreated group. In other words, piroxicam reduced as much as 48 % of glutamate release. Infusion of piroxicam during ischemia decreased glutamate release at 80 min as much as by 54 % (230±53.2 % vs 484.3±166.6 %) with a peak value of glutamate being only 46.2 % to that of the ischemic group without piroxicam administration. This indicates that piroxicam can significantly downregulate glutamate release from ischemic rat cortex and striatum.

One of the probable mechanisms for the enhancement of inhibitory synaptic transmission in neurons after ischemia is that presynaptic GABAa receptors help increase GABA release. Large accumulation of GABA in the extracellular space has been demonstrated by in vivo and in vitro studies during ischemia and early after reperfusion. It is known that GABA release is initially by exocytosis and later by reversed uptake through GABA transporters [2, 19]. The initial exocytosis is due to the depolarization by the rundown of Na+ −K+−ATPase. The activation of presynaptic GABAa receptors increases the chloride concentration in the

**Table 2** Effect of piroxicam on in vivo. OH formed in the cortex and striatum

| Group | 2,3-DHBA (pmol/mg) | | 2,5-DHBA (pmol/mg) | |
|---|---|---|---|---|
| | Cortex | Striatum | Cortex | Striatum |
| Control | 0.089±0.12 | 0.051±0.05 | 0.43±0.32 | 0.21±0.01 |
| Untreated* | 0.23±0.04 | 0.15±0.10 | 0.95±0.24 | 0.53±0.42 |
| Treated** | 0.12±0.16 | 0.081±0.54 | 0.51±0.05 | 0.32±0.14 |

Data represented as mean±S.D and $P<0.05$ was considered significant; * versus control and ** versus untreated

**Table 3** Effect of piroxicam on antioxidant level in the cortex and striatum

| Group | GSH (μg/g of wet tissue) | | GPx (U/mg protein) | | Catalase (U/mg protein) | | SOD (U/mg protein) | |
|---|---|---|---|---|---|---|---|---|
| | Cortex | Striatum | Cortex | Striatum | Cortex | Striatum | Cortex | Striatum |
| Control | 9.88±0.12 | 7.89±0.15 | 9.52±1.32 | 8.02±1.0 | 2.70±0.32 | 2.32±0.21 | 3.74±0.15 | 2.012±0.1 |
| Untreated* | 4.32±0.05 | 3.39±1.12 | 5.86±1.24 | 4.32±0.23 | 1.25±0.21 | 1.05±0.11 | 1.42±0.12 | 1.02±0.11 |
| Treated** | 6.82±0.08 | 5.84±1.01 | 7.21±1.15 | 6.42±0.21 | 2.32±0.58 | 2.01±0.31 | 2.65±0.22 | 1.[illegible]±0.[illegible] |

Data represented as mean±S.D and $P<0.05$ was considered significant; * versus control and ** versus untreated

presynaptic terminals. GABA release happens when chloride reversal potential is more positive than the resting membrane potential. Based on this study, the large accumulation of GABA might be, at least in part, due to the activation of presynaptic GABAa receptors located on the GABAergic terminals. In addition, GABAb receptors are activated under ischemic injury, which results in depressed synaptic activity [27]. Whether GABA release will be facilitated or depressed is determined by the interplay among the extent of energy rundown, presynaptic GABAa receptors (inhibitory or excitatory), and presynaptic GABAb receptors. This also explains why GABA and glutamate accumulation only occurs during ischemia and early after reperfusion but not long lasting. Thus, the present study provides a new way to explain the relationship among GABA, GABAa, or GABAb receptors, and GABAergic synaptic transmission after ischemia. The present study could not directly demonstrate that the facilitation of inhibitory synaptic transmission will reduce excitotoxicity and thus protect surviving neurons, considering that excitotoxicity is the major mechanism for postischemic neuronal death. Since some striatal neurons survive ischemia, they must have undergone little or no excitotoxicity. Previous studies showed that neurons have depressed excitatory synaptic transmission and depressed excitability after ischemia [43, 15]. But, we could not equal the depressed excitability to the depressed excitotoxicity. Whether facilitation of inhibitory synaptic transmission in neurons is directly linked to depressed excitotoxicity or by some other mechanisms still needs further investigation.

Further, the intrinsic ·OH free radical scavenging action of piroxicam as revealed by the salicylate-

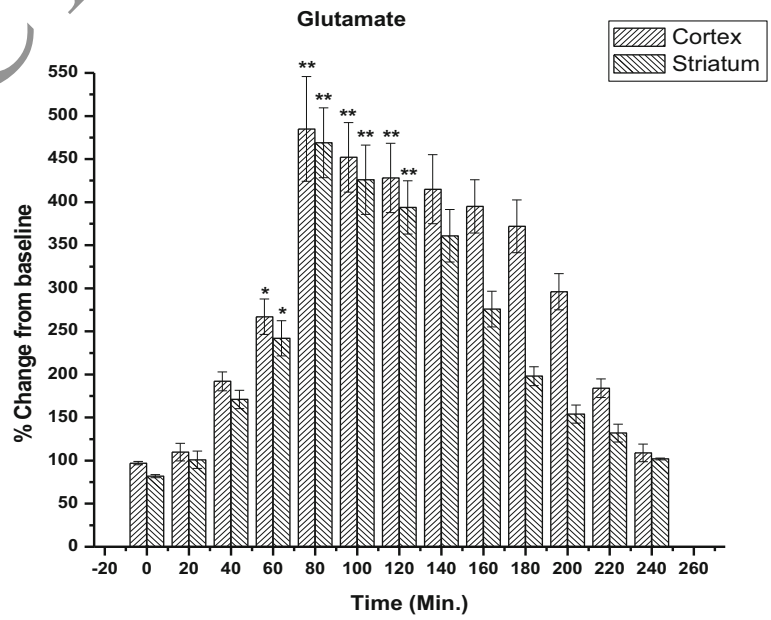


**Fig. 4** Time course optimization of glutamate release in cortical and striatal region of untreated rats. Data represent the mean±SD of a percentage change from baseline, which is the mean of the first two samples versus each corresponding value in the [illegible] group

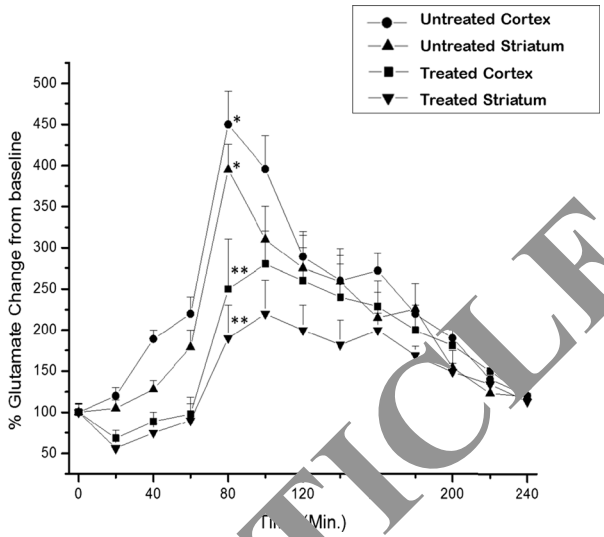


**Fig. 5** Effect of piroxicam on glutamate release. Piroxicam practicably decreased glutamate level in the rat cortex and striatum after occlusion of MCA from basal level. Each point is the mean±S.D. compared with untreated group

trapping method to detect extracellular ·OH by measurement of the stable adducts 2,3- and 2,5- DHBA in the dialysates was also a clue to justify its role in reducing oxidative stress in the striatal environment conducive to the production of oxidants due to ischemic insult (Table 2).

There lies a remarkable complexity of interaction between brain endothelial cells and parenchymal cells, antioxidant enzyme system in capillary endothelial cells also insight an important role in controlling the hypoxic ischemic brain injury [29]. Using a cell culture model of the blood–brain barrier (BBB), some authors have investigated the brain capillary endothelial cell (especially antioxidative defense system), response to hypoxia [45]. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) belong to the members of enzymatic antioxidative defense mechanisms against reactive oxygen species (ROS) and protect macromolecules, cells, and cell membranes from peroxidative damage [22]. SOD catalyzes the dismutation of superoxide anion radical into oxygen and hydrogen peroxide that in turn, can be removed by GSH-Px and catalase (CAT) [11].

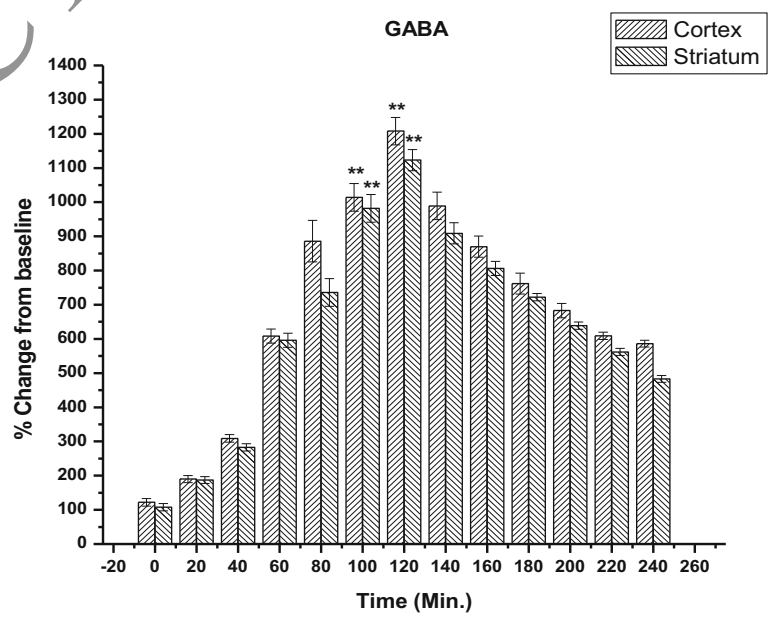


**Fig. 6** Time course optimization of GABA release in cortical and striatal region of untreated rats. Data represent the mean±S.D. of a percentage change from baseline, which is the mean of the first two samples versus each corresponding value in the group

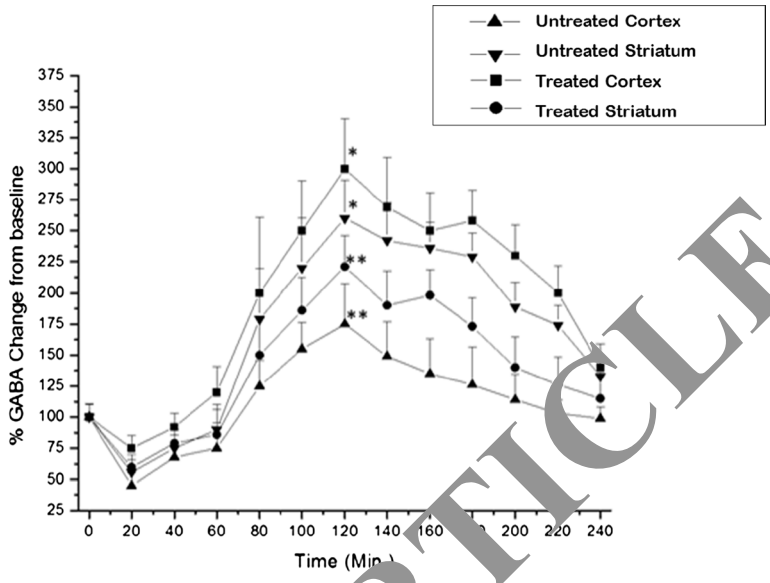


**Fig. 7** Effect of piroxicam on GABA release. Piroxicam practicably increased the level of GABA in the rat cortex and striatum after occlusion of MCA from baseline. Each point is the mean±S.D. compared with untreated group

ROS are unavoidable products of metabolism, and in excess, they can be dangerous for tissues and cells. SOD and GSH-Px, among others, protect tissues and cells against ROS. In our study, we observed that with the induction of ischemic–reperfusion tissue injury, total SOD, catalase, GPx, and GSH reductase activity in brain decreased, which is also in accordance with the past studies [11]. Their results demonstrated a consistent decrease in the activities of SOD, CAT, GPx, and GSH reductase, suggesting the increase in the susceptibility of BBB to oxidative damage during reoxygenation. Our data shows that piroxicam in the optimized dose was able to potentiate the intrinsic antioxidant defense system by increasing the intrinsic antioxidant status which may be one of the most potential properties of piroxicam that can be explored in cerebral ischemia (Table 3).

In the present study, the facilitation of inhibitory synaptic transmission by piroxicam protects striatal neurons against ischemic insults. However, we could not prove the causal link between facilitation of inhibitory synaptic transmission and the selective survival of neurons after ischemia. This study has demonstrated that excessively released glutamate during ischemic stimulation can be diminished by the enhanced activity of presynaptic GABA release. Our work provides a new insight into understanding of the interactions of GABA and glutamate in cortex and striatum during ischemia.

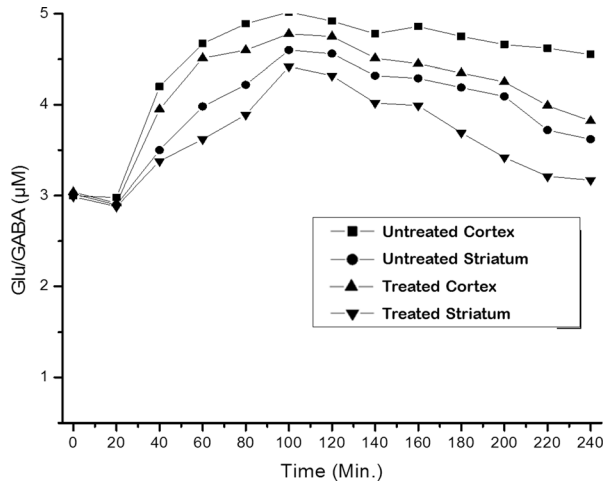


**Fig. 8** Effects of piroxicam (10 mg/kg) on ischemia-reperfusion evoked changes in the ratio of Glu to GABA in cortex and striatum

Our results demonstrate that pretreatment of piroxicam provides preconditioning of the brain to combat ischemia induced increase in extracellular glutamate and oxidative stress. It promotes inhibition of the extracellular glutamate by enhancing GABA level and also decreases the oxidative stress. This property of piroxicam can be explored for future novel therapeutics with other antistroke therapy available till date.

## References


1. Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126
2. Allen NJ, Rossi DJ, Attwell D (2004) Sequential release of GABA by exocytosis and reversed uptake leads to neuronal swelling in simulated ischemia of hippocampal slices. J Neurosci 24:3837–3849
3. Arundine M, Tymianski M (2004) Molecular mechanisms of glutamate dependent neurodegeneration in ischemia and traumatic brain injury. Cell Mol Life Sci 61:657–668
4. Bhattacharya P, Pandey AK, Paul S, Patnaik R (2012) Cognitive effects of NSAIDs in cerebral ischemia: a hypothesis exploring mechanical action mediated pharmacotherapy. Med Hypotheses 79(1):393–395
5. Bhattacharya P, Pandey AK, Paul S, Patnaik R (2012) Combination therapy of Ifenprodil with Piroxicam may be an effective therapeutic intervention in cerebral stroke: a hypothesis. Med Hypotheses 79(1):516–518
6. Bhattacharya P, Pandey AK, Paul S, Patnaik R (2012) Neuroprotective potential of Piroxicam in cerebral ischemia: an in-silico evaluation of the hypothesis to explore its therapeutic efficacy by inhibition of aquaporin-4 and acid sensing ion channel 1a. Med Hypotheses 79(1):352–357
7. Bhattacharya P, Pandey AK, Shukla S, Paul S, Patnaik R (2013) Neuroprotection by μ-calpain and matrix metalloproteinases inhibition by Piroxicam in cerebral ischemia: an in silico study. Mol Chem Res 22(11): 5112–5119
8. Bhattacharya P, Pandey AK, Paul S, Patnaik R, Yavagal D (2013) Aquaporin-4 inhibition mediates Piroxicam-induced neuroprotection against focal cerebral ischemia/reperfusion injury in rodents. PLoS One 8(9):e73481.1371
9. Shankaranarayana Rao BS, Raju TR, Meti BL (1998) Self-stimulation of lateral hypothalamus and ventral tegmentum increases the levels of noradrenaline, dopamine, glutamate, and AChE activity, but not 5- Hydroxytryptamine and GABA levels in hippocampus and motor cortex. Neurochem Res 23(8):1053–1059
10. Butcher SP, Bullock R, Graham DI, McCulloch J (1990) Correlation between amino acid release and neuropathologic outcome in rat brain following middle cerebral artery occlusion. Stroke 21:1727–1733
11. Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527–535
12. Chiueh CC, Miyake H, Peng MT (1993) Role of dopamine autoxidation, hydroxyl radical generation, and calcium overload in underlying mechanisms involved in MPTP-induced parkinsonism. Adv Neurol 60:251–258
13. Costa C, Leone G, Saulle E, Pisani F, Bernardi G, Calabresi P (2004) Coactivation of GABA(A) and GABA(B) receptor results in neuroprotection during in vitro ischemia. Stroke 35:596–600
14. Coyne L, Jiping S, Patten D, Halliwell RF (2007) Characterization of the interaction between fenamates and hippocampal neuron GABAA receptors. Neurochem Int 51(6–7):440–446
15. Deng P, Zhang Y, Xu ZC (2008) Inhibition of Ih in striatal cholinergic interneurons early after transient forebrain ischemia. J Cereb Blood Flow Metab 28:939–947
16. Djuricic B (2002) Glutamate in brain: transmitter and poison. Glas Srp Akad Nauka 47:55–76
17. Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22(9):391–397
18. Fujimoto S, Katsuki H, Kume T, Kaneko S, Akaike A (2004) Mechanisms of oxygen glucose deprivation induced glutamate release from cerebrocortical slice cultures. Neurosci Res 50:179–187
19. Globus MY, Busto R, Dietrich WD, Martinez E, Valdes I, Ginsberg MD (1988) Effect of ischemia on the in vivo release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. J Neurochem 51: 1455–1464
20. Green AR, Hainsworth AH, Jackson DM (2000) GABA potentiation: a logical pharmacological approach for the treatment of acute ischemic stroke. Neuropharmacol 39:1483–1494
21. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T (2009) Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke 40: 1849–1857
22. Halliwel B (1987) Oxidants and human disease: some new concepts. Fed Am Soc Exp Biol 1:358–366
23. Heiss WD, Grond M, Thiel A, von Stockhausen HM, Rudolf J, Ghaemi M, Löttgen J, Stenzel C, Pawlik G (1998) Tissue at risk of infarction rescued by early reperfusion: a positron emission tomography study in systemic recombinant tissue plasminogen activator thrombolysis of acute stroke. J Cereb Blood Flow Metab 18:1298–1307
24. Hauss-Wegrzynizk B, Vraniak P, Wenk GL (1999) The effects of a novel NSAID on chronic neuroinflammation are age dependent. Neurobiol Aging 20(3):305–313
25. Jollow DJ, Mitchell JR, Zampaglione N (1974) Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 11:151–169
26. Jin R, Yang GJ, Li GH (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol 87: 779–789
27. Kuramoto N, Wilkins ME, Fairfax BP, Revilla-Sanchez R, Terunuma M, Tamaki K, Iemata M, Warren N, Couve A, Calver A (2007) Phospho-dependent functional modulation of GABA(B) receptors by the metabolic sensor AMP-dependent protein kinase. Neuron 53:233–247
28. Hong K-S, Saver JL (2009) Quantifying the value of stroke disability outcomes WHO global burden of disease project

disability weights for each level of the modified rankin scale. Stroke 40:3828–3833
29. Lievre V, Bechuwe P, Bianchi A, Bossenmeyer P, Koziel V, Franck VP, Nicolos P, Dauca MBM, Daval JC (2001) Intracellular generation of free radicals and modifications of detoxifying enzymes in cultured neurons from the developing rat forebrain in response to transient hypoxia. Neuroscience 105:287–297
30. Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84–91
31. Louzada PR, Lima AC, Mendonca-Silva DL, Noel F, Mello FG, Ferreira ST (2004) Taurine prevents the neurotoxicity of beta-amyloid and glutamate receptor agonists: activation of GABA receptors and possible implications for Alzheimer's disease and other neurological disorders. FASEB J 18:511–518
32. Luo C, Guo LJ (2005) Effect of taurine on acute ischemia-induced neuron apoptosis in rats. Chin Pharmacol Bull 90: 1057–1061
33. Matsumoto N, Kumamoto E, Furue H, Yoshimura M (2003) GABA-mediated inhibition of glutamate release during ischemia in substantia gelatinosa of the adult rat. J Neurophysiol 89:257–264
34. Mergenthaler P, Dirnagl U, Meisel A (2004) Pathophysiology of stroke: lessons from animal models. Metab Brain Dis 19: 151–167
35. Nakagomi T, Sasaki T, Kirino T, Tamura A, Noguchi M, Saito I, Takakura K (1989) Effect of cyclooxygenase and lipoxygenase inhibitors on delayed neuronal death in the Gerbil Hippocampus. Stroke 20:925–929
36. Nelson RM, Green AR, Lambert DG, Hainsworth AH (2000) On the regulation of ischaemia-induced glutamate efflux from rat cortex by GABA; in vitro studies with GA[illegible] clomethiazole and pentobarbitone. Br J Pharmacol 130: 1124–1130
37. Ng TK, Yung KK (2001) Differential expression of GABA (B) R1 and GABA (B) R2 receptor immunoreactivity in neurochemically identified neurons of the rat neostriatum. J Comp Neurol 433:458–470
38. Nishizawa Y (2001) Glutamate release and neuronal damage in ischemia. Life Sci 69:369–381
39. Oberley LW, Spitz DR (1984) Assay of superoxide dismutase activity in tumor tissue. Methods Enzymol 105:457–464
40. Ouyang CH, Guo LJ, Liu Q, Qu L (2004) Effect of -aminobutyric acid on amino acids and calcium levels in rat brain of acute incomplete global cerebral ischemia. Chin J Pharmacol Toxicol 18:248–252
41. Ouyang C, Ouyang LC, Guoa L, Lua Q, Xua X, Wang H (2007) Enhanced activity of GABA receptors inhibits glutamate release induced by focal cerebral ischemia in rat striatum. Neurosci Lett 420:174–178
42. Okun E, Griffioen KJ, Lathia JD, Tang SC, Mattson MP, Arumugam TV (2009) Toll-like receptors in neurodegeneration. Brain Res Rev 59:278–292
43. Pang ZP, Deng P, Ruan YW, Xu ZC (2002) Depression of fast excitatory synaptic transmission in large aspiny neurons of the neostriatum after transient forebrain ischemia. J Neurosci 22: 10948–10957
44. Pende M, Lanza M, Bonanno G, Raiteri M (1993) Release of endogenous glutamic and asparatic acids from cerebrocortical synaptosomes and its modulation through activation of -aminobutyric acidB (GABAB) receptor subtypes. Brain Res 604:325–330
45. Phillis JW, O'Regan MH (2003) Characterization of modes of release of amino acids in the ischemic/reperfused rat cerebral cortex. Neurochem Int 43:461–467
46. Smith WS (2004) Pathophysiology of focal cerebral ischemia: a therapeutic perspective. J Vasc Interv Neurol 15:S3–S12
47. Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10:290–293
48. Saver JL, Albers GW, Dunn B, Johnston KC, Fisher M (2009) Stroke Therapy Academic Industry Roundtable (STAIR) recommendations for extended window acute stroke therapy trials. Stroke 40:2594–2600
49. Tanaka E, Niiyama S, Uematsu K, Yokomizo Y, Higashi H (2002) The presynaptic modulation of glutamate release and membrane dysfunction induced by in vitro ischemia in rat hippocampal CA1 neurons. Life Sci 72:363–374
50. Tanaka S, Tsuchida A, Kiuchi Y, Oguchi K, Numazawa S, Yoshida T (2003) GABAergic modulation of hippocampal glutamatergic neurons: an in vivo microdialysis study. Eur J Pharmacol 465:61–67
51. Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173–183
52. Tuttolomondo A, Di Raimondo D, di Sciacca R, Pinto A, Licata G (2008) Inflammatory cytokines in acute ischemic stroke. Curr Pharm Des 14:3574–3589
53. Vartiainen N, Huang CY, Salminen A, Goldsteins G, Chan PH, Koistinaho J (2001) Piroxicam and NS-398 rescue neurones from hypoxia/reoxygenation damage by a mechanism independent of cyclo-oxygenase inhibition. J Neurochem 76:480–489
54. Wang Q, Tang XN, Yenari MA (2007) The inflammatory response in stroke. J Neuroimmunol 184:53–68
55. Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang XY, Berg GI, Koziol JA (2007) Microglial activation and matrix protease generation during focal cerebral ischemia. Stroke 38:646–651