Neuroprotective effects of GYKI 52466 on experimental spinal cord injury in rats

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Object. The toxic effects of glutamate in the central nervous system are well known. This neurotoxicity occurs through metabotropic and ionotropic receptors, the latter group composed of N-methyl-D-aspartate, α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA), and kainate receptors. The authors investigated the neuroprotective effects of GYKI 52466, a 2,3-benzodiazepine that is a selective and potent AMPA receptor antagonist, in a rat spinal cord trauma model.

Methods. Sixty Wistar albino rats were studied in three groups of 20 animals each: sham-operated controls (Group 1); spinal cord-injured rats (Group 2); and spinal cord-injured plus GYKI 52466-treated rats (Group 3). In Groups 2 and 3, spinal cord injury (SCI) was induced at the thoracic level by applying an aneurysm clip to the cord for 1 minute. One minute after the clip was removed, the rats in Group 3 received an intraperitoneal injection of 15 mg/kg GYKI 52466. Responses to injury and treatment were evaluated based on biochemical parameters (lipid peroxidation and adenosine 5'-triphosphate [ATP] levels in tissue), and on light and transmission electron microscopy findings in cord tissue collected at different times post-SCI. Five rats from each group underwent assessment of functional recovery at 1, 3, and 5 days after SCI; evaluation was performed using the inclined-plane technique and Tarlov motor grading scale.

The mean lipid peroxidation levels in Groups 1 and 2 were 21.73 ± 4.35 and 35.53 ± 2.99 nmol/g of wet tissue, respectively. The level in Group 3 was 27.98 ± 3.93 nmol/g of wet tissue, which was significantly lower than that in Group 2 (p < 0.01). The mean ATP levels in Groups 1 and 2 were 166.21 ± 25.57 and 41.72 ± 12.28 nmol/g of wet tissue, respectively. The ATP level in Group 3 was 85.82 ± 13.92 nmol/g of wet tissue, which was significantly higher than that in Group 2 (p < 0.01). Light microscopic examination of Group 2 tissues showed hemorrhage, necrosis, polymorphonuclear leukocyte infiltration, and vascular thrombi. In contrast, the examination of Group 3 tissues showed limited hemorrhage and no necrosis or vascular thrombi. The most prominent findings in Group 2 were hemorrhage and necrosis, whereas the most prominent findings in Group 3 were focal hemorrhage and leukocyte infiltration. Electron microscopy demonstrated that GYKI 52466 protected the neurons, myelin, axons, and intracellular organelles. The mean inclined-plane angles in Groups 1, 2, and 3 were 65°, 40 to 45°, and 55°, respectively. Motor scale results in all groups showed a similar trend.

Conclusions. The findings in this rat model suggest that GYKI 52466 may provide significant therapeutic protection from secondary damage after acute SCI. This agent may be a viable alternative treatment for SCI.

KEY WORDS • α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor • kainate receptor • spinal cord injury • 2,3-benzodiazepine • GYKI 52466

R ESEARCH has clearly established that glutamate is the most important excitatory neurotransmitter in the mammalian central nervous system. In extensive reports several authors have documented its excitotoxic effects both in vivo and in vitro. Glutamate acts through metabotropic and ionotropic receptors, with the latter group composed of three subtypes named after three selective agonists: NMDA, AMPA, and KA receptors. There is, however, considerable debate about the pharmacological and electrophysiological distinctions between AMPA and KA receptors. Agrawal and Fellings have demonstrated the presence of AMPA and KA receptors in periaxonal astrocytes in spinal cord white matter, which suggests that these cells play a role in glutamatergic white matter injury. The authors of studies in animals have shown that blockade of AMPA and KA receptors, but not NMDA receptors, enhances the recovery of compound action potentials in the posterior column of the spinal cord as well as reducing functional and histopathological deficits after acute SCI. The AMPA receptors provide additional binding sites for the different classes of benzodiazepines, including the...
2,3-benzodiazepines.6 The molecule GYKI 52466 is one of the latter types and has been described in detail by Tarnawa, et al.26 This agent differs pharmacologically from conventional 1,4-benzodiazepines, such as diazepam, in that it lacks sedative–hypnotic activity and does not bind to benzodiazepine receptors.10,11,26 It acts as a selective noncompetitive AMPA receptor antagonist and also has anticonvulsant and muscle-relaxant properties.26 The main advantage of GYKI 52466 stems from its ability to bind to targets other than glutamate-specific sites. This capability means that it remains efficacious during a neurological insult, regardless of the levels of endogenous agonists that are present.23 Because of these binding properties, its prolonged use as neuroprotective treatment would not influence normal glutamatergic activity.6 Furthermore, GYKI 52466 does not interact with the γ-aminobutyric acid receptor complex.3,6,28 The authors of previous reports have indicated that GYKI 52466 is efficacious in the treatment of various cerebral pathological entities, including ischemia and seizures.10,22 Smith and Meldrum23 have investigated the cerebroprotective efficacy of GYKI 52466 during the onset of stroke in a rat model. They showed that administration of this agent within 1 hour of the ischemic insult reduced cortical infarct volume and caused no associated side effects.23 Our aim in this investigation was to assess the neuroprotective effects of GYKI 52466 after traumatic SCI in a rat model. To the best of our knowledge, this is the first study that has focused on the potential neuroprotective effect of this agent in SCI.

Materials and Methods

A total of 63 adult Wistar albino rats weighing 250 to 300 g were used. Three were replacements for animals that died during surgery. A normal diet was provided throughout the study period. Prior to surgery, each animal received an anesthetic intramuscular injection of 60 mg/kg of ketamine (Ketalar; Parke-Davis, Eczacibaşı, Istanbul, Turkey) and 9 mg/kg of xylazine (Rompun; Bayer, Istanbul, Turkey). The posterior region was shaved and cleaned with povidone iodine solution. Additional doses of ketamine and xylazine were required intraoperatively in some cases. Body temperature, maintained at 37°C throughout the procedure with a heating pad and lamp, was monitored with a rectal thermometer. Arterial pressure and heart rate were also monitored continuously. Three-level laminectomies (T6–8) were performed with the aid of a surgical microscope under 10 magnification. Care was taken to avoid damaging the dura mater.

Traumatic Injury Model

The study involved evaluation of spinal cord samples and postinjury functional recovery in three groups of rats. The SCI model has been described.18 Briefly, after completion of the thoracic laminectomies, an aneurysm clip with approximately 50 g closing pressure was applied extradurally to the spinal cord for 60 seconds and then removed.

For the functional recovery experiments, the trauma was created at severity of 50 g·cm using the weight-drop technique to induce paraparesis.4 A 5-mm-diameter cylindrical glass tube was positioned at a 90° angle to the surface of the dura mater, and a 5-g cylindrical constant weight was dropped from 10 cm height through the tube onto the exposed spinal cord.

Experimental Protocol

Sixty rats were randomly divided into three groups of 20 animals each.

Group 1: Sham-Operated Controls. A skin incision was made, the paravertebral muscles were dissected, and the laminae were exposed. As previously noted, the laminectomies were performed at T6–8. Once these were completed, the paravertebral muscles and the skin were closed with No. 3.0 silk sutures. Two hours after the operation, nine animals were given lethal intraperitoneal injections of pentobarbital. The area where the laminectomies had been performed was exposed and approximately 2 cm of spinal cord was excised under the microscope. The dura was dissected from the cord. Spinal cord samples obtained in six of these nine rats were embedded in liquid nitrogen and transported to the Biochemical Research Laboratory for lipid peroxidation and tissue ATP studies. The cord samples acquired in the other three rats were fixed in 10% formaldehyde solution for light microscopic examination. At 24 hours postinjury, another six animals were killed and spinal cord samples were removed for light microscopic and three were fixed in glutaraldehyde for TEM examination. The remaining five animals in the sham-operated group were used for functional recovery experiments. Neurological status in these rats was assessed at 1, 3, and 5 days postinjury by using the inclined-plane technique and a modified version of the Tarlov motor grading scale.23

Group 2: Trauma-Only Controls. Laminectomies were performed and SCI induced as previously detailed. The rats were treated with physiological serum and dimethyl sulfoxide. Spinal cord samples were collected and processed as described for Group 1. Nine animals were killed at 2 hours postinjury, and six and three spinal cord specimens were processed for biochemical analysis and light microscopic examination, respectively. At 24 hours postinjury, six more rats were killed and three cords each were prepared for light and electron microscopic studies, respectively. The remaining five rats were used to assess functional recovery.

Group 3: Trauma and Treatment Rats. Each rat underwent the same laminectomy and trauma procedures conducted in Group 2. The GYKI 52466 (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide (10% 0.1 mM HCl). One minute after the clip was removed, each rat received an intraperitoneal injection of 15 mg/kg GYKI 52466. This same treatment was repeated 12 hours later. The rats were killed, and spinal cord specimens were removed and prepared as previously stated. As in the other groups, five animals were used for functional recovery experiments.

Biochemical Analysis

Lipid peroxidation was assessed based on the MDA content of the tissue, as measured by the thiobarbituric acid method.27 The MDA was measured as nanomole per gram of wet tissue in the spinal cord. Tissue ATP levels were determined using a modified version of the Adams method1 and a commercially available enzyme kit (Sigma Chemical Co.). The ATP was also measured as nanomole per gram of wet tissue.

Light Microscopy Study

As previously noted, the spinal cord specimens obtained at 2 and 24 hours postinjury were prepared for histological study. Each sample was fixed in 10% neutral formaldehyde solution and embedded in paraffin. Five-micrometer-thick coronal sections of the injured region were cut, and these were mounted on slides and stained with hematoxylin and eosin. The slides were examined under a light microscope at ×10 and ×40 magnification.

Transmission Electron Microscopy Study

Tissue samples obtained at 24 hours postinjury were examined by TEM. For this, the spinal cord tissue was first fixed in 2.5% glutaraldehyde and then postfixed in OsO4 for 1 hour. After dehydration in a series of graded alcohol baths, each specimen was treated with propylene and then embedded in Araldite N. Semithin sections were obtained and stained with toluidine blue to identify the segments of cord that were desired for thin sectioning. The thin sections of the injured cord were counterstained with uranyl acetate and lead citrate. Each section was then examined and photographed under a Zeiss electron microscope.
Neuroprotective effect of GYKI 52466

**Summary of inclined-plane results in all three groups**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hrs</td>
<td>65.0 ± 2.5</td>
<td>51.5 ± 2.3</td>
<td>58 ± 2.7</td>
</tr>
<tr>
<td>3 days</td>
<td>64.0 ± 1.5</td>
<td>44.0 ± 2.2</td>
<td>53 ± 2.7</td>
</tr>
<tr>
<td>5 days</td>
<td>64.5 ± 2.2</td>
<td>45.5 ± 3.3</td>
<td>62 ± 2.4</td>
</tr>
</tbody>
</table>

*Values are presented as the means ± SDs.

**Lipid Peroxidation Levels**

At 2 hours after SCI, the respective mean concentrations of MDA in tissues from Groups 1, 2, and 3 were 21.73 ± 4.35, 35.53 ± 2.99, and 27.98 ± 3.93 nmol/g of wet tissue, respectively (Fig. 1 left). The MDA level in Group 2 (trauma only) was significantly higher than that in Group 1 (p < 0.01). The lipid peroxidation level in Group 3 (trauma combined with GYKI 52466 treatment) was significantly lower than that in Group 2 (p < 0.01).

**Levels of ATP**

At 2 hours postinjury, the respective mean ATP activity levels in Groups 1, 2, and 3 were 166.21 ± 21.73, 24.57, 41.72 ± 12.28, and 85.82 ± 13.92 nmol/g of wet tissue (Fig. 1 right). The ATP activity level in Group 2 was significantly lower than that in Group 1 (p < 0.01), and the level in Group 3 was significantly higher than that in Group 2 (p < 0.01).

**Motor Evaluation**

At 3 days postinjury, the respective mean motor scores in Groups 1, 2, and 3 were 5 ± 0.0, 1 ± 0.0, and 2.2 ± 1.1 (Table 2). Group 3 scored significantly better than Group 2 (p < 0.01; Kruskal–Wallis one-way analysis of variance).

**Functional Recovery Experiments and Assessment**

Anesthesia in these rats was induced using halothane instead of ketamine to avoid potential ketamine-related error for neuroprotective effects. As detailed in the Traumatic Injury Model section SCI was produced using the weight-drop technique. This method was chosen instead of clip compression because it generates a better paraparesis model in which to evaluate an animal’s neurological performance.

Each rat’s functional recovery was assessed using objective and subjective tests at 1, 3, and 5 days after the operation. The inclined-plane technique was used as an objective test, and the modified Tarlov motor grading scale was used as a subjective test. The latter is a five-tier scale with the following classification: Grade 5, able to walk normally; Grade 4, able to walk with mild spasticity or incoordination of the hind limbs; Grade 3, able to stand but unable to walk; Grade 2, minimal voluntary hind limb movements, but unable to stand; and Grade 1, no voluntary hind limb movement.

**Statistical Analysis**

Analysis of variance was conducted to compare the group findings. Statistical significance was set at a probability value less than 0.01. Results were presented as the mean ± SD where applicable.

**Inclined-Plane Results**

The results of inclined-plane testing are summarized in Table 1. In Group 2 the inclined-plane angle at 3 days postinjury ranged from 40 to 45° (mean 44 ± 2.2°). In Group 3 the inclined-plane angle at 3 days postinjury ranged from 50 to 55° (mean 53 ± 2.7°). Comparison of the mean values revealed a significant difference between Groups 2 and 3 (p < 0.01).

**Light Microscopy Findings**

As expected, light microscopic examination of cord samples from Group 1 showed normal findings.

In Group 2 tissue samples we observed disruption of the normal gray and white matter arrangement and even complete loss of normal architecture in some areas. Hemorrhage and congestion were prominent in the samples obtained at 2 hours postinjury (Fig. 2 upper). The speci-
mens obtained at 24 hours postinjury showed hemorrhagic necrosis and vascular thrombosis. In addition, the traumatized cord was infiltrated with polymorphonuclear leukocytes, erythrocytes, and macrophages (Fig. 2 lower). Cystic vacuolar degeneration and edema were prominent in both the gray and white matter zones.

In Group 3, evaluation of tissues obtained at all stages postinjury showed some change from normal spinal cord appearance. At 2 hours postinjury, the hemorrhage and congestion were less severe and more focal than in Group 2 (Fig. 3 upper). The samples collected 24 hours after injury showed no hemorrhagic necrosis or vascular thrombi (Fig. 3 lower). Some areas of the gray and white matter showed a few polymorphonuclear leukocytes and cystic vacuolar degeneration according to the time period postinjury.

Transmission Electron Microscopy Findings

As expected, TEM study of the cord specimens obtained in Group 1 showed normal gray and white matter (Fig. 4).

In Group 2 tissue samples we found typical myelin and axonal degeneration, as well as severe edema. Degenerated configuration of myelin sheaths and axons was noted. Most of the axons were shrunken and disintegrated, granular in appearance, or lacking structure, and some had no myelin sheath (Fig. 5 upper). The white matter showed prominent outer and inner vesicular degeneration and displacement of axons. There were many large clefts between myelin layers. Another noteworthy neuronal change in the Group 2 tissues was intracytoplasmic edema. The cells in the gray matter, particularly the neurons, exhibited marked edema and their cell membranes were damaged. No cristae or other organelles were observed in these cells (Fig. 5 lower). The neuronal cytoplasm contained large vacuoles. There was marked accumulation of fibrin and erythrocytes throughout the cord tissue. Outer and inner vesicular degeneration was observed in almost all samples. All the traumatized zones of the cord showed accumulation of polymorphonuclear leukocytes and macrophages, indicating an inflammatory response. Perivascular edema, shrinkage of endothelial cells, myelin debris, and loss of some axons were also noted.

In Group 3, normal cellular structure was preserved, and the only obvious abnormalities were mild edema and a few erythrocytes in the gray matter. In contrast to the findings in Group 2, there was no fibrin accumulation and no outer or inner vesicular degeneration. Treatment with GYKI 52466 appeared to have a protective effect on the periaxonal myelin. Dispersion of myelin sheaths and displacement of axons were rarely observed. The myelin layers were arranged in a regular pattern, but some areas of white matter showed a few myelin clefts and slender, cracked axons. Some of the mitochondria were swollen, but cristae were still present (Fig. 6 upper). In general, the gray matter appeared similar to that in normal rat spinal cord. The neuronal changes were limited, and there was minimal cell membrane damage. The Group 3 cord tissue also showed less intracytoplasmic edema than Group 2. There was no widespread edema in the cords obtained in the treated group, but small areas of mild edema were noted on some TEM slides (Fig. 6 lower). The endothelial cells appeared normal, and vessel lumens were patent. There were no large vacuoles or clearing areas, and no leukocyte infiltration was observed.
Discussion

Agrawal and Fehlings\(^3\) have demonstrated that AMPA/KA receptors are present in astrocytes in spinal cord white matter. Their results suggested that non-NMDA glutamate receptors are involved in traumatic SCI, and they indicated that this damage may be mediated by AMPA and KA receptors on astrocytes. Although activation of AMPA/KA receptors on the cell primarily triggers an influx of sodium from the extracellular space, some subtypes may also make the cell membrane permeable to calcium. Agrawal and Fehlings\(^3\) showed that AMPA receptors are highly calcium permeable. Several reports have documented that KA receptor activation can trigger cellular influxes of sodium and calcium.\(^2,3,8,14,16,24\) Such findings indicate that activation of these receptors is a potential neurotoxicity pathway. Research has demonstrated that extracellular excitatory amino acid concentrations in the spinal cord reach toxic levels 15 minutes after experimental SCI.\(^4\) This rapid rise is the reason we administered GYKI 52466 1 minute after SCI.

Compounds that act as AMPA receptor antagonists are potentially useful for preventing and treating a broad range of acute and chronic neurological disorders.\(^6,10,23\) The GYKI 52466 is the only 2,3-benzodiazepine known to act as a noncompetitive antagonist through negative allosteric modulation.\(^3,6,28\) Furthermore, this agent is hydrophobic and is able to cross the blood–brain barrier, which is a significant advantage with respect to clinical use.\(^10,23\) The authors of several studies have indicated that GYKI 52466 has cerebroprotective and anticonvulsant properties, although no hypnotic sedative effects.\(^6,19,22,28\) In our study, we administered GYKI 52466 by intraperitoneal injection. There were no adverse behavioral side effects; however, we did note a hypnotic sedative effect in some animals.

Hall and Braughler\(^15\) have demonstrated increased tissue levels of lipid peroxidation products at 1 hour after SCI in cats. In line with this, we also found elevated levels of lipid peroxide at 2 hours after SCI in all the damaged rat spinal cords we studied. Comparison of the trauma-only and the trauma-and-treatment groups showed that intraperitoneal administration of GYKI 52466 was
Agrawal and Fehlings detected AMPA and KA receptors to some degree. As previously mentioned, axon, myelin, and intracellular organelles (Figs. 3–6).

GYKI 52466 administration after SCI protects neurons, ATP in the Group 3 specimens. ATP production and would explain the higher levels of intracellular organelles were absent in Group 2 but were higher than those in Group 2 (p < 0.01). Additionally, TEM examination showed that mitochondria and other intracellular organelles were absent in Group 2 but were present with intact cristae in Group 3. The latter indicates ATP production and would explain the higher levels of ATP in the Group 3 specimens.

Overall, our light and TEM results demonstrated that GYKI 52466 administration after SCI protects neurons, axon, myelin, and intracellular organelles (Figs. 3–6). This treatment also blocked the development of intracystoplasmic edema to some degree. As previously mentioned, Agrawal and Fehlings detected AMPA and KA receptors on periaxonal astrocytes, which suggested that these cells may have some function in secondary glutamatergic neurological injury. Periaxonal glia, and astrocytes in particular, play an important role in the modulation of axonal signaling and the regulation of extracellular ionic disturbances. These actions require energy. Although the exact mechanism underlying the neuroprotective action of GYKI 52466 is not fully understood, we believe that its function as an AMPA/KA receptor antagonist is important. Additionally, we suspect that this agent’s significant relaxant effect on smooth muscle in spinal vessel walls would lead to increased local spinal cord blood flow, another form of neuroprotection.

In this study, we used inclined-plane testing and the modified Tarlov motor grading scale to assess functional recovery in rats at 1, 3, and 5 days after SCI. These tests can provide valuable information about the severity of SCI and responses to treatment. Both the inclined-plane results and the motor grades obtained in the animals in Group 2 (trauma only) were poorer than those in the GYKI 52466–treated animals (Table 1). On average, at 5 days postinjury the rats treated with GYKI 52466 were able to maintain their position at a 62° inclined-plane angle and their motor score was 2.8, whereas the corresponding means in Group 2 were 45° and 1. The differences in neurological function between Groups 2 and 3 were statistically significant at all three time points (p < 0.01 for all). The neurological performance of rats in Group 3 (trauma combined with treatment) immediately after SCI was inferior to that of the sham-operated control group. The performance levels of Group 3 rats, however, returned to near normal by Day 5 postinjury, demonstrating the neuroprotective effect of GYKI 52466.

Conclusions

The results of this study offer strong evidence that GYKI 52466 provides neuroprotection after SCI. This effect in a rat model suggests a possible therapeutic role for this agent in the treatment of secondary damage after SCI in humans.

References

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