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# Glutamine Synthetase Induced Spinal Seizures in Rats

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Glutamine synthetase (GS) is a key enzyme in the regulation of glutamate neurotransmission in the central nervous system. It is responsible for converting glutamate to glutamine, consuming one ATP and NH3 in the process. Glutamate is neurotoxic when it accumulates in extracellular fluids. We investigated the effects of GS in both a spinal cord injury (SCI) model and normal rats. 0.1-ml of low (2-$\mu$M) and high (55-$\mu$M) concentrations of GS were applied, intrathecally, to the spinal cord of rats under pentobarbital anesthesia. Immediately after an intrathecal injection into the L1-L3 space, the rats developed convulsive movements. These movements initially consisted of myoclonic twitches of the paravertebral muscles close to the injection site, repeated tonic and clonic contractions and extensions of the hind limbs (hind limb seizures) that spread to the fore limbs, and finally rotational axial movements of the body. An EMG of the paravertebral muscles, fore and hind limbs, showed the extent of the muscle activities. GS (2-$\mu$M) caused spinal seizures in the rats after the SCI, and GS (6-$\mu$M) produced seizures in the uninjured anesthetized rats. Denatured GS (70℃, 1 hour) also produced spinal seizures, although higher concentrations were required. We suggest that GS may be directly blocking the release of GABA, or the receptors, in the spinal cord.

**Key Words:** Glutamine synthetase, spinal seizure, EMG, molecular effect

## INTRODUCTION

Glutamine synthetase (GS) regulates the metabolism of glutamate in the central nervous system (CNS). It catalyzes the synthesis of glutamine from glutamate, and is responsible for the detoxification of ammonia in the brain.[1] GS is crucial for nitrogen homeostasis, as glutamine is a constituent of proteins, and serves as a nitrogen source for several biosynthetic pathways.[2] It is located in the glial fraction of the brain, primarily in the astrocytes.[3-5] Glutamine synthesized by GS in the astrocytes is transported into the neurons, where it serves as a precursor for the neurotransmitters, glutamate and $\gamma$-aminobutyric acid.[6,7] GS is of particular importance in the brain because it is the only known pathway for the synthesis of glutamine.

GS has been reported to be neuroprotective,[8] possibly due to catalysis of glutamate and the prevention of excitotoxicity.[9] The neuroprotective effect of GS is proportional to the inhibition or induction of its activity, and strongly implicates glutamate catalysis as the mechanism of this neuroprotection.[10]

During our investigations on the potential effects of GS as a neuroprotective agent in acute spinal cord injury, we discovered that it was a potent seizure-producing protein when applied locally to the spinal cord. Furthermore, GS appears to produce seizures in the spinal cord, even when denatured, and when its activity as an enzyme is blocked.

Spinal seizures can be elicited by electrical stimulation,[11] or drugs, such as penicillin and strychnine,[12,13] and the intrathecal injection of morphine induces myoclonic seizures,[14] but there have been no report on GS producing spinal seizures.

The purpose of the present study was to show that GS produces spinal seizures, and suggest possible mechanisms for its action.

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## MATERIALS AND METHODS

Fifty-four Long-Evans hooded adult rats (300-500 g) were used in the experiments. They were deeply anesthetized with pentobarbital (50 mg/kg for female, 65 mg/kg for male, i.p.). At these doses, the rats showed no withdrawal reflexes to firm pinches of the tail or hindpaws. The spinal cord was exposed through a skin incision and a laminectomy at L1 - L3. We injured the spinal cord by dropping a 2.50 cm, 10 g rod (25 g · cm) onto the T 10 thoracic cord exposed by the laminectomy. The impactor contact surface was 2.5 mm in diameter, approximately the diameter of the spinal cord of a 300 g rat. The vertebral columns were mechanically stabilized with two spinal clamps, fixing the vertebrae on either side of the impact site. Although the vertebral column was clamped close to the impact site, the vertebral column continued to show slight movements due to respirations during the impact. The spinal cord compression was, therefore, estimated by subtracting the rod and vertebral movements during impact. An electrical contact circuit monitored the contact between the impactor head and the cord. These signals, along with a 1 msec time reference signal, were recorded with a computer (IBM AT) to give the actual impact height, velocity, and compression of the spinal cord.

A catheter was inserted into the subdural space, and advanced approximately 1.5 cm to the thoracic region and the insertion site was closed with "Krazy" glue. To confirm that the dural closure had been effective, we injected 0.1 ml of saline, and watched the insertion site for leakage, if there was no leakage, we then injected the GS solution over a 30 - 60 second period.

The sheep GS was purchased from Sigma Chemical Company (St. Louis, MO, USA). The activity of the GS was expressed in units, defined as the amount of enzyme that will convert one micromole of glutamate to glutamine in 15 minutes at 37℃. According to the company-supplied information on the product, 1-mg of GS has approximately 1 unit of enzyme activity. Because the GS was prepared in a potassium (K) buffer during the manufacturing process, the GS contains a large amount of potassium, which we eliminated by dialysis in Ringer's solution. The GS solution (100 units/2 ml) was dialyzed in 500 ml of Ringer's solution using Slid-A-Lyzer cassettes (Pierce Chemical Co, Rockford, IL, USA) with membranes that allowed the passage of molecules with a MW of less than 10 kDa. The procedure was conducted overnight at 4℃, and the Ringer's solution changed once to ensure complete dialysis. The dialyzed GS solution was then divided into 0.5-ml aliquots, and lyophilized using a concentrator (Savant Instruments, Holbrook, NY, USA).

Each aliquot, containing GS, was then mixed with a liposome solution. The liposomes were prepared by dissolving Type-IIs phosphatidylcholine, from soybeans, (Sigma, St. Louis, MO, USA) in water at 4℃, overnight, at a concentration of 20-mg/ml. These samples were then sonicated, centrifuged at 10,000 g the following day and diluted 1:10 in TE. Liposome solution was added to each aliquot to achieve a concentration of 0.2 units GS per microliter. The aliquots were stored at -70℃ for later use.

Bacterial GS (Sigma, St. Louis, MO, USA) was used in some of the experiments, in the other experiments, we denatured the GS by placing it in a water bath at 70℃ for one hour. We also blocked the enzyme activity with L-methionine sulfoximine (MSO; Sigma). The MSO was added to the GS solution at a concentration 10 X higher than that of the GS, along with 250-mM ATP (adenosine 5'-triphosphate, Sigma). After incubation at 37℃ for 2 hours the mixture was dialyzed at 4℃ for 24 hours to remove the excess MSO and ATP.

The concentrations of the GS ranged from 0.1 to 55 $\mu$M. 0.1 ml of the solution was injected intrathecally. The injections were stopped as soon as the seizures appeared, or else the whole 0.1 ml was injected. To assess the amount of GS injected, and presence of the GS in the spinal cord, we removed the spinal cords 15 minutes after the injection, and froze them at -70℃ for later analyses.

To document the seizure activity, we recorded the electromyographic activity in the triceps, paravertebral and gastrocnemius muscles, with needle electrodes. The PowerLab System (AD Instruments, CA, USA) was used to amplify and digitize the recorded EMG signals before, during

and after the intrathecal injection of the GS solution.

To assess the GS activity, we used high-performance liquid chromatography (HPLC), to measure the conversion of glutamate to glutamine, with a mobile phase flow rate of 0.6 ml/min, and a total run time of 60 min. The samples were composed of 20.60 ml deionized water, 17.20 ml 100-mM imidazole HCL Buffer, with a pH of 7.1 at 37℃ (Sigma, adjust to pH 7.1 at 37℃ with 1 M HCL), 1.80-ml 3-M sodium glutamate, 1.80-ml 250-mM adenosine 5'-triphosphate (ATP, freshly prepared), 3.55-ml 900-mM magnesium chloride, 0.90-ml 1-M potassium chloride and 1.80-ml 1.2-M ammonium chloride. From this sample solution, 2.70-ml was obtained and made 10-$\mu$M concentration of DL-Norvaline for obtaining standard curve. We placed 0.5 unit/0.1cc (10-$\mu$M) GS into each test tube. To evaluate the MSO activity, we mixed 200-$\mu$M/0.1 ml MSO and 20-$\mu$M/0.1 ml, and added 0.1 ml of this mixture to the test tube.

## RESULTS

The intrathecal administration of a high dose of sheep GS (10-$\mu$M/0.1-ml) caused spinal seizures in the rats following the spinal cord injury. The seizures started with paravertebral muscle twitching, piloerection and, eventually, hind limb extensions, with repeated tonic and clonic contractions that spread to the fore limbs, accompanied by rotational movement along the longitudinal axis of the body. The paravertebral muscle twitching always started in the lumbar area, and spread rostrally over a 1-2 minutes period, and the seizure would cause respiratory arrest. The low dose of sheep GS (0.1-$\mu$M/0.1 ml) tended to slow respiration. After the administration of a 1$\mu$M/0.1 ml sheep GS intrathecal injection, the respiration became deeper and slower. The paravertebral muscle twitching and hind limb seizures started after a 2$\mu$M/0.1ml intrathecally administration of the sheep GS. The seizure was partial, localized and continued for 4 to 5 minutes. The intrathecal administration of sheep GS (10$\mu$M/0.1 ml) in the normal rats also caused spinal seizures shortly after the injection. The seizure patterns were similar to those in the SCI rats but not usually as severe. We repeated each of the experiments twice, to confirm their reproducibility. Phosphatydilcholine and phosphate buffer solutions did not cause seizures.

To determine whether the seizures were due to glutamate or glutamine, we injected solutions of both intrathecally. The glutamate solutions did not produce similar seizure activity in rats following the SCI. An extremely high concentration (3M/0.1 ml) of glutamate caused only mild paravertebral muscle twitching and ipsilateral hind limb extensions. The activity was typically localized in the lumbar area, and continued for no longer than 5 minutes following the injection. Glutamine was injected intrathecally into the SCI and normal rats in low (100$\mu$M/0.1ml) and high (1M/0.1ml) doses. An intrathecal injection of 10-mM/0.1ml glutamine solutions increased the respiration and heart rate but produce no muscle activity at concentrations <1 M/0.1-ml. An intraspinal injection of glutamine using a microinjector, caused no seizure until 10-mM /0.1ml had been injected.

The bacterial GS (10$\mu$M/0.1 ml) induced seizure activity in rats following the SCI. Compared to the sheep GS, however, the seizures were localized in the hind limbs and paravertebral muscles, and were milder and short-lived. An intrathecal administration of the bacterial GS (10$\mu$M/0.1 ml) to the normal rats reduced the respiration rate, and produced localized, mild twitching of the paravertebral muscles.

The denatured (70℃, 1hour) sheep GS (10$\mu$M) administered to the SCI rats produced seizures, which began in the paravertebral area. After paravertebral muscle twitching, the seizure spread to the ipsilateral hind limb, but generally localized and mild. Higher concentrations of denatured sheep GS (55$\mu$M/0.1 ml) caused spinal seizures, resembling those produced by 10$\mu$M/0.1 ml of the sheep GS. Low (0.1$\mu$M/0.1 ml) to high (55$\mu$M/0.1 ml) doses of the denatured GS (n=8) were administered intrathecally to the normal rats. The seizures started immediately after the injection, but were mild and localized only in the paravertebral area and the hind limb on the same side. Lower concentration of the denatured sheep GS

(0.1 μM) caused only hind limb muscle twitching. In one case (GS without PC, after 5 μM injection) the animal showed sudden respiratory arrest, and died. The MSO-inactivated GS solutions (sheep) also produced seizures, but at relatively higher concentrations (6 μM/0.1 ml). The seizure patterns were similar to those with the active GS solutions (Table 1).

To assess the ability of GS to produce brain seizures, we applied it to the brain of the normal rats. A microcatheter was placed in the temporal subdural space. Increasing concentrations of GS caused no visible seizure activity until the 60-μM/0.1 ml levels. The seizures started with contralateral periauricular muscle twitching, and spread to the perioral muscles. After 30 to 60 seconds of partial seizures, generalized tonic clonic seizures started. Such seizures usually lasted 3 to 5 minutes. Compared to the spinal injections of the sheep GS, the seizures were mild, and temporally limited, despite the ten fold higher concentrations of GS.

To confirm the seizure activity, we evaluate the muscle activity using EMG. Immediately after the injection, typical multi-spiked seizure waves were observed in the paravertebral muscles,followed by those in the hind and fore limbs (Fig. 1).

To assure the activity of the GS, we evaluate the level of glutamate and glutamine using HPLC. HPLC assays were performed on the samples to

**Table 1.** The Effect of Intrathecal Administration of GS and Several Agents

| Agent | Concentration (μM/ml) | Spinal seizure | Respiratory* Depression | Seizure pattern | SCI or normal |
|---|---|---|---|---|---|
| Sheep GS | 0.1( n=2) | - | + | - | SCI |
| | 2 (n=2) | + | + | Localized, paravertebral Muscle & hindlimb | SCI |
| | 10 (n=2) | + | + | Generalized tonic-clonic Respiration holding | SCI |
| | 10 (n=2) | + | + | Generalized tonic-clonic Less severe than SCI | normal |
| Bacterial GS | 10 (n=2) | + | + | Localized, paravertebral m. | SCI & normal |
| Sheep GS | 0.1 (n=2) | + | - | Localized, hindlimb | normal |
| (denatured) | 55 (n=2) | + | + | Generalized tonic-clonic | SCI |
| Bacterial GS | 20 (n=2) | + | - | Very mild localized, | SCI |
| (denatured) | | | | Paravertebral m. | |
| Sheep GS | 6 (n=2) | + | + | Generalized tonic-clonic, mild | SCI |
| with MSO | | | | | |
| Glutamate | 10 mM (n=2) | - | + | - | SCI |
| | 3M (n=2) | + | + | Localized, hindlimb | SCI |
| Glutamine | 10 mM (n=2) | - | + | - | SCI |
| | 1M (n=2) | - | + | - | SCI |
| PC | | - | - | - | SCI |
| PBS | | - | - | - | SCI |

*Determined by significant reduction in the respiration rate, as observed by watching the rats.
SCI, spinal cord injury; PC, phosphatydilcholine; PBS, phosphate buffer solution.

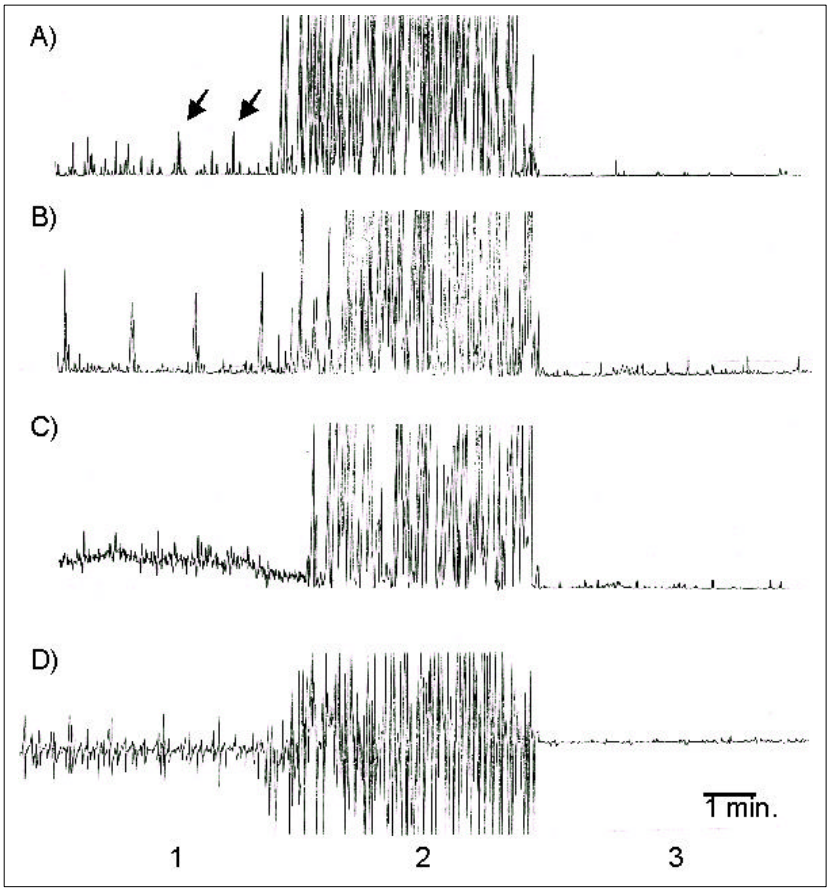


**Fig. 1.** Effects of GS on rat spinal cord by intrathecal injection. Immediately after GS injection, multi-spike seizure waves were seen on the paravertebral muscles (A-2), followed by the hind limbs (B) and the fore limbs (C). After 1 - 2 minutes of severe seizures, the rat died (A-3). A-C: after calibration of (real wave)2; D: real EMG wave; Arrows: electrocardiograph (ECG).

confirm the GS solutions activities. Active solutions of GS converted the glutamate to glutamine, consuming ATP and NH3 in the process. The inactivated solution (denatured GS or MSO-treated GS solutions) showed no glutamate conversion (Fig. 2).

## DISCUSSION

GS is expressed at basal levels in most tissues, with the exception of those in the central nervous system (CNS), where its expression is elevated. In the CNS, GS is localized to the Muller glia of the retina, astrocytes, and some oligodendroglia.[3,15,16] In the astrocytes, the primary GS-expressing cell type in the spinal cord, GS plays a major role in the "small glutamate compartment", catalyzing glutamate after it is released from the neurons during a synaptic transmission, which is taken up by the astrocytes.[17] The Glutamine re-enters the neurons, where it is recycled to glutamate for neurotransmission. GABA ($\gamma$-aminobutyric acid) can also enter this pathway, as it is taken up by the astrocytes, where it is converted to glutamate through the tricarboxylic acid (TCA) cycle. This is mirrored by the reverse reaction, which occurs after glutamine is taken up by GABA-ergic neurons and converted to glutamate. GS is the limiting factor in this cycle, which is also known as the "glutamate shuttle". Several investigators have hypothesized that GS is responsible for regulation of the glutamate levels in synapses, and is also partly involved in the regulation of GABA.[18] Genetically epilepsy-prone rats have significantly lower brain GS activity than normal rats, which has been proposed to result in the increased seizure susceptibility exhibited by these animals.[19] Seizure-prone gerbils have also been shown to have a lower brain GS activity than normal gerbils.[20] An important difference between the spinal epileptiform activity and cortical seizures is the absence of post-ictal depression following the spinal seizures. Another difference

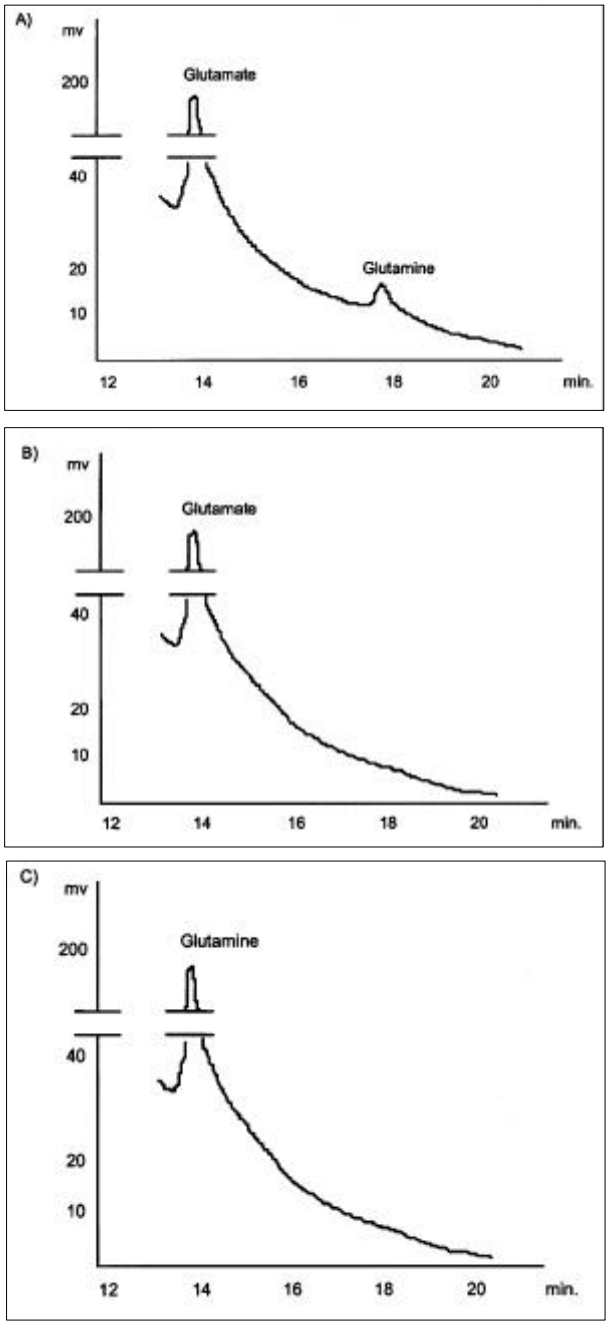


**Fig. 2.** Analysis of GS activity using HPLC. The GS activity was tested by HPLC to observe for the conversion of glutamate to glutamine. Each sample was incubated for 24 hours at 37(C. The concentration of glutamate and glutamine used was 3M. (A) Conversion of glutamate to glutamine by 0.5 units of GS consuming ATP and NH3. (B) Denatured GS did not convert glutamate to glutamine. (C) GS did not convert glutamine to glutamate.

in the pattern of seizure activity generated in spinal cord, and cortical gray matter, is the temporal irregularity of the spinal interictal discharges, compared to the cortical "spikes" induced by penicillin.[21]

In our study, GS initiated spinal seizures in the muscles close to the injection site, initially in the paravertebral muscles. The seizures then radiated to the hind limbs and spread proximally to the cervical spinal cord, where the fore limbs become involved. When it reaches the upper cervical cord, it causes respiratory arrest. During the terminal stages, the rats show axial rotation and other activities suggestive of brain stem involvement. There were no difference in the seizure patterns between the normal and SCI rats, following the administration of GS.

Lothman and Somjen studied spinal seizures provoked by the administration of penicillin. They believed that the ventral horns were the primary focus of the seizure activity induced by the topical and systemic administration of penicillin. They also found that the paroxysmal depolarization of the primary afferent terminals was more prominent in the muscle afferent fibers, which terminated in the ventral motor nuclei, and were less noticeable in the cutaneous afferents terminating in dorsal spinal gray matter. The persistence of spinal seizures depends on the continued presence of penicillin in the spinal cord. The topical application of penicillinase promptly, and completely, stopped the seizures.[21] Strychnine resembles penicillin in favoring the simultaneous coactivation of antagonistic muscles. In other respects penicillin and strychnine differ. Strychnine is known to block the reciprocal postsynaptic inhibition upon which penicillin has no effect. Furthermore, strychnine does not induce tonic seizures, only clonic paroxysmal activity ("tetanus"), which may persist for hours, with a frequency of 10- to 30/sec, compared to the 4 - 6/sec during the clonic phase of the penicillin-induced seizures.[13,21]

Despite numerous studies, the mechanisms of thesespinal seizures remain unclear. Two major hypotheses on the genesis of spinal seizures have been proposed. The synaptic release of excitatory amino acids (e.g. L-glutamate, L-aspartate) and their subsequent activation of specific receptors sensitive to the action of NMDA underlie the spinal convulsions.[22] It is generally agreed that the convulsions induced by the alkaloid are fully

explained by its antagonism of the spinal post-synaptic inhibition mediated by glycine.

We considered the possible mechanisms by which the GS may cause the spinal seizures. Firstly, the ammonium clearance following the administration of GS may cause a seizure: secondly, the enzymatic activity of GS may be responsible for the seizures: or finally, the GS interacts with the receptors in the spinal cord.

The HPLC assays showed that the GS converted glutamate to glutamine, consuming ATP and NH3 in the process. This reaction, however, is slow and relatively inefficient in solution. We were also unable to demonstrate that the GS catalyzes conversion, when applied to the spinal cord, will cause the seizure activity within seconds. The rapidity of this action rules out the slower mechanisms, such as the ammonium or glutamate clearance from the extracellular space. Furthermore, an intrathecal injection of glutamate caused no spinal seizures until a 2-M concentration was used. Likewise, an intrathecal injection of a very high (1 M) concentration of glutamine caused no seizures. A control phosphatidylcholine and phosphate buffer solution also caused no seizures. We therefore concluded that the seizure-genic properties of GS might not be related to its enzymic activity. To test this, we compared the seizure-genic effects of sheep and bacterial GS, denatured, and MSO-inhibited GS solutions. Although the higher concentrations of the bacterial, denatured, and MSO-inhibited GS, were necessary for the induction of the seizures, they did produce severe seizures similar to those produced by the active sheep GS.

We preferentially think that GS may be directly blocking the inhibitory receptors (GABAA, glycine, 5-HT1a and Histamine H1 receptor). GABA depresses the axonal conduction in the neonatal dorsal column, and is released by injured spinal neurons, which may diffuse to the white matter, and contribute to secondary axonal damage. Bicucullin (GABAA receptor antagonist) blocked the GABAA receptors in the hemicords in a neonatal rat hypoxia model.[23] In these experiments, bicucullin remarkably protected, and preserved, the axonal conduction under conditions of profound and prolonged hypoxia. In terms of a high concentration in the spinal cord and brain stem, and its' rapid reaction, glycine might be related to the spinal seizures caused by the GS.

Thus, spinal seizures, following the administration of GS, are caused by different mechanisms when they occur in the brain, and further investigation will be needed for their elucidation.

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