Research Article

NMDA Receptor-Mediated Neuroprotective Effect of the Scutellaria baicalensis Georgi Extract on the Excitotoxic Neuronal Cell Death in Primary Rat Cortical Cell Cultures

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Received 10 January 2014; Accepted 4 April 2014; Published 21 May 2014

Academic Editor: Thomas E. Salt

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The objective of the current research work was to evaluate the neuroprotective effect of the ethanol extract of Scutellaria baicalensis (S.B.) on the excitotoxic neuronal cell death in primary rat cortical cell cultures. The inhibitory effects of the extract were qualitatively and quantitatively estimated by phase-contrast microscopy and lactate dehydrogenase (LDH) assays. The extract exhibited a potent and dose-dependent inhibition of the glutamate-induced excitotoxicity in the culture media. Further, using radioligand binding assays, it was observed that the inhibitory effect of the extract was more potent and selective for the N-methyl-D-aspartate (NMDA) receptor-mediated toxicity. The S.B. ethanol extract competed with [3H]MDL105,519 for the specific binding to the NMDA receptor glycine site with 50% inhibition occurring at 35.1 μg/mL. Further, NMDA receptor inactivation by the S.B. ethanol extract was concluded from the decreasing binding capability of [3H] MK-801 in the presence of the extract. Thus, S.B. extract exhibited neuroprotection against excitotoxic cell death, and this neuroprotection was mediated through the inhibition of NMDA receptor function by interacting with the glycine binding site of the NMDA receptor. Phytochemical analysis of the bioactive extract revealed the presence of six phytochemical constituents including baicalein, baicalin, wogonin, wogonoside, scutellarin, and Oroxylin A.

1. Introduction

N-Methyl-D-aspartate (NMDA) receptors have a well-defined role in neuronal plasticity. Excitotoxic neuronal cell death can occur if these receptors are overactivated. As such, NMDA antagonists (NMDA inhibitors) are thought to play a crucial role in neuroprotection. However, it has been reported that NMDA receptors also have an important role in enhancing neuronal survival. Thus, it follows that NMDAR antagonists not only will protect from excitotoxicity but would also reduce prosurvival activity of NMDAR. Hence, the recognition of the switches regulating prosurvival vis-à-vis proexcitotoxic outcome of NMDAR stimulation may lead to development of NMDAR antagonists that specifically block the excitotoxicity while augmenting the protective NMDAR signaling. Glutamate is the principal excitatory neurotransmitter in the CNS. It stimulates various types of receptors including the N-methyl-D-aspartate receptors (NMDAR). NMDAR form calcium-permeable ion channels and are principal mediators of the excitotoxic cell death following excessive release of glutamate after different forms of CNS insults [1–5].

Scutellaria baicalensis (Labiateae family) is a plant sometimes referred to as Huang Qin or Scutellariae radix (root). Scutellaria baicalensis (Chinese skullcap) is a traditional Chinese medicine for the purposes of cardiovascular and cognitive health as well as longevity [6–8]. It has been reported to possess strong neuroprotective properties. Scutellaria baicalensis is a component of various combination therapies (from TCM) including Ger-Gen-Chyn-Tang [9],
Soshiho-tang [10], and Shuanghuanglian [11]. Various phytochemical components have been identified in *S. baicalensis* aerial and root parts. Baicalin (baicalin-7-glucoronide) and its aglycone baicalein as well as another glycoside known as baicalein-7-O-glucoside have been reported in *S. baicalensis*. In addition, wogonoside (wogonin-7-glucuronide) and its aglycone wogonin as well as another glycoside known as wogonin-5-O-glucoside have also been reported in it. Some other phytoconstituents reported in *S. baicalensis* are Oroxylin (5,7-dihydroxy-6-methoxyflavone) and its glucoside, neoaicalein, scutellarin, and isoscutellarin; chrysin; skullcap flavone; apigenin; luteolin; 6-hydroxyluteolin, and so forth [12–16].

The phytochemicals present in *Scutellaria* species have been reported to show a range of neuroprotective effects. Wogonin inhibited inflammatory activation of microglia by reduced cytotoxicity towards cocultured PC-12 neurons, supporting an *in vitro* neuroprotective role of this flavonoid. The efficacy of wogonin was further demonstrated in two experimental brain injury models. In the 4- vessel occlusion model of transient global ischemia, wogonin decreased the death rate of hippocampal neurons, the induction of iNOS, and TNF-β in hippocampus, whereas, in the kainate injection model, this flavonoid markedly protected from excitotoxic brain injury. Similarly, baicalein attenuated the NO production by suppressing iNOS induction, in LPS-activated BV-2 mouse microglial cells, besides reducing apoptotic cell death and NF-κB activation [17–19].

2. Materials and Methods

2.1. Materials. The plant material (roots) of *Scutellaria baicalensis* was collected from a local region of Hangzhou and was authenticated by a well-known botanist. Minimum essential medium (MEM), horse serum, and fetal calf serum were obtained from Gibco. Multiwell plates were bought from Falcon. Laminin, poly-L-lysine, L-glutamine, Glu, glucose, essential medium (MEM), horseserum, andfetal calfserum and was authenticated by a well-known botanist. Minimum medium supplemented with glucose (25 mM), fetal calf serum (5%), horse serum (5%), and glutamine (5 mM). After 14–16 days in the culture medium, the cells were used for the experiment.

2.4. Induction of Neuronal Cell Excitotoxicity and Their Assessment. Earle's balanced salt solution (EBSS) was used to rinse the cultured neuronal cells before the excitotoxic injuries were induced by exposure to 350 μM NMDA or Glu concentration in Mg²⁺-free EBSS for 20 min. After the induction of excitotoxicity, the neuronal cultures were rinsed and maintained at 37°C for 20–24 hours in glucose supplemented MEM. Then, the neuronal cell cultures were treated for 30 min with 350 μM NMDA or Glu in the presence of 1, 10, 25, 50, and 100 μg/mL concentrations of the extract in order to assess the effect of the S.B. ethanol extract on NMDA or Glu-induced neuronal injury. Stock sample solutions of the ethanol extract were prepared in dimethyl sulfoxide (DMSO). Lactate dehydrogenase (LDH) assay and phase-contrast microscopy were used to estimate quantitative and qualitative extent of the neuronal damage, respectively. As described previously [20], neuronal damage quantification was estimated by measuring lactate dehydrogenase (LDH) activity released into the culture medium. Background LDH release was determined by using sister neuronal cultures in each experiment and then subtracted from the values. The final concentration of the vehicle (not more than 0.5%) exhibited no effect on cell excitotoxicity.

2.5. Synaptic Membranes for Receptor Binding Studies. For receptor binding studies, the synaptic membranes were prepared from forebrains of male Sprague-Dawley rats as reported earlier [21]. After centrifugation, the forebrains were homogenized at 1500 ×g for 15 min. The supernatant was collected and centrifuged at 3000 ×g for 30 min. The pellet stored at −70°C overnight was liquefied at room temperature and then again suspended in 25 mM Tris-acetate (pH 7.0) containing 0.05% Triton X-100. The solution was incubated at 37°C for 15 min and centrifuged at 4000 ×g for 15 min. Finally, the pellet was resuspended to give a protein concentration of 1.5 mg/mL, determined by DC Protein Assay Kit (Bio-Rad).

2.6. Binding Studies with [³H]MDL 105,519, [³H]MDL 105,519 binding assay was carried out in 96-well plates. Incubation of the synaptic membranes (25 μg/well) at 25°C for 20 min in a mixture containing 4 nM [³H]MDL 105,519 and different concentrations of the S.B. extract along with 25 mM Tris-acetate was carried out. This reaction mixture was then washed thoroughly with the 0.6 mL of ice-cold 25 mM Tris-acetate buffer by filtration using Whatman GF/A glass fiber filter. Then, the filter was wrapped with MeltiLex, sealed in a sample bag, and then counted by MicroBeta TriLux (Microplate Scintillation and Luminescence Counter, PerkinElmer) at a counting efficiency of 30–40%. Nonspecific
binding, which was determined in the presence of 1 mM glycine, was less than 10% of total binding.

2.7. Binding Studies with $[^3H]MK-801$. For this purpose, the synaptic membranes (25 µg/mL) were incubated for 40 min in a 2 mL reaction mixture containing 4 nM $[^3H]MK-801$, 0.2 µM Glu, 2 mM glycine, and different concentrations of the ethanol S.B. extract along with 25 mM Tris-acetate buffer (PH 70). Then, the reaction was terminated by filtering through Whatman GF/B glass fiber filter and the bound radioactivity was determined by a liquid scintillation counter (Hidex 300 SL, USA) at a counting efficiency of 50–55%. Nonspecific binding, determined in the presence of 200 µM MK-801, was less than 10% of the total binding.

3. Liquid Chromatography-Tandem Mass Spectrometry (LC-ESI-MSMS)/HPLC Analysis

LC-MS equipment (LC-MS QqQ-6410B Agilent Technologies) consisted of a chromatographic system coupled with an Agilent Triple Quad mass spectrometer fitted with an ESI source. MS conditions were the following: nebulizer gas 45 Ps, gas temperature 325 °C, capillary voltage 4000 V, and MS range 100–1200 Da; MSn spectra were obtained using both positive and negative modes.

HPLC analysis was carried out by an Agilent 1260 infinity series. A Chromolith RP-18e column (4.6 mm ID, 50 mm length) (Merck) was used. Gradient elution of the samples was performed using 0.1% formic acid (eluent A) and methanol (eluent B). The gradient elution initial conditions were 45% of eluent B with linear gradient to 60% from 2 to 10 min, followed by linear gradient to 70% of eluent B at 35 min, and then linear gradient to 99% of eluent B at 38 min, with this proportion being maintained for 2 min. The column was then returned to the initial condition at 40 min and maintained until the end of the run at 42 min. The flow rate was 1 mL/min. The sample injection volume was 10 µL.

3.1. Statistical Analysis. The experiments were done in triplicate and the data were processed by nonlinear regression analysis using GraphPad software, USA, for the calculation of IC$_{50}$ values. All the results were expressed as mean ± SEM. Statistical significance was considered at $P ≤ 0.05$.

4. Results

4.1. Assessment of Neuronal Excitotoxicity. After the exposure of cultured rat cortical neuronal cultures to 35 µM Glu or NMDA for 20 min, acute neuronal swelling, breakage in dendrocytes, and the indistinct nuclear shape were observed by phase-contrast microscopy (Figure 1). This neuronal injury became more intense with increase in incubation time, ultimately resulting in enhanced neuronal damage and finally neuronal cell death. The objective of the current research work was to evaluate the neuroprotective effect of the ethanol extract of S. baicalensis as claimed in the traditional Chinese medicine, where this plant has been used against various neurological disorders. Our results demonstrated that when the neuronal cell cultures were exposed for 20 min to Glu (350 µM) in the presence of different concentrations of the S.B. extract, the Glu-induced excitotoxicity was dramatically inhibited in a dose-dependent pattern showing maximum inhibition at 100 µg/mL of the extract (Figure 1). The qualitative estimation of Glu-induced neuronal cell death was evaluated by phase-contrast microscopy, whereas the degree of
neuronal cell death was quantitatively estimated by calculating LDH release activities given off by the damaged neurons out into the culture media. The IC$_{50}$ values of the extract were found to be 60.01 and 28.60 $\mu$g/mL, respectively, for Glu and NMDA-induced excitotoxicity, respectively (Figures 1 and 2).

4.2. Selective Inhibition of NMDA Receptor-Mediated Excitotoxicity. As the Glu-induced excitotoxicity is arbitrated through a number of Glu-receptor subtypes, in order to estimate whether the S.B. extract inhibits the NMDA receptor-mediated toxicity, the cell cultures were subjected to 350 $\mu$M NMDA for 20 min. NMDA-induced excitotoxicity is already reported in the literature [22]. In the current study, we found that neuronal damage induced by NMDA and the neuronal deformation was morphologically similar to that produced by Glu. The S.B. extract exhibited a potent inhibition of NMDA-induced excitotoxicity as well. As is evident from the graph (Figure 2), the NMDA-inhibition curve exhibited a more pronounced shift than Glu-inhibition curve, indicating a more powerful and selective inhibitory effect of the extract on NMDA receptor-mediated excitotoxicity. The IC$_{50}$ value of the extract in this case was found to be 28.6 $\mu$g/mL. The extract showed a dose-dependent inhibition, and at a concentration of 100 $\mu$g/mL, almost 90–95% of the neurons were secured from the excitotoxic insults. As can be seen in Figure 1, after the treatment with 100 $\mu$g/mL S.B. extract + 350 $\mu$M Glu, the neurons showed regular and distinct shape under phase-contrast microscopy unlike in 350 $\mu$M Glu culture. Thus, these results indicate that the ethanol S.B. extract displays a neuroprotective effect against Glu or NMDA-induced excitotoxic neuronal cell death and more importantly this neuroprotective effect is principally arbitrated through NMDA receptors.

4.3. $[^3]$HMDL 105,519 Binding Studies. In the present study, further experiments were carried out to confirm whether the S.B. extract interacts with the glycine site of NMDA receptor. For this purpose, $[^3]$HMDL 105,519 radioligand receptor binding assay was carried out where $[^3]$HMDL 105,519 acts as a selective glycine site antagonist. The results indicated that S.B. extract competitively inhibited the binding of $[^3]$HMDL 105,519 to the glycine receptor site (Figure 3). It was observed that, at a concentration of 100 $\mu$g/mL of the extract, more than 90% of the binding of $[^3]$HMDL 105,519 was displaced by the extract. The IC$_{50}$ value was found to be 35.1 $\mu$g/mL.

4.4. $[^3]$HMK-801 Binding Studies. As can be seen in Figure 4, the specific binding of $[^3]$HMK-801 was appreciably inhibited by the S.B. extract. The IC$_{50}$ value of the extract was found to be 65.1 $\mu$g/mL. At a concentration of 100 $\mu$g/mL, $[^3]$HMK-801 binding with the receptor site was around 20% of the control binding calculated in the absence of the extract. Based on these two radioligand binding assays, we can conclude that the S.B. extract probably inhibits the NMDA receptor by knocking out glycine from its binding site. It has been

![Graph](image1.png)

**Figure 2:** Inhibition of N-methyl-D-aspartate (NMDA) or glutamate- (Glu-) induced excitotoxicity by the ethanol extract of *Scutellaria baicalensis* (S.B.). Lactate dehydrogenase (LDH) activities given off by the damaged neurons into the culture media were measured at 20–24h after the exposure. Data were calculated as percent of control LDH activity released into the Glu-treated culture medium. Cultures were exposed for 20 min to 350 mM Glu or NMDA in the presence of various concentrations (1, 10, 25, 50, and 100 $\mu$g/mL) of the AGR extract and maintained as described in the experimental part.

![Graph](image2.png)

**Figure 3:** *Scutellaria baicalensis* (S.B.) extract inhibits the specific binding of $[^3]$HMDL 105,519 to the glycine site of the N-methyl-D-aspartate (NMDA) receptor. Different concentrations of the extract were employed. Nonspecific binding determined in the presence of 2 mM glycine was subtracted from the total binding. Data were calculated as percent of the control binding measured in the absence of the extract.
10, 25, 50, and 100 receptor. Different concentrations of the extract (here we used control binding). These phytochemicals vone), baicalin (baicalein-7-O-glucuronide), and wogonin various flavonoids such as baicalein (5,6,7-trihydroxyfla-

Figure 4: The ethanol extract of Scutellaria baicalensis inhibits the binding of [3H]MK-801 to the N-methyl-D-aspartate (NMDA) receptor. Different concentrations of the extract (here we used 10, 25, 50, and 100 μg/mL) were employed. Nonspecific binding determined in the presence of 200 μM MK-801 was subtracted from the total binding. Data were calculated as percent of the control binding measured in the absence of the extract. Each point represents the mean ± S.D. * P < 0.05; ** P < 0.01 (compared with control binding).

already reported in the literature that assessment of [3H]MK-801 binding is a hallmark of the functional state of NMDA receptors [23].

4.5. LC-ESI-MSMS Analysis/HPLC Analysis. The phytochemical analysis of the S. baicalensis ethanol extract was carried out by LC-ESI-MS in combination with HPLC-DAD analytical techniques. The extract was run under both positive and negative ESI-MS conditions and it showed several major and minor ionic fragments. The six chemical constituents identified were baicalein, baicalin, wogonin, wogonoside, scutellarin, and Oroxylin A (Figure 5). These phytochemicals present in the root part of S. baicalensis have already been reported previously in this and other plant species of the Scutellaria genus. The total ion MS chromatogram (TIC), HPLC profile, and HPLC-3D plot are shown in Figures 6, 7, and 8, respectively. Fragmentation of the major peaks was used for the identification of compounds. The identification of the chemical compounds was also carried out by comparing the molecular ion peaks along with the MS fragmentation pattern with those of the literature.

5. Discussion

Scutellaria baicalensis Georgi has been reported to contain various flavonoids such as baicalein (5,6,7-trihydroxyflavone), baicalin (baicalein-7-O-glucuronide), and wogonin (5,7-dihydroxy-8-methoxyflavone). These phytochemicals are proven potent antioxidants and have been reported to quench reactive oxygen species (ROS) to protect neuronal cells from oxidative damage in cerebral ischemia/reperfusion. These flavonoids have also been reported to inhibit lipid peroxidation of neuronal membranes and to prevent Glu-induced excitotoxicity. Wogonin, as an inhibitor of the CNS inflammation, has been reported to suppress NO (nitric oxide) production and iNOS (inducible nitric oxide synthase) activation in cultured rat astrocytes. Bai-

6. Conclusion

In conclusion, our study convincingly demonstrated that the neuroprotective effect of the S.B. ethanol extract on the Glu or NMDA induced excitotoxicity was mediated through the blockade of NMDA receptors. So this extract can act as NMDA receptor antagonist and these may find potential applications for the discovery of neuroprotective agents from plant sources.
Figure 5: Phytochemical compounds identified in the bioactive ethanol extract of the root of *Scutellaria baicalensis* Georgi. These compounds were identified using LC-ESI-MS analysis.

Figure 6: LC-MS chromatogram of the bioactive ethanol extract of the root of *Scutellaria baicalensis* Georgi.

Figure 7: HPLC chromatogram of the ethanol extract of *Scutellaria baicalensis*.

Figure 8: HPLC-3D plot of the ethanol extract of *Scutellaria baicalensis*.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

This research was supported by Zhejiang Province Traditional Chinese Medicine Scientific Research Plans (Project no. 2013ZA077) and Zhejiang Province Education Department Scientific Research Projects (Project no. Y201328185).

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