ORIGINAL RESEARCH

Ginsenoside Rd Protects Neurons Against Glutamate-Induced Excitotoxicity by Inhibiting Ca^{2+} Influx

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Abstract Our previous studies have demonstrated that ginsenoside Rd (GSRd), one of the principal ingredients of *Pana notoginseng*, has neuroprotective effects against ischemic stroke. However, the possible mechanism(s) underlying the neuroprotection of GSRd is/are still largely unknown. In this study, we treated glutamate-injured cultured rat hippocampal neurons with different concentrations of GSRd, and then examined the changes in neuronal apoptosis and intracellular free Ca^{2+} concentration. Our MTT assay showed that GSRd significantly increased the survival of neurons injured by glutamate in a dose-dependent manner. Consistently, TUNEL and Caspase-3 staining showed that GSRd attenuated glutamate-induced cell death. Furthermore, calcium imaging assay revealed that GSRd significantly attenuated the glutamate-induced increase of intracellular free Ca^{2+} and also inhibited NMDA-triggered Ca^{2+} influx. Thus, the present study demonstrates that GSRd protects the cultured hippocampal neurons against glutamate-induced excitotoxicity, and that this neuroprotective effect may result from the inhibitory effects of GSRd on Ca^{2+} influx.

Keywords Ginsenoside Rd · Glutamate · Excitotoxicity · Calcium influx · NMDA receptor

Introduction

Notoginseng, the root of *Pana notoginseng* (Burk.) F.H. Chen (Araliaceae), is a traditional Chinese herbal medicine and has widely been used in Asia for thousands of years. Modern pharmacological researches have demonstrated that notoginseng can treat cardiovascular and cerebrovascular diseases because of its effects of promoting blood circulation, regulating blood pressure, improving ventricular diastolic function, and so on (Ohtani et al. 1987; Yoshikawa et al. 1997; Wang et al. 2006b; Xia et al. 2011). Saponins are the main active ingredients in notoginseng, commonly known as ginsenosides and notoginsenosides (Wang et al. 2006a). Ginsenoside Rd (GSRd, Fig. 1) is one of the major active components of ginsenosides (Nah et al. 2007; Yang et al. 2007a).

Recently, GSRd has been found to have various pharmacological effects, such as anti-convulsion (Lian et al. 2006), anti-aging (Zhao et al. 2009), and regulation of immune response (Yang et al. 2007b). In our randomized, double-blind, placebo-controlled, phase II multicenter trial, we found that GSRd shows efficacy and safety for the treatment of acute ischemic stroke (Liu et al. 2009). Moreover, we found that GSRd can attenuate the cytotoxicity of PC12 cells induced by hydrogen peroxide, protect against the injury of cultured hippocampal neurons induced by oxygen–glucose deprivation (Ye et al. 2008; Ye et al. 2009), and attenuate apoptosis and inflammation after

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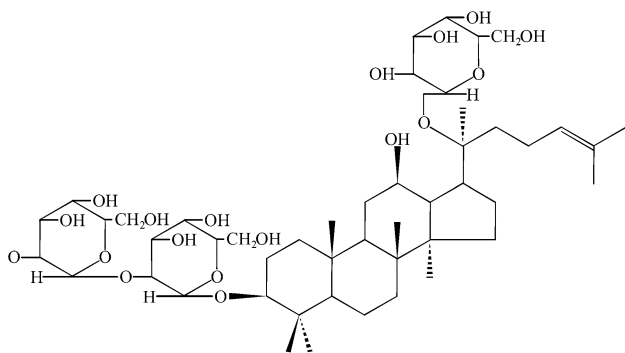University, 17 Changle Xi Road, Xi'an 710032, China

Fig. 1 The chemical structure of ginsenoside Rd. The molecular formula is $C_{48}H_{82}O_{18}\cdot3H_2O$, and the molecular weight is 1001

transient focal ischemia (Ye et al. 2011a; Ye et al. 2011b). All of these results indicate that GSRd may be a promising neuroprotectant with distinctive advantages for the treatment of ischemic stroke. However, we still lack knowledge about the mechanism(s) underlying this assumed role. Previous studies have reported that GSRd inhibited Ca^{2+} entry through receptor-operated Ca^{2+} channels (ROCC) and store-operated Ca^{2+} channels (SOCC), without affecting voltage-dependent Ca^{2+} channels (VDCC) in vascular smooth muscle cells (Guan et al. 2006). In addition, GSRd can reverse basilar hypertrophic remodeling in stroke-prone renovascular hypertensive rats by inhibiting voltage-independent Ca^{2+} entry (Cai et al. 2009). These results suggest that GSRd may serve as a potential Ca^{2+} channel blocker.

Glutamate receptor-mediated neuronal excitotoxicity is one of most important factors responsible for CNS injury. Among glutamate receptors, it has been accepted that the NMDA receptor, one of the ionotropic glutamate receptors, plays the major role in glutamate-induced excitotoxicity. The NMDA receptor is a receptor-gated ion channel and has high Ca^{2+} permeability. Under pathological conditions, such as ischemia and hypoxia, excessive glutamate is released and accumulated, subsequently over-stimulating the NMDA receptor and causing a large amount of Ca^{2+} influx, which leads to calcium overload and eventually results in excitotoxicity (Choi and Rothman 1990; Ankarcrona et al. 1995; Lo et al. 2003; Lipton 2006). A line of evidence shows that several ginsenosides, such as GSRg2, GSRb1, GSRg3, and GSRd, can protect against glutamate-induced injury of PC12 cells or cultured cortical neurons (Kim et al. 1998; Li et al. 2007; Li et al. 2010). Given that GSRd may be a potential Ca^{2+} channel blocker in the vascular smooth muscle cells (Guan et al. 2006), it is interesting to explore whether GSRd also serves as a blocker of the NMDA receptor consequently exerting its neuroprotective effects by inhibiting Ca^{2+} influx.

In the present study, we injured cultured hippocampal neurons with high concentrations of glutamate, and observed the effects of GSRd on the cell death and Ca^{2+} influx. Our data showed that GSRd significantly attenuated glutamate-induced neurotoxicity and inhibited Ca^{2+} influx.

Materials and Methods

Materials

Ginsenoside Rd with a purity of 98% was obtained from Tai-He Biopharmaceutical Co. Ltd. (Guangzhou, China). GSRd stock solutions were prepared in saline containing 10% 1,3-propanediol (v/v). The reagents for cell culture were purchased from GIBCO (Grand Island, NY, USA). Cleaved Caspase-3 antibody and in situ cell death detection (TUNEL) kit were from Cell Signaling Technology (Danvers, MA, USA) and Roche Diagnostics GmbH (Mannheim, Germany), respectively. Fluo-4/AM and Pluronic F-127 were purchased from Invitrogen (Eugene, OR, USA). Poly-L-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-glutamic acid, N-methyl-D-aspartic acid (NMDA), and Dizocilpine maleate (MK-801) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Grouping

Hippocampal cell cultures were prepared from embryonic day 18 SD rat embryos as previously described (Kaech and Banker 2006). In brief, hippocampi were isolated and dissociated into cell suspensions by trypsinization and mechanical trituration. The dissociated cells were plated onto poly-L-lysine-coated 96-well plates, coverslips, or 35-mm culture dishes at the density of $1–5 \times 10^5$ cells/cm². The cells were cultured in Neurobasal medium containing 2% B27, 0.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C and 5% CO_2. Culture medium was changed twice a week. Experiments were performed after 10–14 days of culture.

Four experimental groups were established: (1) Control group, in which the neurons were untreated with any drugs; (2) Glutamate group, in which the neurons were treated with 500 μM glutamate for 3 h, and then maintained in culture medium for 6 h (Ankarcrona et al. 1995); (3) GSRd group, in which the neurons were co-treated with 500 μM glutamate and GSRd at different concentrations (0.1, 1, or 10 μM) for 3 h followed by 6 h of treatment with GSRd alone; and (4) MK-801 group, in which 10 μM MK-801 was used to replace GSRd in the GSRd group.

MTT Assay

Cell viability was examined by MTT assay as previously described (Denizot and Lang 1986). In brief, hippocampal neurons were seeded in 96-well plates at a density of

5×10^5 cells per well. After experimental treatments, 10 µl of MTT solution was added to each well at a final concentration 5 mg/ml. After 4 h of incubation at 37°C, the medium was replaced with 150 µl dimethyl sulfoxide to dissolve the formazan crystals. Then, the plates were shaken for 10 min, and the absorbance was assessed at 490 nm using a microplate reader. Absorbance was represented as a percentage of control.

TUNEL Assay and Caspase-3 Immunostaining

TUNEL and Caspase-3 stainings were used for determining apoptotic cells. Neurons grown on glass coverslips were fixed in 4% paraformaldehyde for 30 min at room temperature. For TUNEL staining, neurons were manipulated according to the introduction of the kit. For Casaspase-3 immunostaining, the cells were incubated using rabbit anti-cleaved Caspase-3 antibody (1:500) overnight at 4°C. After washing three times in PBS, the cells were incubated using biotinylated anti-rabbit IgG (1:200) for 2 h, and then using Cy2-conjuncted streptavidin (1:300) for 1 h. All the cells were counterstained with Hoechst 33342. TUNEL- or Caspase-3-positive cells were observed and counted under a fluorescence microscope (Leica, Germany).

Intracellular Ca^{2+} Imaging

Intracellular calcium concentration $[Ca^{2+}]_i$ was determined by monitoring the fluorescence intensity of a calcium indicator Fluo-4/AM under a confocal laser scanning microscope (Olympus, Tokyo, Japan). In brief, neurons were incubated in HEPES buffer containing 5 µM Fluo-4/AM and 0.001% Pluronic F-127 for 50 min at 37°C. After being washed three times with HEPES buffer, the neurons were incubated at 37°C in the same solution for 30 min to allow for complete de-esterification of Fluo-4/AM. The cells were transferred into a recording chamber mounted on the confocal laser scanning microscope. The fluorescent images were scanned using indicated wavelength settings (excitation at 488 nm and emission at 525 nm). The fluorescence intensity was recorded every 10 s and quantified by dynamic single-cell analysis, as described by previous studies (Rubart et al. 2003; Zhou et al. 2008; Samways et al. 2009). In each group, at least 10 neurons were recorded from three independent assays. $[Ca^{2+}]_i$ was represented by the relative fluorescence intensity, $\Delta F/F_0 = (F-F_0)/F_0$, where F is the fluorescence intensity measured after drug application, and F_0 is the baseline (Samways et al. 2009).

Statistical Analysis

Data were expressed as mean ± SEM and compared using ANOVA followed by LSD post test. The software SPSS

13.0 was used to analyze all these data, and statistical significance was defined as $P < 0.05$.

Results

GSRd Increases Cell Viability After Glutamate-Induced Excitotoxicity

The MTT assay was employed to assess the cell viability of glutamate-injured hippocampal neurons (Fig. 2). Compared with the medium control group, 500 µM glutamate significantly decreased the cell viability to 48.70 ± 1.39% ($P < 0.01$). Treatment of glutamate-injured neurons with 0.1, 1, and 10 µM GSRd increased the cell viability to 63.35 ± 0.92% ($P < 0.05$), 86.33 ± 1.34% ($P < 0.01$), and 91.41 ± 1.78% ($P < 0.01$), respectively. The NMDA receptor antagonist MK-801 protected glutamate-injured neurons and increased the cell viability to 95.35 ± 1.33%. In addition, we found that GSRd or propanediol vehicle did not affect the survival of normal hippocampal neurons (data not shown), consistent with our previous study (Ye et al. 2009).

GSRd Decreases Glutamate-Induced Apoptosis

TUNEL staining was performed to determine glutamate-induced apoptosis of hippocampal neurons (Fig. 3a, b). In the control group, only a few TUNEL-positive cells were observed. 500 µM glutamate markedly increased the
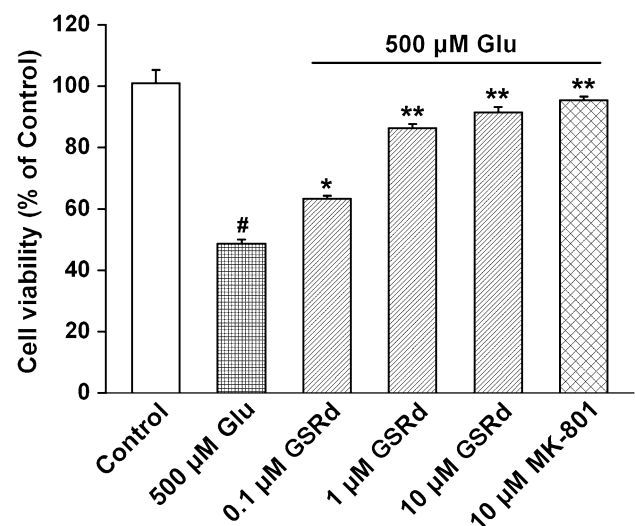

Fig. 2 The effects of GSRd on cell viability of hippocampal neurons after glutamate exposure. MTT assay showed that GSRd ameliorated the cell viability in a dose-dependence manner after glutamate-induced cytotoxicity. The data were presented relative to the control and shown as mean ± SEM. Glu, glutamate. #$P < 0.05$ versus the control group; *$P < 0.05$, **$P < 0.01$ versus glutamate group

number of TUNEL-positive cells to $48.01 \pm 1.00\%$ ($P < 0.05$, vs the control group) while 10 μM GSRd and MK-801 attenuated the number of glutamate-induced apoptotic cells to $23.82 \pm 1.31\%$ ($P < 0.05$, vs Glutamate group), and $20.82 \pm 0.57\%$ ($P < 0.05$, vs Glutamate group), respectively. In addition, cleaved Caspase-3 expression was also examined to determine apoptosis (Fig. 3c, d). Similar to the results of TUNEL staining, GSRd and MK-801 remarkably decreased the number of glutamate-induced apoptotic cells ($P < 0.05$). These results suggest that GSRd can protect hippocampal neurons against glutamate-induced neurotoxicity.

GSRd Attenuates Glutamate- and NMDA-Induced Ca^{2+} Influx

Since intracellular Ca^{2+} overload plays an important role in the process of glutamate receptor-mediated excitotoxicity, we then explored whether GSRd affected $[Ca^{2+}]_i$ induced by glutamate stimulation. Calcium imaging results (Fig. 4) showed that compared with the control group, 500 μM glutamate triggered a rapid increase of intracellular Fluo-4 fluorescence intensity by about 2.5 folds within 2 min and by 3.5 folds afterward. Compared with glutamate group, 10 μM GSRd markedly reduced glutamate-induced fluorescence intensity by more than fivefolds after drug administration. In addition, MK-801 also inhibited the fluorescence intensity increase stimulated by glutamate.

To further confirm that GSRd suppresses Ca^{2+} overload during excitotoxicity, we directly treated the hippocampal neurons with NMDA, a specific agonist of the NMDA receptor and observed the effects of GSRd on NMDA-triggered Ca^{2+} influx. Our calcium imaging results (Fig. 5) showed that 100 μM NMDA significantly increased intracellular Fluo-4 fluorescence intensity by about 1.5–2-folds, compared with the control group. Compared with NMDA group, GSRd significantly decreased NMDA-induced fluorescence intensity by two–fourfolds. Similar results were observed when MK-801 was added (Fig. 5). These results indicate that GSRd may inhibit glutamate- and NMDA-triggered Ca^{2+} influx and subsequently attenuate excitotoxicity.

Discussion

Our previous in vitro and in vivo studies have demonstrated that GSRd has neuroprotective effects on injured neurons (Ye et al. 2008; Liu et al. 2009; Ye et al. 2009; Ye et al. 2011a; Ye et al. 2011b). In the present study, we showed that GSRd attenuated glutamate- and NMDA-induced

excitotoxic injury of cultured rat hippocampal neurons by inhibiting Ca^{2+} influx.

Glutamate-induced excitotoxicity is one of the main factors responsible for the neuronal death in a variety of CNS disorders. It is therefore reasonable to explore whether the neuroprotective effects of GSRd result from the inhibition of glutamate-triggered excitotoxicity. Based on a previous report (Ankarcrona et al. 1995), we exposed cultured hippocampal neurons to high concentration (500 μM) of glutamate for 3 h followed by 6-h recovery in normal medium. In this model, both necrosis and apoptosis occurred in the neuronal death, and it can correctly imitate the excitotoxicity after acute ischemic stroke. Our results showed that GSRd markedly ameliorated glutamate-induced decrease of cell viability in a dose-dependent manner. Moreover, glutamate-induced apoptosis was also attenuated after treatment with GSRd, consistent with a previous study (Li et al. 2010). Therefore, the present data indicate that GSRd attenuates glutamate-induced excitotoxic injury.

After CNS injury, excessive accumulation of glutamate can over-stimulate glutamate receptors and subsequently trigger intracellular Ca^{2+} overload, which initiates a series of downstream lethal events including oxidative stress, mitochondrial dysfunction, and inflammation (Dirnagl et al. 1999; Szydlowska and Tymianski 2010). In the present study, we took advantage of calcium imaging techniques to show that GSRd significantly attenuated glutamate-induced increase of intracellular calcium, namely, by blocking Ca^{2+} overload. This finding may account for the results of our previous studies, in which GSRd was shown to suppress oxidative stress-induced impairment and stabilize the MMP in oxygen–glucose deprivation-injured rat hippocampal neurons (Ye et al. 2009), hydrogen peroxide-injured PC12 cell line (Ye et al. 2008), and MCAO-induced transient focal ischemia in rats (Ye et al. 2011a; Ye et al. 2011b). Similarly, another study has also shown that GSRd has an antioxidative effect on hydrogen peroxide-injured cultured astrocytes (Lopez et al. 2007). Although the latter study indicates that GSRd might function as an ROS scavenger to exert its protective effects, there is no direct evidence so far that GSRd directly scavenges free radicals.

Glutamate activates three classes of ionotropic receptors, namely, NMDA, AMPA, and kainite receptors, among which the NMDA receptors are mainly responsible for glutamate-induced excitotoxicity because of their high Ca^{2+} permeability (Dingledine et al. 1999; Paoletti and Neyton 2007). In order to investigate whether Ca^{2+} influx blockage by GSRd is mediated by affecting NMDA receptors, we examined the effects of MK-801, a non-competitive NMDA receptor antagonist which binds to a site within the ion channel on $[Ca^{2+}]_i$ after exposure to glutamate. An NMDA
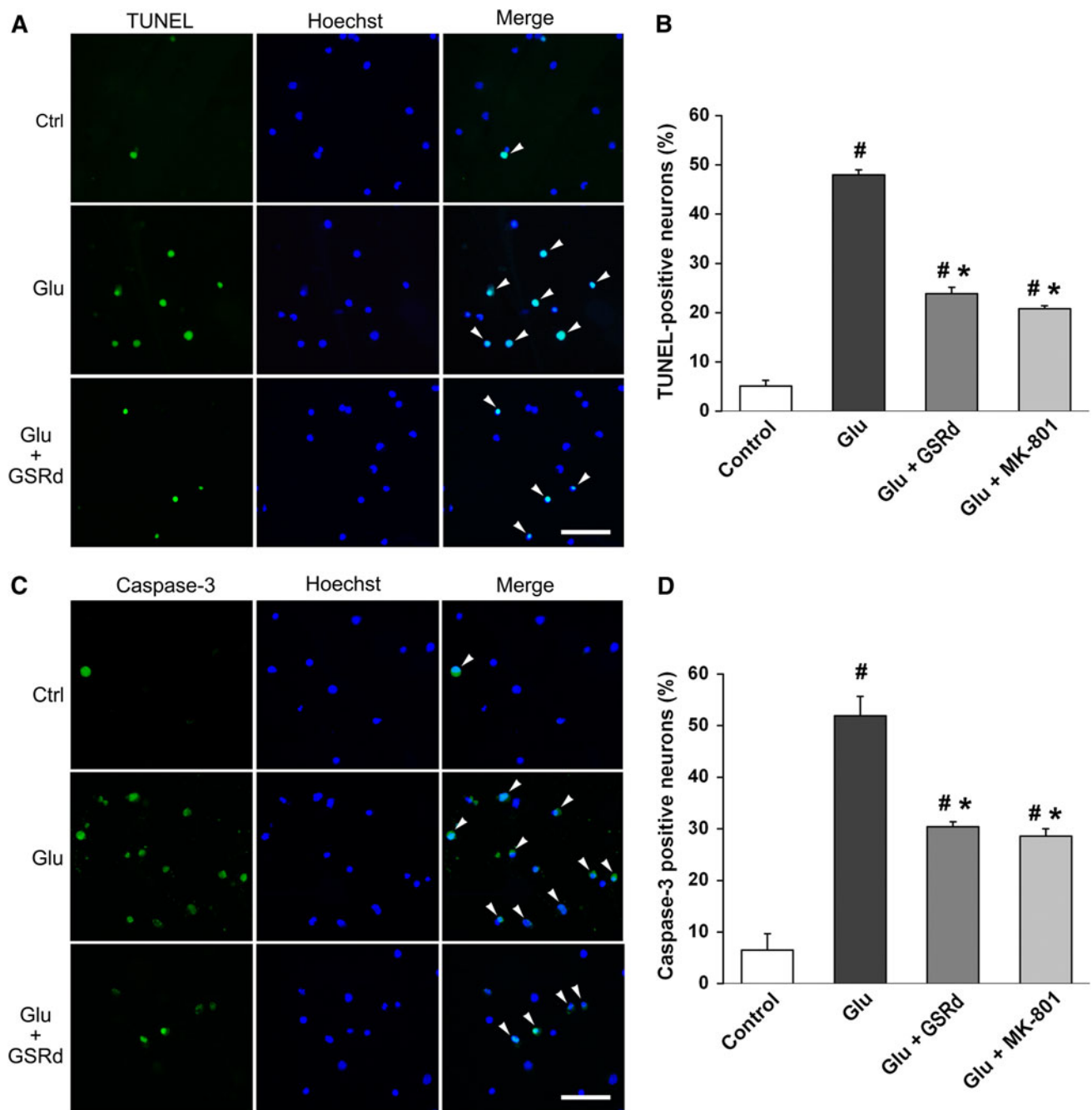
Fig. 3 Effects of GSRd on glutamate-induced apoptosis determined by TUNEL staining and Caspase-3 immunocytochemistry. **a** Representative photomicrographs illustrate TUNEL (*green*) staining in the control, glutamate, and GSRd groups (*left panels*). Hoechst 33342 (*blue*) staining indicates the total cell number (*middle panels*). The merge panels show the double-stained neurons with TUNEL and Hoechst 33342 indicated by *arrowheads* (*right panels*). *Scale bar*: 50 μm. **b** Quantitation of TUNEL-positive neurons in different groups. #$P < 0.05$ versus the control group; *$P < 0.05$ versus glutamate group. **c** Representative photomicrographs show Caspase-3 (*green*) staining in the control, glutamate, and GSRd groups (*left panels*). Hoechst 33342 (*blue*) staining shows the total cell number (*middle panels*). The merge panels show the double-stained neurons with Caspase-3 and Hoechst 33342 indicated by *arrowheads* (*right panels*). *Scale bar*: 50 μm. **d** Quantitation of Caspase-3-positive neurons in different groups. #$P < 0.05$ versus the control group; *$P < 0.05$ versus glutamate group

receptor agonist was also utilized to test this possibility. Our results showed that GSRd attenuated glutamate-/NMDA-induced increase of $[Ca^{2+}]_i$ to the same extent as MK-801.

These results imply that GSRd may affect NMDA receptors to exert its neuroprotection. A line of evidence has shown that GSRd can inhibit (α-adrenoceptor-operated Ca^{2+} influx
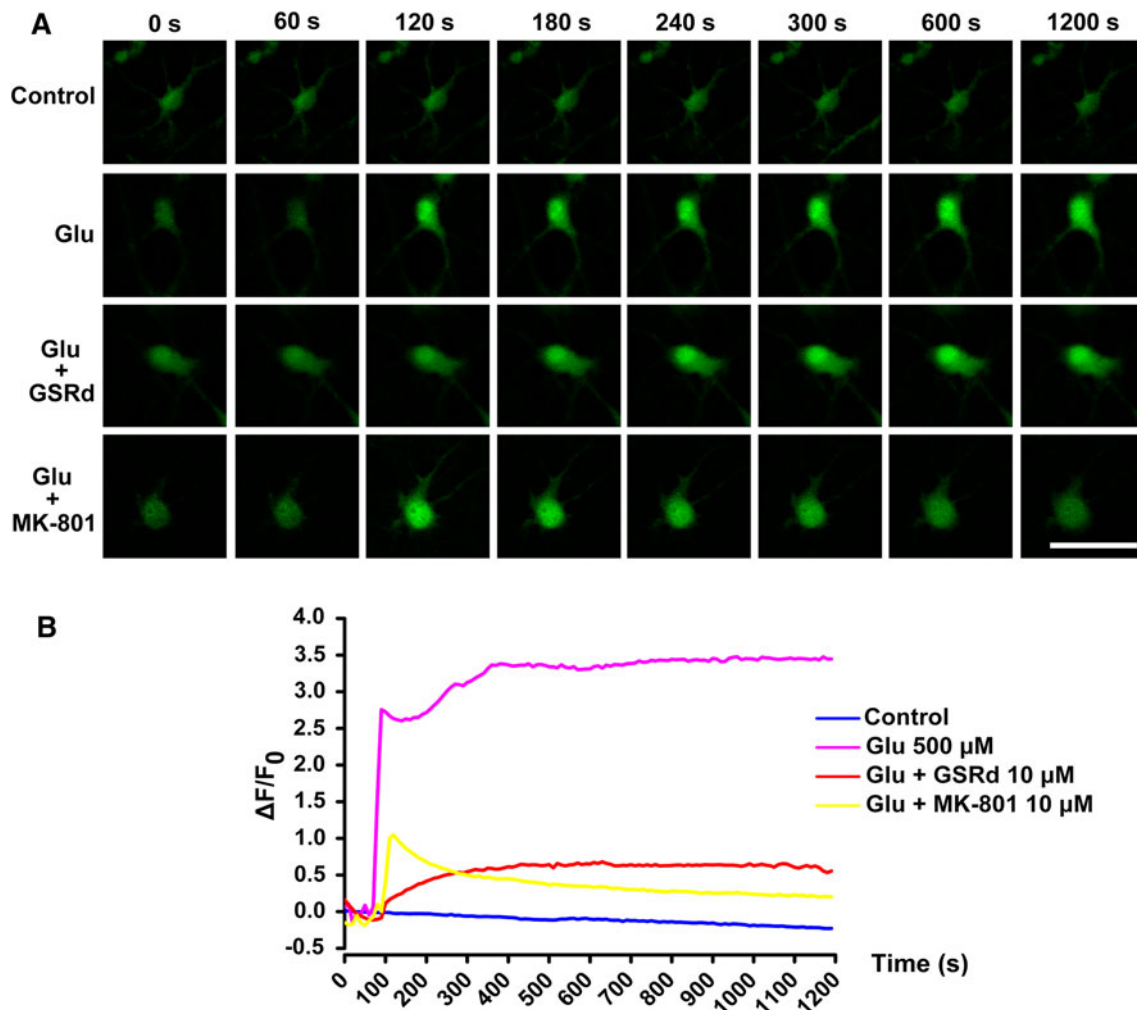
Fig. 4 Effects of GSRd on glutamate-induced calcium influx in cultured hippocampal neurons. **a** Representative panels show the dynamic changes of intracellular Fluo-4 fluorescence intensity in the control, glutamate, GSRd, and MK-801 groups at different time points (0, 60, 120, 180, 240, 300, 600, and 1200 s). *Scale bar* 50 μm.

b Representative traces show the real-time dynamic changes of relative fluorescence intensity on a single neuron from each group. Note that GSRd and MK-801 significantly attenutate glutamate-induced increase in fluorescence intensity

without affecting KCl-induced increase of intracellular Ca^{2+} in vascular smooth muscle cells (Guan et al. 2006), and block voltage-independent Ca^{2+} entry to reverse basilar hypertrophic remodeling in stroke-prone renovascular hypertensive rats (Cai et al. 2009). These cited studies and our present findings all suggest that GSRd may be served as a selective receptor-gated Ca^{2+} channel blocker.

To date, numerous clinical trials targeting glutamate receptors have failed because of their inefficacy or intolerable side effects in stroke patients (Kalia et al. 2008). In contrast, our phase II (Liu et al. 2009) and III (unpublished data) multicenter clinical trials show the efficacy and safety of GSRd in treating acute ischemic stroke. This encourages us to explore the possible mechanism(s) underlying GSRd neuroprotection. In the present study, we revealed that GSRd protected neurons against excitotoxicity by inhibiting

Ca^{2+} influx triggered by glutamate or NMDA. Given the facts that GSRg3 and GSRh2, the members of proto-panaxadiol dammarane glycosides same as GSRd, can antagonize NMDA receptors (Kim et al. 2002; Kim et al. 2004; Lee et al. 2006), we propose that GSRd may also serve as a direct modulator in regulating NMDA receptor functions. However, the data presented cannot exclude the possibility that GSRd may indirectly regulate glutamate-/NMDA-induced Ca^{2+} influx, which involves the release of Ca^{2+} from intracellular store mediated by G-protein signaling pathway or transient receptor potential channels. Therefore, further studies are required to clarify regulatory mechanisms of GSRd on Ca^{2+} influx mediated by glutamate receptors.

In summary, the present study provides the evidence that GSRd protects rat hippocampal neurons against
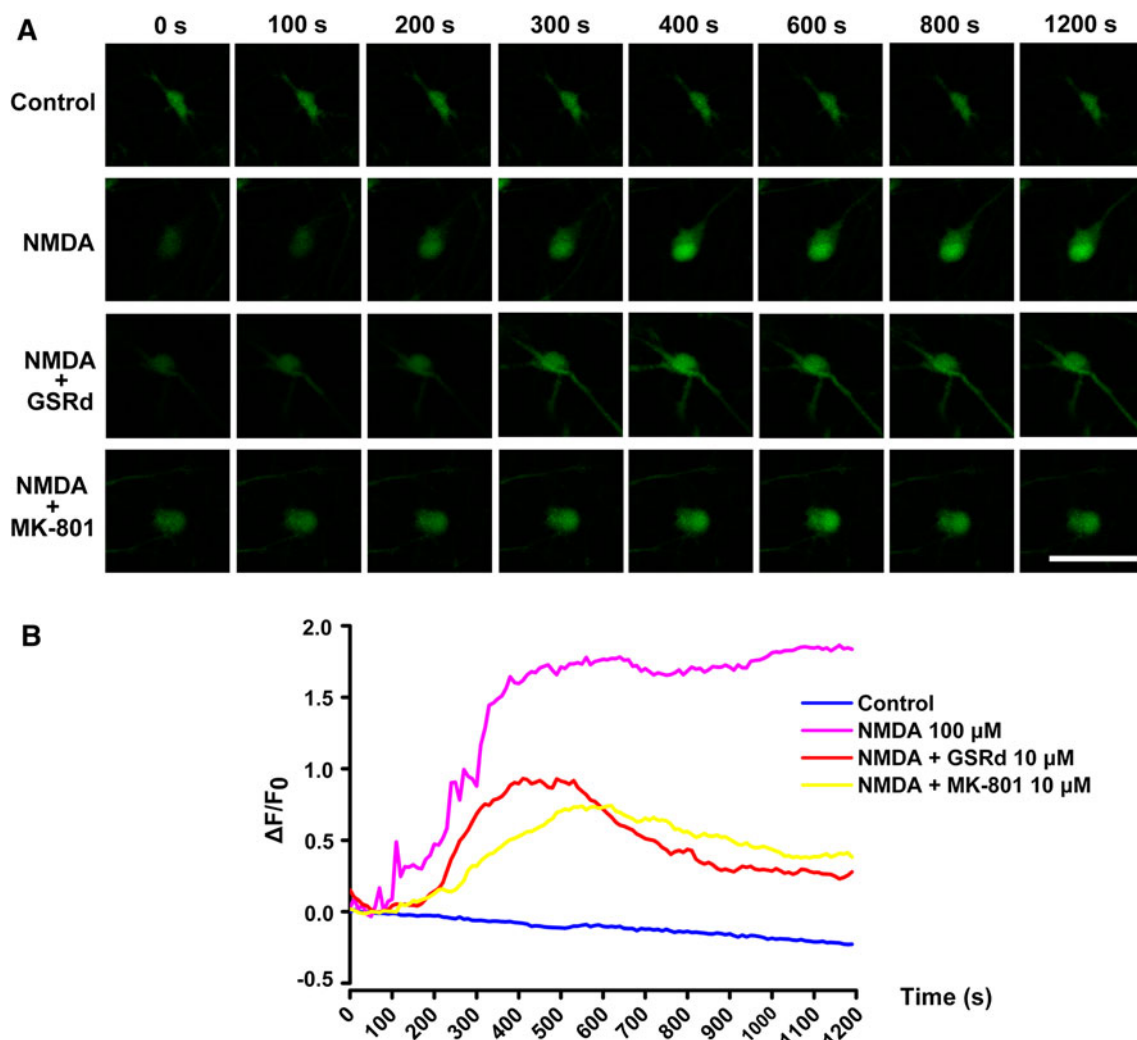
Fig. 5 Effects of GSRd on NMDA-induced calcium influx in cultured hippocampal neurons. **a** Representative panels show the dynamic changes of intracellular Fluo-4 fluorescence intensity in the control, NMDA, GSRd, and MK-801 groups at different time points (0, 100, 200, 300, 400, 600, 800, and 1200 s). *Scale bar* 50 μm.

b Representative traces show the real-time dynamic changes of relative fluorescence intensity on a single neuron from each group. Note that GSRd and MK-801 markedly attenutate NMDA-induced increase in fluorescence intensity

glutamate-induced excitotoxicity, possibly by attenuating Ca^{2+} influx.

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