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Sulforhodamine 101 induces long-term potentiation of intrinsic excitability and synaptic efficacy in hippocampal CA1 pyramidal neurons

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Abstract

Sulforhodamine 101 (SR101) has been extensively used for investigation as a specific marker for astroglia *in vivo* and activity-dependent dye for monitoring regulated exocytosis. Here, we report that SR101 has bioactive effects on neuronal activity. Perfusion of slices with SR101 (1 μ M) for 10 min induced long-term potentiation of intrinsic neuronal excitability (LTP-IE) and a long-lasting increase in evoked EPSCs (eEPSCs) in CA1 pyramidal neurons in hippocampal slices. The increase in intrinsic neuronal excitability was a result of negative shifts in the action potential (AP) threshold. The *N*-methyl *D*-aspartate receptor (NMDAR) antagonist, AP-5 (50 μ M), blocked SR101-induced LTP-IE, but glutamate receptor blockers, AP-5 (50 μ M), MCPG (200 μ M), and MSOP (100 μ M), only partially blocked SR101-induced potentiation of eEPSCs. SR101 induced an enhancement of evoked synaptic NMDAR currents, suggesting that SR101 enhances activation of synaptic NMDARs. SR101-induced LTP-IE and potentiation of synaptic transmission triggered spontaneous neuronal firing in slices and *in vivo* epileptic seizures. Our results suggest that SR101 is an epileptogenic agent that long-lastingly lowers the AP threshold to increase intrinsic neuronal excitability and enhances the synaptic efficacy to increase synaptic inputs. As such, SR101 can be used as an experimental tool to induce epileptic seizures.

Keywords

SR101 dye; action potential threshold; EPSC; intrinsic excitability; NMDA receptor; seizure

Sulforhodamine 101 (SR101), as an amphoteric rhodamine, is a water-soluble red fluorescent dye that has been used extensively for investigating neuronal morphology (Schneider, 1989; Ehinger et al., 1994; Kjaerulff et al., 1994; Miller et al., 2001), preparing fluorescent liposomes (VanderMeulen et al., 1992; Skopinskaia et al., 2000), monitoring regulated exocytosis and endocytosis (Wang and Goren, 1987; Keifer et al., 1992; McLaren

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et al., 1993; Kjaerulff et al., 1994; Takahashi et al., 2002), and quenching the fluorescence background of FM 1-43 in slice experiments (Pyle et al., 1999; Winterer et al., 2006). Recently, SR101 has been reported to be a specific marker of astroglia *in vivo* (Nimmerjahn et al., 2004; Winship et al., 2007) and in slices (Kafitz et al., 2008). However, effects of SR101 on neuronal activities have not been examined thoroughly, especially for SR101-induced long-term changes in neuronal excitability and synaptic efficacy. In this study, we examined long-term effects of SR101 on synaptic efficacy and intrinsic neuronal excitability. We found that perfusion of slices with SR101 (1 μ M) for 10 min induced long-term potentiation of intrinsic excitability (LTP-IE) and synaptic efficacy mainly through enhancing activation of synaptic glutamate NMDARs. Furthermore, injection of SR101 into the brain of rats induced epileptic seizures *in vivo*.

EXPERIMENTAL PROCEDURES

Slice Preparation

Brain slices were prepared as described previously (Kang et al., 1998). Briefly, 15- to 25-day-old (P15-P25) Sprague-Dawley rats were anaesthetized with sodium pentobarbitone (55 mg/kg) and then decapitated. Transverse brain slices of 300 μ m thickness were cut with a vibratome (TPI, St Louis, MO, USA) in a cutting solution containing (mM): 2.5 KCl, 1.25 NaH_2PO_4 , 10 MgSO_4 , 0.5 CaCl_2 , 10 glucose, 26 NaHCO_3 and 230 sucrose. Slices containing the hippocampus were incubated in artificial cerebrospinal fluid (ACSF) gassed with 5% CO_2 /95% O_2 for 1-4 hours and then transferred to a recording chamber (1.5 ml) that was perfused continually (3 ml/min) with ACSF gassed with 5% CO_2 /95% O_2 at room temperature (23-24°C). ACSF contained (mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 2 MgCl_2 , 2 CaCl_2 , 10 glucose and 26 NaHCO_3 (pH at 7.4 when gassed with 5% CO_2 /95% O_2).

Whole-cell Patch-clamp Recording

Cells were visualized with a 60 $\times$ /0.90 W water immersion lens on an Olympus BX51 upright microscope (Olympus Optical Co., NY, USA) equipped with infra-red differential interference contrast (IR-DIC) optics. Patch electrodes with a resistance of 4 - 7 $\text{M}\Omega$ were pulled from KG-33 glass capillaries (inner diameter 1.0 mm, outer diameter. 1.5 mm, Garner Glass Co., Claremont, CA, USA) using a P-97 electrode puller (Sutter Instrument Co., Novato, CA, USA). Pyramidal neurons in the CA1 pyramidal layer were identified by their DIC morphology and electrophysiological properties as described previously (Kang et al., 1998). Pyramidal neurons were voltage-clamped at -60 mV or current-clamped without holding currents (Hamill et al., 1981) using an Axopatch 200B (Axon Instruments Inc.). If recordings in cells showed a seal resistance < 5 $\text{G}\Omega$, a holding current > -100 pA, or an increase in the series resistance >10% of control, the data were rejected. The patch pipette filling solution for whole-cell recording contained (in mM): 123 K-gluconate, 10 KCl, 1 MgCl_2 , 10 HEPES, 1 EGTA, 0.1 CaCl_2 , 1 ATP, 0.2 GTP, and 4 glucose (pH adjusted to 7.2 with KOH). To obtain evoked excitatory postsynaptic currents (eEPSCs), a bipolar cluster electrode (FHC, Bowdoin, ME, USA) was placed in the CA1 striatum radiatum to stimulate Schaffer collateral fibers. A single stimulus with 0.8-1.0 μ A intensity and 0.1 ms duration was delivered every 12 second. A negative current injection (-5 mV, 100 ms) was given prior to each stimulus to monitor changes in the membrane input resistance. The input resistance was calculated by the equation: $R_{\text{in}} = \Delta V / \Delta I$, where ΔV is the change in the membrane potential and ΔI the injected current. Pair-pulse stimuli were used to evoke synaptic NMDAR currents and the interval between two pair-pulse stimuli was 80 ms. Recorded signals were filtered through an 8-pole Bessel low-pass filter with 2 kHz cut-off frequency and sampled using the PCLAMP 10.0 acquisition program (Axon Instruments Inc.) with an analog-digital point sample interval of 50 or 100 μ s. The somatic AP threshold

was identified as the membrane potential of the start point of the first AP induced by depolarization steps (Figure 1A, dotted line). The series resistance was compensated by adjusting the series resistance compensation of Axopatch 200B. The value of membrane potentials in this paper was not adjusted by the pipette liquid junction potential that was 13.7 ± 0.1 mV in our experimental conditions according to the methods reported previously (Neher, 1992). Afterhyperpolarization (AHP) was induced by a train of five action potentials evoked by five depolarizing current injections (50 pA and 15 ms duration at 15 ms intervals) and measured from the negative peak to the baseline.

Injection SR101 into the brain of rats

Adult rats (250–300 g) were anesthetized using ketamine (60 mg/kg) and xylazine (10 mg/kg) and then positioned in a stereotaxic frame. The skull was exposed and small holes were drilled to permit insertion of injection pipettes and EEG electrodes. The position of the hole for injection was at stereotaxic coordinates with respect to bregma of AP, -4.0 mm; ML, 4.0 mm; and DV, -3.0 mm. Two 0.075 mm diameter platinum EEG electrodes (MS303/9, Plastics One, Roanoke, VA) were surgically implanted above the dura over the sensorimotor cortex (3.0 – 3.5 mm posterior to bregma, 3.0 – 3.0 mm lateral to midline). After intrahippocampal injection of SR101, electroencephalography (EEG) was continuously recorded up to one week by a DP-311 differential amplifier (Warner Instruments, Hamden, CT) with a low-frequency filter at 0.1 Hz and high-frequency filter at 100 Hz, and pCLAMP 9.2 program (Axon Instruments, Sunnyvale, CA) with an interval of 200 μ s. Rats were also videotaped for behavioral analysis using a PhenoTyper video-based monitoring system (Noldus Information Technology, Leesburg, VA) with Studio software 10 (Pinnacle Systems, Mountain View, CA).

Data Analysis and Chemicals

Data were analyzed using Clampfit 10.0 (Axon Instruments Inc., CA, USA), Origin 6.0 (OriginLab Co., Northampton, MA, USA), and CorelDraw 12.0 (Corel Co. Ontario, Canada) programs. Statistical data are presented as means $\pm$ SEM unless otherwise indicated. DL-2-Amino-5-phosphonovaleric acid (AP-5), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX), ($\pm$)- α -Methyl-(4-carboxyphenyl)glycine (MCPG), 7-hydroxyiminocyclopropan[b]chromen-1 α -carboxylic acid ethyl ester (CPCCOEt), and (R,S)- α -methylserine-O-phosphate (MSOP) were purchased from Tocris Cookson Ltd (Ellisville, MI, USA). SR101, QX-314, bicuculline, and other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).

RESULTS

SR101 induces LTP-IE

To examine effects of SR101 on neuronal activity, we performed whole-cell current-clamp recording in CA1 pyramidal neurons to measure the AP threshold with current-injection steps of 10 pA (Fig. 1A, bottom lines). The AP threshold was defined as the membrane potential at which an AP starts (Fig. 1A, dotted line). Under the control conditions, the AP threshold in pyramidal neurons is in a range of -40 to -52 mV ($n = 10$ neurons). Perfusion of slices with SR101 (1 μ M) for 10 min induced a long-term negative shift in the AP threshold (Fig. 1A-C). SR101-induced negative shifts in the AP threshold (> 5 mV which is the maximal change in the time control) were observed in 100% of tested pyramidal neurons. The AP threshold continued to decrease after the end of SR101 application (Fig. 1A-C, $\bullet$), and attained its maximal decrease in 120 – 140 min. The resting membrane potential (RMP) of neurons showed minor changes during 140 min recording (Fig. 1B and C, $\square$), suggesting that changes in the AP threshold are not due to RMP shifts resulting from junction potential between the pipette solution and intracellular solution. SR101-induced

decrease in the AP threshold was not due to the long-time recording because the time control recording showed relative stable AP threshold (Fig. 1C, ○) and RMP (Fig. 1C, □). These results suggest that SR101 induces LTP-IE. SR101 also induced delayed (>30 min) spontaneous APs in 100% (10 of 10) of tested pyramidal neurons (Fig. 2A). Pooled data indicated that SR101 significantly increased the frequency of sAPs (Fig. 2B, Left panel), but did not depolarize the resting membrane potential of pyramidal neurons (Fig. 1C, ■), suggesting that SR101 induces spontaneous neuronal firing by increasing intrinsic neuronal excitability and synaptic inputs rather than by depolarizing the cell membrane. The dose-response curve (Fig. 2B, Right panel) showed that EC_{50} is $0.22 \pm 0.12 \mu\text{M}$. To test the role of presynaptic neurons in SR101-induced LTP-IE, we examined SR101-induced AP threshold shifts in slices, CA3 of which was cut off. SR101 induced AP threshold shifts in slices without CA3 (Fig. 2C, CA3-cut) were not significantly different from those in whole hippocampus (Fig. 2C, Whole, $P = 0.53$, Student's t -test), suggesting that LTP-IE is not due to changes in presynaptic neurons. Another form of LTP-IE, activity-dependent inhibition of afterhyperpolarization (AHP), has been previously reported (Saar et al., 1998, 2001; Ireland and Abraham, 2002; Melyan et al., 2002; Sourdet et al., 2003; Melyan et al., 2004). We examined the effect of SR101 on AHP induced by five APs that were evoked by five stimuli. In contrast to correlated stimulation that induces changes in both the AP threshold and AHP (Xu et al., 2005), SR101 did not induce significant changes in AHP (Fig. 2D and E, AHP, control: $2.9 \pm 0.2 \text{ mV}$; SR101: $2.7 \pm 0.2 \text{ mV}$; $P = 0.12$, paired t -test, $n = 10$ cells). The increased input resistance has also been reported to contribute to LTP-IE (Campanac et al., 2008; Xu et al., 2005). We next tested the effect of SR101 on the input resistance (R_{in}). SR101 did not significantly alter the input resistance (Fig. 2E, R_{in} , $P = 0.11$, paired t -test, $n = 10$ cells), suggesting that SR101-induced LTP-IE does not involve changes in the input resistance.

Previous studies have indicated that in CA1 pyramidal neurons, glutamate NMDA receptors (NMDARs) are involved in induction of activity-dependent LTP-IE in CA1 pyramidal neurons (Campanac et al., 2008; Daoudal and Debanne, 2003; Xu et al., 2005). To test whether NMDARs are involved in SR101-induced LTP-IE, we used the NMDAR antagonist, AP-5. In the presence of AP-5 ($50 \mu\text{M}$), SR101-induced negative shifts of the AP threshold were significantly reduced (Fig. 3A and B, □, $P < 0.001$, twoway ANOVA, $n = 5$ cells for each group). The AP threshold changes in the presence of APV (Fig. 3B, □, 130 min: $-2.8 \pm 0.7 \text{ mV}$) were not significantly different from time control (Fig. 1C, ○, $2.9 \pm 0.5 \text{ mV}$, $P = 0.83$, Student's t -test, $n = 5$ cells for each group). The results suggest that activation of NMDARs is involved in SR101-induced LTP-IE. To test the time course of NMDAR activation during and after SR101 application in induction of LTP-IE, we applied AP-5 during or after SR101 application. Application of AP-5 started at 30 min after SR101 application significantly reduce the late portion of LTP-IE (Fig. 3C and D, □, 130 min: $-7.2 \pm 0.7 \text{ mV}$, $P < 0.05$ compared with no AP-5, Student's t -test, $n = 5$ cells for each group). AP-5 applied only during SR101 application (washing out AP-5 after SR101 application) induced a late negative shift in the AP threshold (Fig. 3D, ○, 130 min: $-5.4 \pm 0.5 \text{ mV}$) that is significantly different from AP-5 application during whole experiments (Fig. 3B, □, 130 min: $-3.1 \pm 0.4 \text{ mV}$, $P < 0.05$, Student's t -test, $n = 5$ cells for each group). These results suggest that continuing activation of NMDARs leads to the progressive development of LTP-IE.

SR101 induces a long-term increase in eEPSCs

To test effects of SR101 on excitatory synaptic transmission, we recorded evoked EPSCs (eEPSCs) in CA1 pyramidal neurons by stimulating Schaffer collateral fibers. Bicuculline ($10 \mu\text{M}$) was added to the ACSF solution to block GABAergic synaptic transmission during experiments. To monitor changes in the access resistance (R_a), a -5 mV hyperpolarizing

step was given prior to each fiber stimulus (Fig. 4A, R_a). Following SR101 application, the amplitude of eEPSCs increased with time and attained the plateau value about 25 - 30 min following SR101 perfusion (Fig. 4C, ●). The increase lasted for more than 60 min (Fig. 4C, ●), and the long-lasting increase in eEPSCs was significantly different from the time control (Fig. 4C, ○, $P < 0.001$, two-way ANOVA, $n = 8$ cells for each group). The rising time and decay constant of eEPSCs were not altered by SR101 (Fig. 4C, Insert, Rise and Decay, $P = 0.12$ and 0.29 , respectively, paired t -test, $n = 8$ cells)

To test whether glutamate receptors are involved in SR101-induced potentiation of eEPSCs, MCPG (200 μ M), MSOP (100 μ M), and AP-5 (50 μ M) were added into the perfusion solution. In the presence of these blockers, SR101 still induced increases in eEPSCs (Fig. 4B and C, □), but with a reduced amplitude compared with the absence of the blockers ($P < 0.01$, two-way ANOVA, $n = 8$ cells). These results suggest that glutamate receptor antagonists only partially blocked SR101-induced potentiation of eEPSCs, and a relative minor portion was independent on activation of glutamate receptors. To further evaluate the role of NMDA receptors in SR101-induced potentiation of eEPSCs, we used the NMDAR antagonist AP-5 (50 μ M) alone. AP-5 partially blocked SR101-induced potentiation of eEPSCs (Fig. 4C, ■, $P < 0.01$, two-way ANOVA, $n = 5$ cells) similarly as AP-5 together with MCPG and MSOP did (Fig. 4C, □), suggesting that the major portion of SR101-induced potentiation of eEPSCs involves activation of NMDA receptors.

SR101 enhances synaptic NMDAR currents

A number of studies have shown that induction of LTP and LTP-IE (negative shift of the AP threshold) in CA1 pyramidal neurons depends on activation of NMDARs (Bliss and Collingridge, 1993; Nicoll and Malenka, 1999; Choi et al., 2000; Xu et al., 2005). To test whether SR101 influences activation of synaptic NMDARs, we recorded synaptic NMDAR currents evoked by stimulating Schaffer collateral fibers in the ACSF solution containing 5 mM Ca^{2+} , 0 mM Mg^{2+} , bicuculline (30 μ M) and CNQX (10 μ M). Since NMDAR currents contain Ca^{2+} currents (MacDermott et al., 1986) and Mg^{2+} voltage-dependently blocks NMDARs (Mayer et al., 1984), increased extracellular Ca^{2+} (from 2 mM to 5 mM) and decreased Mg^{2+} (from 2 mM to 0 mM) enhanced the amplitude of NMDAR currents. Pair-pulse stimuli with an 80 ms interval were used to evoke synaptic NMDAR currents. During the control period, paired NMDAR currents (Fig. 5A, 1, A1 and A2) were recorded with the mean amplitude of 14.6 ± 3.4 pA (A1) and 28.0 ± 5.1 pA (A2). Following application of SR101 (1 μ M, 10 min), NMDAR currents were significantly enhanced in 10 - 20 min following application SR101 (Fig. 5A and B, 3, $P < 0.01$ for both A1 and A2, paired t -test, $n = 7$ cells). The enhancement of synaptic NMDAR currents lasted longer than 60 min (Fig. 5B, 70-80 min compared with 0-10 min, $P < 0.05$, paired t -test), in accordance with progressive induction of LTP-IE. The rising time was significantly enhanced by SR101 (Fig. 5A, Insert, Rise, $P < 0.05$, paired t -test, $n = 7$ cells), but the decay constant was not altered (Fig. 5A, Insert, Decay, $P = 0.97$, paired t -test). The current enhancement by SR101 was mediated by NMDA receptor/channels because AP-5 abolished the increased current (Fig. 5A, AP-5). The time course of normalized synaptic NMDAR currents showed that synaptic NMDAR currents (A1 and A2) were enhanced similarly (Fig. 5B, ● and ○), and significantly different from the time control group (Fig. 5B, ◇, $P < 0.001$, two-way ANOVA, $n = 7$ cells for each group). We further examined the role of mGluR activation in SR101-induced increases in evoked synaptic NMDAR currents. In the presence of MCPG (200 μ M), MSOP (100 μ M), and the selective mGluR1 antagonist CPCCOEt (100 μ M), SR101 induced a similar enhancement of evoked synaptic NMDAR currents (Fig. 5C and D, □, $P = 0.45$ at 30 min compared with no mGluR blockers, Student t -test, $n = 5$ and 7 cells, respectively), suggesting that activation of mGluRs is not involved in SR101-induced enhancement of synaptic NMDAR currents.

SR101 loading of astrocytes does not induce LTP-IE

Since SR101 loads astrocytes, we next tested whether loading of astrocytes with SR101 is involved in SR101-induced LTP-IE by performing dual whole-cell recordings in pairs of astrocytes and pyramidal neurons. Patch pipettes for astrocytes were filled with the pipette solution containing 10 μ M SR101. Infusion of SR101 into astrocytes did not induce significant changes in the AP threshold (Fig. 6A and B, $\square$) comparing with the time control (Fig. 6A and B, $\circ$, $P = 0.37$, two-way-ANOVA, $n = 8$ cells for each group), suggesting that SR101-loading of astrocytes does not induce LTP-IE. Perfusion of slices with a SR101-fixable analog, Texas Red-hydrazide (10 μ M), induced a similar negative shift in the AP threshold (Fig. 6B, TR), supporting the idea that these dyes directly influence intrinsic neuronal excitability.

SR101 induces epileptic seizures

Since SR101 increases intrinsic neuronal excitability, synaptic inputs, and spontaneous firing in slices, we next tested whether SR101 induces epileptic seizures *in vivo* by injecting SR101 (10 μ M, 2 μ l or 5 μ l) into the hippocampus of rats. After intrahippocampal injection of SR101, EEGs were continuously monitored up to one week. EEG showed that seizure activities appeared after 30 - 60 min and recurred for days after intrahippocampal injection of 5 μ l or 2 μ l of 10 μ M SR101 (Fig. 7A-D, SR101) in all tested rats. Behaviors of rats showed limb jerkin and spasms. As a control, injection of ACSF (2 μ l or 5 μ l) into the hippocampus of rats did not induce any seizure activities (Fig. 7A-D, ACSF). These results suggest that SR101 can be used as an experimental tool to induce epileptic seizures.

DISCUSSION

Sulforhodamine 101 (SR101) has recently been identified as a cell type-selective fluorescent marker of astrocytes, both *in vivo* and in slice preparations. As a result, its use as an experimental tool has rapidly accelerated, based on SR101 as a phenotypic reporter. In the present study, we have found that SR101 is also a potent bioactive compound. In particular, we observed that SR101 induced LTP-IE, increases in synaptic efficacy, and spontaneous firing in hippocampal slices. Strikingly, the concentration of SR101 that caused sustained changes in neuronal excitability was 10-25-fold lower than that typically used to tag live astrocytes. Moreover, injection of SR101 into the adult rat hippocampus evoked epileptic seizures.

One of the key observations in this study was that SR101 increased intrinsic neuronal excitability (LTP-IE). The membrane potential threshold for evoking action potentials decreased slowly and reached its lowest level 120 - 140 min after 10 min exposure to 1 μ M SR101. The reduction of the AP threshold was remarkably consistent, and all cells studied exhibiting a significant decrease in the AP threshold. Neuronal excitability increased because the AP threshold fell from -48.6 ± 0.9 mV to -61.5 ± 2.5 mV. Thus, the threshold for generating AP was just 3 mV higher than the resting membrane potential (-64.7 ± 0.6 mV). In addition, SR101 also induced a long-lasting increase in eEPSCs that further increases synaptic inputs and spontaneous firing. SR101-induced LTP-IE is distinct from LTP-IE resulted from activity-dependent inhibition of afterhyperpolarization (AHP) (Saar et al., 1998, 2001; Ireland and Abraham, 2002; Melyan et al., 2002; Sourdet et al., 2003; Melyan et al., 2004) and increase of input resistance (Campanac et al., 2008). However, SR101 induced LTP-IE shared the similarity of NMDAR-dependency with correlated stimulation-induced LTP-IE (Xu et al., 2005).

SR101 potentiated synaptic NMDAR currents evoked by stimulating Schaffer collateral fibers, supporting that SR101 enhances activation of synaptic NMDARs. The long-lasting

increase in synaptic NMDAR currents (Fig. 5B) suggests that continuing activation of NMDARs induces slowly and progressively developed LTP-IE. Since activation of NMDARs requires synaptic release of glutamate, the SR101 effect depends on spontaneous synaptic activity. Thus, SR101 potentiates activation of NMDARs in a long-lasting manner, leading to progressive development of LTP-IE. The blockade of the late portion of LTP-IE by AP-5 applied after SR101 application (Fig. 3D, □) and the induction of the late portion of LTP-IE by washing out AP-5 (Fig. 3D, ○) support this idea. These results also suggest that SR101 potentiates activation of NMDARs after washing out SR101, implying that SR101 may affect NMDARs intracellularly. SR101 may enhance synaptic NMDAR currents by directly affecting gating property of NMDARs. The results that the rising time of NMDAR currents was increased by SR101 and the SR101 analog, Texas Red-hydrazide, induced a similar LTP-IE support this idea. Astrocytic release of gliotransmitters induced by loading SR101 seems not involved because patching astrocytes with SR101 did not induce LTP-IE (Fig. 6). The intracellular pathway that couples activation of NMDARs to voltage-gated sodium channels is unknown yet. The possible pathway is that NMDAR-mediated increases in intracellular Ca^{2+} activate Ca^{2+} -dependent Cam/Kinases and/or PKA pathways, which in turn modulate voltage-gated sodium channels.

The mechanism underlying SR101-induced potentiation of eEPSCs is more complicated, because glutamate receptor antagonists only partially inhibited the major portion of the potentiation (Fig. 4B and C, □). The major portion of SR101-induced potentiation of eEPSCs involves activation of NMDARs because AP-5 inhibited this portion of the potentiation (Fig. 4C, ■). The pathways involved in glutamate receptor-independent portion were not fully explored in this study. It is possible that SR101 also directly enhancing activation of AMPA receptors in the dendritic membrane of pyramidal neurons by affecting either gating or phosphorylation of AMPARs. Thus, additional studies are required to dissect how SR101 induces the glutamate receptor-independent portion of the potentiation.

It is in this study documented that SR101 induced LTP-IE, increases in synaptic efficacy, and epileptic seizures. The bioactive properties of SR101 may permit its use in creating animal models of epileptic seizures. However, the bioactive effects of SR101 also raise concerns with regard to the use of SR101 as a specific marker of astrocytes where neuronal functions are studied.

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Comprehensive list of abbreviations

SR101	Sulforhodamine 101
LTP-IE	long-term potentiation of intrinsic excitability
AP	action potential
NMDAR	<i>N</i> -methyl <i>D</i> -aspartate receptor
mGluR	
metabotropic	
glutamate receptor	
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

IR-DIC	infra-red differential inference contrast
EEG	electroencephalography
AP-5	DL-2-Amino-5-phosphonovaleric acid
CNQX	6-cyano-2,3-dihydroxy-7-nitro-quinoxaline
MCPG	($\pm$)- α -Methyl-(4-carboxyphenyl)glycine
MSOP	(R,S)- α -methylserine-O-phosphate
CPCCOEt	7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester
AHP	afterhyperpolarization
EPSC	excitatory post-synaptic current
ACSF	artificial cerebrospinal fluid

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Fig. 1. SR101 induces LTP-IE

(A) A representative recording showing that SR101 shifted the threshold of action potential to the more negative value (dotted line). Bottom lines indicate current injection steps (numbers in pA). (B) The AP threshold (●) and RMP (□) from data in A were plotted against the time. (C) The mean change in the AP threshold against the time. Recording without SR101 application was used as the time control (T-Con and RMP T-con). $P < 0.001$ compared with the time control group, twoway ANOVA, $n = 5$ cells for each group.

Fig. 2. SR101 induces spontaneous APs

(A) Whole-cell current-clamp recording from a representative pyramidal neuron showing no spontaneous APs during control (Con) and a high frequency of spontaneous APs 50 min after application of SR101. (B) Left panel, the mean frequency of sAPs (AP Freq) during control (Con), 50 - 60 min after application of SR101 (SR101), and 50 - 60 min after recording without application of SR101 (T-Con). The number indicates the sample number. **, $P < 0.01$ compared with control or time control, paired t -test. Right panel, the dose-response curve of SR101 effects on spontaneous spikes. $n = 5$ cells for each concentration. The line is Sigmoidal fitting. (C) SR101-induced changes in the AP threshold in CA1 pyramidal neurons in whole hippocampus (Whole) and CA3-cut off hippocampus (CA3-cut). (D) AHP (arrowheads) during control (Con) and 50 min after SR101 application (SR101). AHP was induced by a train of five action potentials evoked by five depolarizing current injections (50 pA and 15 ms duration at 15 ms intervals) and measured from the negative peak (arrowhead) to the baseline (dotted line). (E) The mean AHP (AHP) and the mean input resistance (R_{in}) during control (Con) and 50 min after application of SR101 (SR101).

Fig. 3. AP-5 blocks SR101-induced LTP-IE

(A) SR101-induced changes in the AP threshold in the presence of AP-5 (50 μ M) from a representative neuron. (B) Statistical data of SR101-induced changes in the AP threshold in the absence (●) or presence of AP-5 (□). (C) SR101-induced changes in the AP threshold with AP-5 application started at 30 min after SR101 application from a representative neuron. (D) SR101-induced changes in the AP threshold when AP-5 was applied after SR101-induction (Post-AP-5, □) or during SR101-induction (AP-5 wash, ○).

Fig. 4. SR101 induces an increase in eEPSCs

(A) Representative evoked EPSCs during control (1), during application of SR101 (2) and 50 min after SR101 application (3). Slices were perfused with the ACSF solution added with bicuculline (10 μM). (B) Representative recording of SR101-induced increase in eEPSCs in the presence of AP-5 (50 μM), MCPG (200 μM), and MSOP (100 μM). (C) Statistical data for SR101-induced potentiation of eEPSCs in the absence (●) or presence of AP-5, MCPG, and MSOP (□) or AP-5 alone (■). $P < 0.01$ between SR101 and T-Con, SR101 and blockers, or blockers and T-con, two-way-ANOVA, $n = 8$ cells for each group. Insert panel, the mean rising time (Rise) and decay constant of eEPSCs before (Con) and after perfusion of SR101 (SR101).

Fig. 5. SR101 increases synaptic NMDAR currents

(A) Recording traces from a representative neuron showing pair-pulse-evoked synaptic NMDAR currents (A1 and A2) during control (1), application of SR101 (2), and 10 min after the end of SR101 application (3). Application of AP-5 (50 μ M) following SR101 application blocked NMDAR currents (AP-5). $V_H = -60$ mV. Insert panel, the mean rising time (50-90%, Rise) and decay constant (10-90%, Decay) before (Con) and after application of SR101 (SR101). (B) The mean normalized synaptic NMDAR currents (eEPSC_{NMDA}) evoked by the first (●) and second stimuli (○). $n = 7$ cells. The time control without SR101 application was also presented (T-Con). (C) Recordings from a representative neuron showing SR101-induced increase in synaptic NMDAR currents in the presence of mGluR antagonists, MCPG (200 μ M), MSOP (100 μ M), and CPCCOEt (100 μ M) at the time indicated in D. (D) Statistical data showing that mGluR antagonists did not block SR101-induced increase in synaptic NMDAR currents. $n = 5$ cells.

Fig. 6. SR101-loading of astrocytes is not involved

(A) Whole-cell recording from a representative pyramidal neuron during control (Con) or 90 min after patching an astrocyte near its dendrites with SR101 (10 μ M). (B) Statistical data showing that infusion of SR101 (10 μ M) into astrocytes ($\square$) did not significantly change the AP threshold, comparing with the time control ($\circ$). $n = 8$ pairs. Closed circles represent changes in the AP threshold induced by perfusion of Texas Red-hydrazide (TR, 10 μ M) for 10 min. $n = 5$ cells.

Fig. 7. Seizure activities induced by intrahippocampal injection of SR101

(A) and (B) Representative sections of EEGs during the fifth day after intrahippocampal injection of 10 μ M SR101 (2 μ l and 5 μ l) or ACSF (2 μ l and 5 μ l). (C) and (D) Frequency (C) and duration (D) of seizure activities during the fifth day after intrahippocampal injection of 10 μ M SR101 (SR101, 2 μ l and 5 μ l) or ACSF (ACSF, 2 μ l and 5 μ l). The number indicates the sample number of rats. * and **, $P < 0.05$ and 0.01 compared with ACSF, Student t -test.