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Experimental Trial

Magnesium L-threonate Prevents and Restores Memory Deficits Associated with Neuropathic Pain by Inhibition of TNF-α

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Background: Clinical studies have shown that about two-thirds of patients with chronic pain suffer from short-term memory (STM) deficits and an effective drug for treatment of the neurological disorder is lacking at present.

Objective: We tested whether chronic oral application of magnesium L-threonate (MgT), which has been shown to improve memory in normal and aging animals by elevating Mg2+ in the brain, could prevent or restore the STM deficits induced by spared nerve injury (SNI), an animal model of chronic neuropathic pain. The mechanisms underlying the effect of MgT on STM deficits were also investigated.

Study Design: The experiments were conducted in a random and double-blind fashion in adult male rats. MgT was administrated via drinking water at a dose of 609 mg/kg/d for 2 weeks, starting either one week before SNI (preventative group) or one week after SNI (therapeutic group), and water without the drug served as control.

Methods: STM was accessed with a novel object recognition test (NORT), followed by recording of long-term potentiation (LTP) in the hippocampus in vivo and the measurement of the expression of tumor necrosis factor-α (TNF-α) with Western Blot or Immunohistochemistrical staining. α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptor (NMDAR) currents were recorded with patch clamp in CA1 neurons in acute and cultured hippocampal slices.

Result: We found that chronic oral application of MgT was able to prevent and restore the deficits of STM and of LTP at CA3-CA1 synapses in the hippocampus induced by SNI. Furthermore, both preventative and therapeutic chronic oral application of MgT blocked the up-regulation of TNF-α in the hippocampus, which has been previously shown to be critical for memory deficits. SNI reduced NMDAR current and the effect was dramatically attenuated by elevating extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$). In cultured hippocampal slices, chronic application of recombinant rat TNF-α (rrTNF-α) for 3 days reduced NMDAR current in a concentration-dependent manner and the effect was again blocked by elevating $[Mg^{2+}]_o$.

Limitations: We showed that oral application of MgT inhibited the over-expression of TNF-α and rescued the dysfunction of the NMDAR, but the causal relationship between them remains elusive.

Conclusions: Our data suggested that oral application of MgT was able to prevent and restore the STM deficits in an animal model of chronic neuropathic pain by reversing the dysfunction of the NMDAR, and normalization of TNF-α expression may play a role in the effect. Oral application of MgT may be a simple and potent means for handling this form of memory deficit.

Key words: Magnesium L-threonate, short-term memory, tumor necrosis factor-α, NMDA receptor, neuropathic pain

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Short term memory (STM), a kind of temporary storage of information, is a necessary component of working memory (1,2). Intact STM is critical for achievements of an individual in modern life (3). Compelling clinical and experimental studies have demonstrated that chronic pain is often accompanied with STM and working memory deficits in human and in animals (4-7). The potential drug for treating this form of memory impairment is lacking at present.

Why do patients with chronic pain often suffer from cognitive deficits? Recently, some pathological changes have been explored in the hippocampus, a brain region critical for memory formation (8), in patients and in animals with chronic pain. It has been shown that the volume of the hippocampus is reduced in patients with chronic low back pain or with complex regional pain syndrome (9). Also, long-term potentiation (LTP), a synaptic model of memory storage (10), is impaired, and neurogenesis and synaptic density are reduced in the hippocampus of rats with spared nerve injury (SNI), an animal model of neuropathic pain (7,9). The mechanisms underlying the pathological changes, however, are not clear. Abundant evidence has shown that several pro-inflammatory cytokines, such as TNF-α and IL-1β, are up-regulated in the hippocampus in the condition of neuropathic pain (11-13). Our recent work has demonstrated that the over-production of TNF-α plays a critical role for the deficits of STM and hippocampal LTP in rats with SNI (7). However, how the inflammatory cytokines impairs the memory process is largely unknown.

Magnesium, a necessary ion, has many indispensable physiological functions (14). Elevation of extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) inhibits the inflammatory response by reduction of inflammatory cytokine (15,16). $[Mg^{2+}]_o$ is an important regulator of synaptic plasticity *in vitro* (17). Recently, a novel magnesium compound [magnesium-L-threonate (MgT)] that can elevate brain magnesium via chronic oral supplementation was developed (18). MgT treatment enhances short- and long-term memory in both young and aged rats by increase of synaptic density and plasticity in the hippocampus (18). However, the molecular mechanisms underlying the beneficial effect of MgT remain elusive.

Allowing for the facts presented above, in the present work we tested whether chronic oral administration of MgT could prevent or restore the STM deficits induced by SNI and found that elevating $[Mg^{2+}]_o$ may rescue the dysfunction of the NMDAR by inhibiting the up-regulation of TNF–α, leading to its preventative and therapeutic effect on memory deficits in animal models of neuropathic pain.

Methods

Animals and Surgery

Adult male Sprague–Dawley rats (3 – 8 w) were obtained from Institute of Experimental Animals of Sun Yat-sen University. The animals were housed in separated cages under the standard light cycle (6:00 – 18:00) with access to food and water ad libitum. The room temperature was maintained at 23 ± 1°C and humidity at 50 – 60%. All experimental procedures were approved by the local committee for animal care and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animals. The SNI was carried out following the procedures described by Decosterd and Woolf (19). Briefly, under sodium pentobarbital anesthesia (40 mg/kg, i.p.), the common peroneal and the tibial nerves were exposed and cut, but the sural nerve remained intact. A complete hemostasis was confirmed and the wound was sutured in 2 layers.

MgT Administration and Experimental Design

MgT (Magceutics Inc. USA) was administered via drinking water (604 mg/kg/d, 50 mg/kg/d elemental magnesium), which has been shown to elevate brain magnesium but not to influence water and food intake or the normal body weight gain in adult rats (18). The concentration of MgT in the drinking water was determined and adjusted based on these parameters to reach the target dose. To determine the preventative and therapeutic effects of MgT STM deficit induced by SNI and to investigate the underlying mechanisms, the experiments were designed as follows (Fig. 1): MgT was administrated for 2 weeks, starting either one week before SNI (preventative group) or one week after SNI (therapeutic group), and the water without the drug served as the control. And then STM was accessed with use of the novel object recognition test (NORT), followed by recording long-term potentiation (LTP) in vivo and detecting TNF-α expression with Western Blot (WB) or Immunohistochemistrical (IHC) staining.

Novel Object Recognition Test

The NORT was used to evaluate the STM ability and carried out following the procedures as described by

Slutsky et al (18). The retention interval is 10 minutes. The experimenters measured the time spent exploring each object. The recognition index was calculated as the ratio of time spent exploring the novel object over total exploration time.

Acute Hippocampal Slice Preparation

The acute hippocampal slices were prepared from 3-week-old Sprague-Dawley rats and recovered in the chamber containing oxygenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF) at 28°C for 5 hours. ACSF contains the following substances (mM): 125 NaCl, 2.5 KCl, 2 $CaCL_2$, 0.8 $MgSO_4$, 1.25 NaH_2PO_4, 26.2 $NaHCO_3$, 25 D-glucose, and bubbled with 5% CO2/95% O_2. The osmolarity of ACSF was 297 – 305 mOsm.

Electrophysiological Recording

Field excitatory postsynaptic potentials (fEPSPs) in CA3–CA1 synapses were recorded *in vivo* following the procedures described previously (20). Electrophysiological criteria (21) were used to determine the optimal electrode placement. The recording electrode was positioned 3.4 mm posterior to the bregma, 2.5 mm lateral to the midline, and the depth of the recording electrode was about 2.2 mm from the dura. The stimulating electrode was positioned at 4.2 mm posterior to the bregma and 3.8 mm lateral to the midline, and about 2.7 mm in depth from the dura. A single square pulse of voltage at low frequency (0.05 Hz, 0.2 ms duration) was used to evoke fEPSPs and the intensity of the test stimulus was adjusted to produce 50 – 55% of maximum response. High-frequency stimulation (HFS, 100 Hz, 50 pulses, 4 trains in 15 second intervals) was used to induce LTP. The intensity of HFS was raised to evoke 75% of maximum fEPSPs amplitude. The amplitudes of fEPSPs were determined off-line by LTP program (www.ltp-program.com). In each experiment, the responses to 9 consecutive test stimuli were averaged. The mean amplitudes of fEPSPs recorded before HFS served as baseline.

Excitatory post-synaptic currents (EPSCs) in CA1 pyramidal neurons evoked by stimulation of the Schaffer collateral-commissural pathway or mini excitatory postsynaptic current (mEPSCs) were recorded with a patch clamp in hippocampal slices. The slices were incubated with ACSF and the recording pipettes (3 – 5 MΩ) were filled with a solution containing (mM): 125 K-gluconate, 3 KCl, 8 NaCl, 0.5 CaCl2, 5 EGTA, 10 HEPES, 2 MgATP, and 0.3 NaGTP (adjusted to pH 7.2 with KOH). Picrotoxin (Sigma) at 100 μM was always present to block GABAA receptor-mediated inhibitory synaptic currents. The

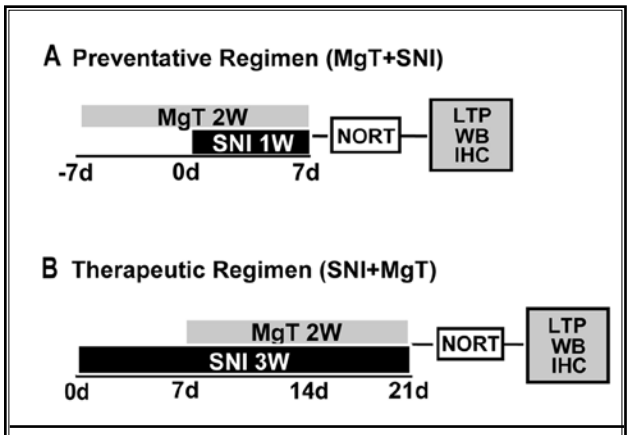

Fig. 1. *Experimental design. A. Preventative regimen of the oral application of MgT. MgT was administrated for 2 weeks, starting one week before spared nerve injury (SNI), followed by novel object recognition test (NORT), long-term potentiation (LTP) recording, Western Blot (WB), and immunohistochemistry (IHC). B. Same as A, but oral administration of MgT started one week after SNI.*

evoked EPSCs (eEPSCs) mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors and NMDA {sp} receptor (NMDAR) were isolated by the voltage clamp, according to Abumaria et al's paper (22). AMPA receptor currents were recorded at –70 mV and the negative peak induced by stimulation of the Schaffer collateral-commissural pathway was considered as the amplitude of AMPA current. And then, NMDAR currents were recorded at +50 mV in the same neuron with the same stimulus and the positive peak at 80 ms after the stimulus artifact was considered as the amplitude of NMDAR current (Fig. 4A insert). The ratio of AMPA current to NMDA current was calculated in each individual neuron. For mEPSCs recording, one μM TTX (Sigma) was applied to the recording solution to block action potential discharges. To record NMDA-mediated mEPSC and AMPA-mediated mEPSC, the AMPA receptor blocker CNQX (20 μM, Sigma) and the NMDA-receptor blocker AP-5 (50 μM, Sigma) were applied to the recording solution, respectively. To observe NMDAR-mediated mEPSC, Mg2+-free ACSF was used.

Immunohistochemistry and Western Blot

Cryostat sections (20 μM) were cut in a cryostat (LEICA CM1900) and processed for IHC staining, as previously described (23). The primary antibody was goat polyoclonal anti-TNF-α antibody (1:200, Santa Cruz), mouse monoclonal neuronal-specific nuclear protein (NeuN, neuronal marker, 1:200; Millipore Bioscience

Research Reagents), glial fibrillary acidic protein (GFAP, astrocyte marker, 1:1000; Cell Signaling Technology), or mouse anti-OX-42 (a microglia marker, 1:100, Abcam). The secondary antibody was IgG labeled with Cy3 (1:400, Jackson Immuno Research) and FITC, respectively (1:400, Jackson Immuno Research).

The Western Blot was carried out following the procedures described by Schafers et al (24), The primary antibody was mouse anti-TNF-α monoclonal antibody (1:200; Santa Cruz) or mouse anti-β-actin polyclonal antibody (1:1000, Cell Signalling Technology).

Organotypic Hippocampus Slice Cultures

Hippocampal slice cultures were prepared from 10-day-old Sprague-Dawley rats, according to the interface culture method (25). Hippocampal slices were cultured on porous (0.4 μm) insert membranes (Millipore, Millicell CM) and transferred to 6-well culture trays (Corning). Each well contained culture medium composed of 50% MEM (Eagle) with Glutamax-1, 25% heat-inactivated horse serum, 25% EBSS, 6.5 mg/mL D-Glucose, 50 U/ml Penicillin, and 50 ug/ml Streptomycin (all from Gibco).

Statistical Analysis

The results of the NORT, IHC, and WB were analyzed with one-way analysis of variance (ANOVA). The data from the basal synaptic transmission, LTP between groups, were analyzed with repeated measures 2-way ANOVA, and post hoc test was used for detailed statistical analysis, as appropriate. In the experiments of patch clamp, eEPSCs were analyzed with Clampfit 9.2 (HEKA Instruments, Germany). The differences in the changes of the NMDA/AMPA ratio between the different groups were tested using one-way ANOVA followed by individual post hoc comparisons. For recording mEPSC, transient current events were analyzed in terms of amplitude and frequency in 60 second-long bins using the Mini-Analysis Program 6.1 (Synaptosoft, USA). Events were identified as synaptic currents (mEPSC) by setting the event detection threshold at 2-fold the baseline noise level and by checking that events had rise times faster than decay times, rise times greater than 0.4 ms, and decay times greater than 1.5 ms. Events not fitting the above parameters were excluded. Amplitudes and frequencies of each cell were averaged with about 40 events and analyzed with one-way ANOVA. Significance of the shift in cumulative probability distributions of interevent interval (IEI) and amplitude was assessed using the non parametric Kolmogorov-Smirnov 2-sample test (KS test). All experiments were statistically analyzed using SPSS software 16.0. Difference was considered significant if $P < 0.05$. Data are presented as mean ± SEM.

Results

Effect of MgT on the Deficits of STM and of LTP in the Hippocampus Induced by SNI

Our previous work has shown that SNI impairs STM and LTP at CA3-CA1 synapses in the hippocampus (7). To test the preventative and therapeutic effects of MgT on memory deficits, the drug was applied orally for 2 weeks, starting either 7 days before or 7 days after SNI, and then STM was tested with NORT, followed by recording of LTP at CA3-CA1 synapses of the hippocampus *in vivo* (Fig. 1).

Consistent with our previous work (7), the recognition index for STM was significantly lower in SNI rats, compared to that in sham-operated rats ($P < 0.01$, 0.43 ± 0.07 vs 0.68 ± 0.02, Fig. 2A). Whereas, the index in the SNI rats pretreated with MgT was significantly higher than that in untreated SNI rats ($P < 0.05$, 0.60 ± 0.03 vs 0.43 ± 0.07, Fig. 2A) and was not different from that in sham-operated rats ($P > 0.05$), indicating that STM deficits induced by SNI was prevented by MgT. To explore the synaptic mechanisms underlying the preventative effect of MgT on memory impairment, we evaluated synaptic plasticity at CA3-CA1 synapses of the hippocampus in vivo following the memory test, and found that both SNI and treatment with MgT did not affect the basal synaptic transmission, as the amplitudes of fEPSPs evoked by different stimulus intensities (stimulus–response curves) were not different among sham-operated, SNI, and SNI pretreated with MgT groups ($P > 0.05$, Fig. 3Aa). The pretreatment with MgT, however, prevented impairment of LTP by SNI. The potentiation of fEPSP induced by HFS in SNI rats was significantly lower, compared with the sham group ($F_{(1, 17)} = 9.377$, $P < 0.01$, Fig. 3Ab, Left). The potentiation in the MgT-pretreated SNI group was significantly higher than that in SNI alone group ($F_{(1, 16)} = 4.877$, $P < 0.05$, Fig. 3Ab, Left) and was not different from that in the sham group ($F_{(1,18)} = 0.313$, $P > 0.05$, Fig. 3Ab, Left). MgT also affected the duration of potentiation. At 60 minutes after HFS the mean amplitude of fEPSP in SNI alone group was no longer different from baseline ($P > 0.05$, 108.21 ± 6.22%, Fig. 3Ab, Right), while those in the sham group and in the MgT-pretreated SNI group were still

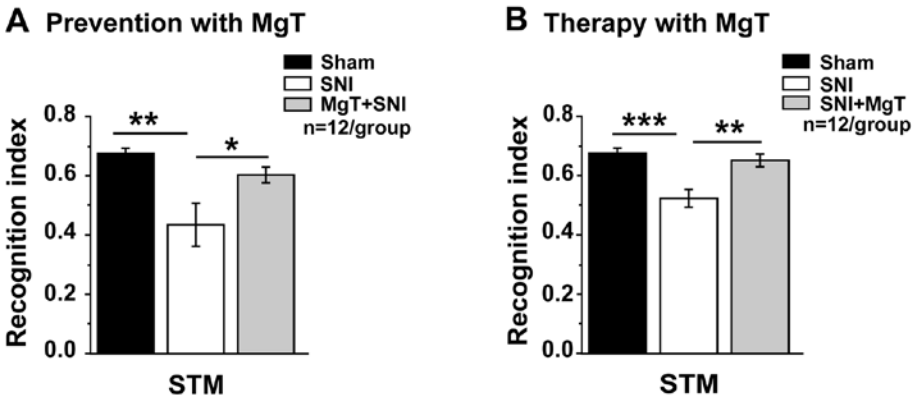

Fig. 2. *The preventative and therapeutic effects of oral administration of MgT on short-term memory (STM) deficits induced by SNI. A. Oral administration of MgT for 2 weeks, starting 7 days before SNI, prevented STM deficits induced by SNI. The recognition indexes of STM accessed with NORT in 3 different groups as indicated are shown. n = 12 in each group. B. Same as A, but oral administration of MgT started 7 days after SNI. *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean ± SEM.*

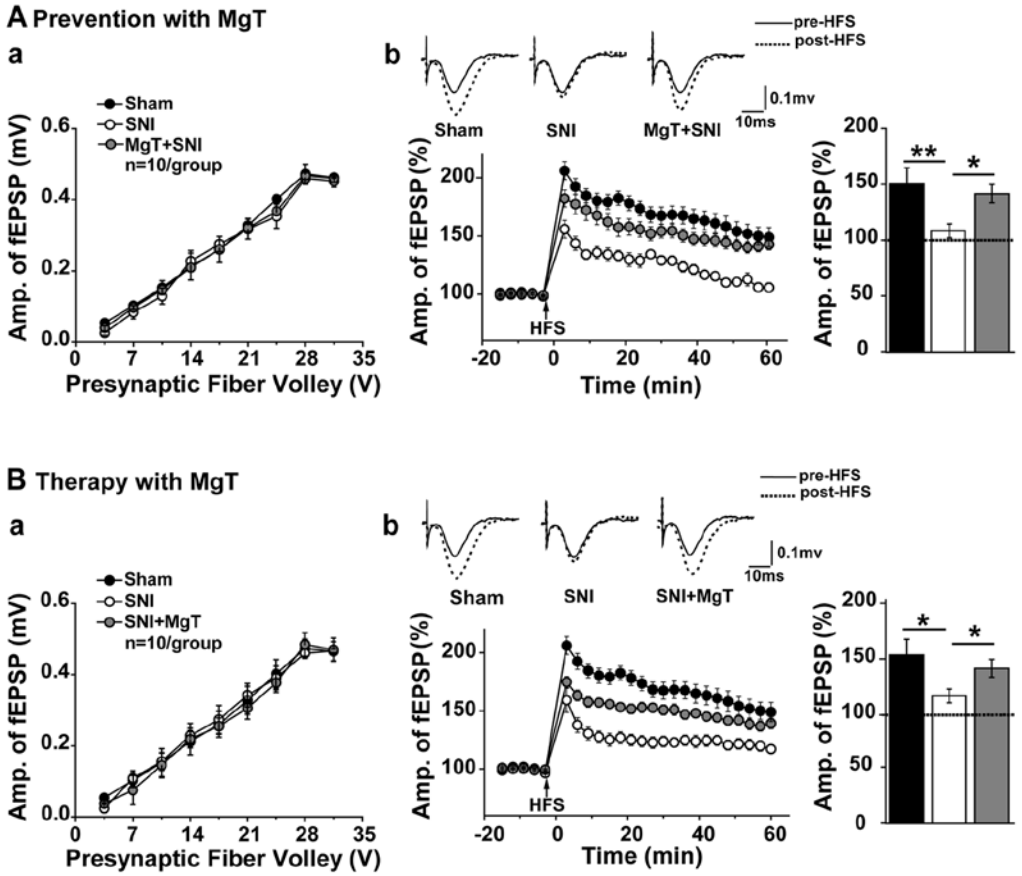

Fig. 3. *Effects of MgT on the basal synaptic transmission and the impairment of LTP induced by SNI. A. Oral administration of MgT did not affect basal synaptic transmission (a), but prevented impairment of LTP at CA3-CA1 synapses induced by SNI (b). Left: The amplitudes of fEPSP are expressed as percentage of baselines. Insets: Representative traces of fEPSP are presented before and after HFS. Right: The mean magnitudes of fEPSP at 60 minutes after HFS in the 3 groups are shown. Dashed lines indicate the normalized basal synaptic responses. B. Same as A, but MgT was administrated 7 days after SNI. *P < 0.05, **P < 0.01. Data are presented as mean ± SEM, n = 10 in each group*

significantly higher than baselines (150.64 ± 14.10 % and 141.63 ± 8.40%, Fig. 3Ab, Right).

Oral administration of MgT for 2 weeks, starting 7 days after SNI, rescued STM impairment produced by SNI. As shown in Fig. 2B, the recognition index for STM was significantly higher in MgT-treated SNI rats than that in the untreated SNI group ($P < 0.01$, 0.65 ± 0.02 vs 0.52 ± 0.03), and was not different from that in the sham group ($P > 0.05$, 0.65 ± 0.02 vs 0.67 ± 0.02). The therapy with MgT also rescued the impairment of LTP caused by SNI (Fig. 3Bb). Repeated measures 2-way ANOVA analysis indicated that the magnitude of potentiation induced by HFS in the MgT-treated SNI group was significantly higher than that in the untreated SNI group ($F_{(1,15)} = 5.089$, $P < 0.05$, Fig. 3Bb, Left) and was not different from that in the sham group ($F_{(1,17)} = 7.612$, $P > 0.05$, Fig. 3Bb, Left). One hour after HFS, the amplitude of fEPSP in the untreated SNI group returned to baseline level (113.87 ± 6.16 %). Whereas the amplitudes in the sham group and in the MgT-treated SNI group was 150.55 ± 4.13 % and 138.55 ± 7.98% of baselines, respectively, and no difference between the 2 groups was detected ($P > 0.05$, Fig. 3Bb, Right), indicating that the impairment of LTP caused by SNI was reversed by therapy with MgT.

Effect of Elevating $[Mg^{2+}]_o$ on the Inhibition of NMDA Receptor Current at CA3-CA1 Synapses Induced by SNI

The NMDA plays an important role in the formation of memory and for LTP in the hippocampus (26,27). It has been shown that elevating magnesium concentrations in the hippocampus improves memory by increasing the synaptic NMDAR current (18). To determine whether a similar mechanism is also involved in the protective effect of MgT on deficits of STM and LTP induced by SNI, the effect of elevating $[Mg^{2+}]_o$ on synaptic NMDA and AMPA receptor currents in hippocampal slices from SNI and sham-operated rats was tested.

In this series of experiments, NMDAR and AMPAR currents in each CA1 pyramidal cell evoked by stimulation of the Schaffer collateral–commissural pathway were recorded in hippocampal slices from sham-operated and SNI rats, and the ratio of NMDAR current to AMPAR current (NMDA/AMPA ratio) was calculated. All slices were incubated with ACSF containing either 0.8 or 1.2 mM $[Mg^{2+}]_o$ for 5 hours before patch clamp recording. We found that when the slices were pre-incubated with ACSF containing 0.8 mM $[Mg^{2+}]_o$, the NMDA/AMPA ratio in slices from SNI rats was significantly lower than that from sham rats ($P < 0.01$, 0.32 ± 0.02 vs. 0.77 ± 0.08, Fig. 4A). However, when $[Mg^{2+}]_o$ increased to 1.2 mM, in slices from SNI rats, the ratio was obviously higher, compared to that recorded in slices incubated with 0.8 mM $[Mg^{2+}]_o$ ($P < 0.01$, 0.65 ± 0.05 vs. 0.32 ± 0.02, Fig. 4A), and was not different from that from sham rats ($P > 0.05$, 0.65 ± 0.05 vs. 0.77 ± 0.08, Fig. 4A).

The reduction of NMDA/AMPA ratio may be attributed to a decrease in NMDAR current or an increase in AMPA receptor current. To determine which one may contribute to our results, mEPSCs mediated by AMPA and NMDA were recorded. As shown in Fig. 4B, SNI did not affect AMPA receptor function, since there is no difference in amplitude and frequency of AMPA-mediated mEPSCs between the sham group and the SNI group, when the slices were pre-incubated with 0.8 mM $[Mg^{2+}]_o$. Elevating $[Mg^{2+}]_o$ also did not affect AMPA receptor function in the SNI group, as the amplitude and frequency of AMPA-mediated mEPSCs in the slices incubated with 1.2 mM $[Mg^{2+}]_o$ were not different from those incubated with 0.8 mM $[Mg^{2+}]_o$ (Fig. 4Ba and b, Table1). In contrast, SNI decreased NMDAR function by reducing the amplitude but not the frequency of NMDA-mediated mEPSCs. The amplitude of NMDA-mediated mEPSCs was significantly lower in SNI rats, compared to that in sham rats (Fig. 4Ca, Table 1), when the slices were pre-incubated with 0.8 mM $[Mg^{2+}]_o$. Whereas, the effect of SNI was abolished by elevating $[Mg^{2+}]_o$ to 1.2 mM. The amplitude was significantly higher in slices pre-incubated with 1.2mM $[Mg^{2+}]_o$ than that with 0.8 mM $[Mg^{2+}]_o$ (Fig. 4Ca). And the frequency of NMDA-mediated mEPSCs was not influenced by either SNI or elevation of $[Mg^{2+}]_o$ (Fig. 4Cb). Together, it is suggested that the dysfunction of the NMDAR caused by SNI could be rescued by elevating $[Mg^{2+}]_o$.

Effect of Oral Application of MgT on the Over Production of TNF-α in the Hippocampus Produced by SNI

Our previous study has shown that the up-regulation of TNF-α is responsible for the impairment of STM and LTP in the hippocampus following SNI (7). It has been reported that elevating magnesium is capable of reducing production of pro-inflammatory cytokines (16). We therefore investigated whether oral administration of MgT could affect the up-regulation of TNF-α in the hippocampus induced by SNI. Western blot with hippocampal tissue showed that the expression of TNF-α increased substantially following SNI ($P < 0.01$, Fig. 5A and B) and the effect was significantly inhibited

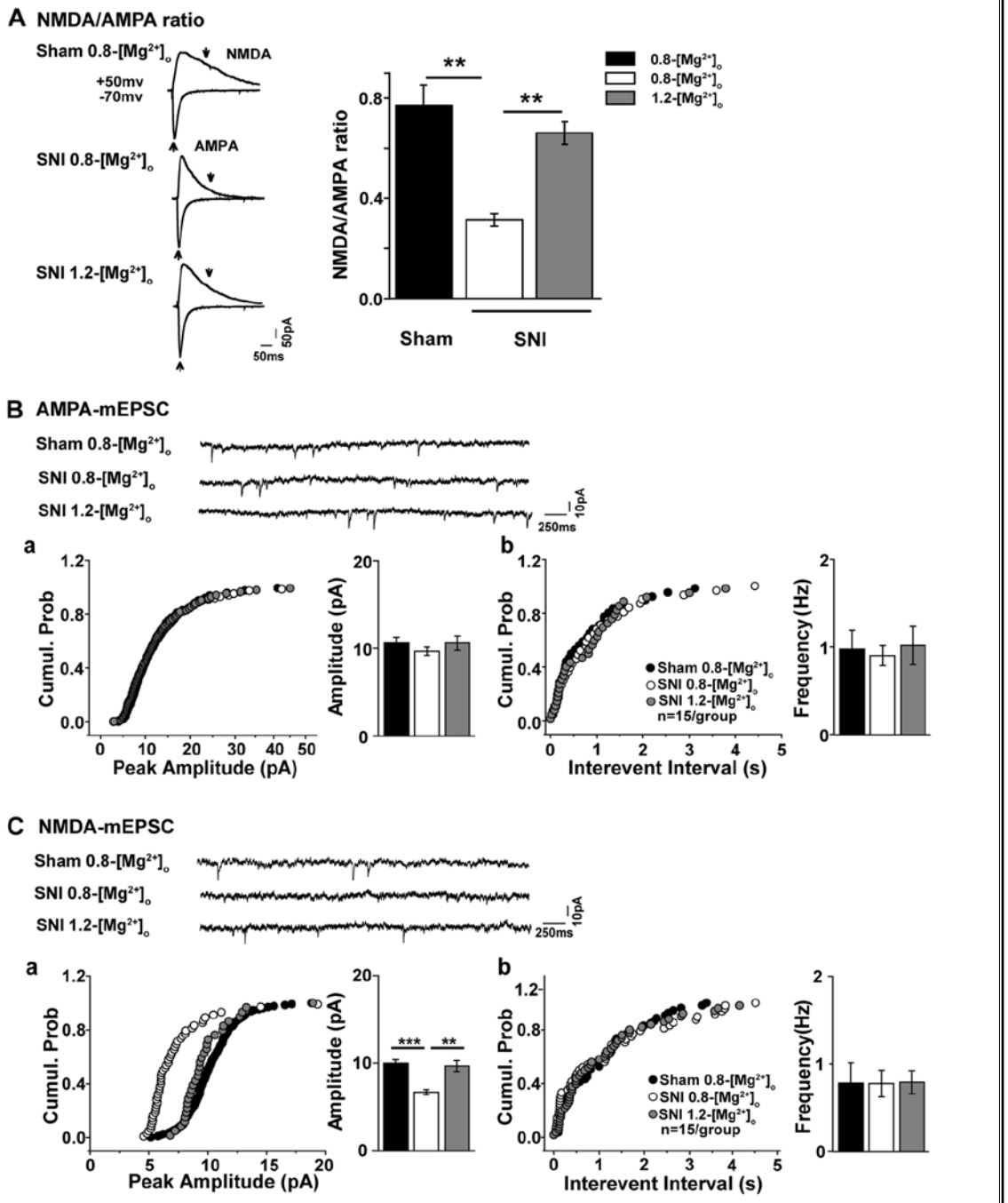

Fig. 4. Effect of elevating $[Mg^{2+}]o$ on synaptic NMDAR current at CA3-CA1 synapses in SNI rats. A. The reduction of NMDA/AMPA current ratio at CA3-CA1 synapses in SNI rats is rescued by elevating $[Mg^{2+}]_o$. Inset, Representative traces of AMPA receptor EPSCs (low trace) and NMDAR EPSCs (up traces) evoked by stimulation of Schaffer collateral–commissural pathway recorded at membrane potentials of -70 or +50 mV. The arrow indicates where the peak amplitudes of AMPA or NMDAR currents were measured. B. Elevating $[Mg^{2+}]_o$ does not influence the amplitude and frequency of AMPA mediated mEPSCs. Inset, Representative traces of AMPA mediated mEPSC. a. Cumulative probability and average amplitude of AMPA mediated mEPSCs. b. Cumulative probability and average frequency of AMPA mediated mEPSCs. C. Elevating $[Mg^{2+}]_o$ increased the amplitude but not frequency of NMDA mediated mEPSC in SNI. n = 15 in each group. $^{**}P < 0.01$, $^{***}P < 0.001$. Data are presented as mean ± SEM.

Table 1 - Elevating $[Mg^{2+}]_o$ revised the decrease of NMDAR current induced by SNI

$[Mg^{2+}]_o$		0.8 mM		1.2 mM
Slice		Sham	SNI	SNI
NMDA/AMPA ratio		0.77±0.08	0.32±0.02 **	0.65±0.05 ##
AMPA mEPSC	Amplitude (pA)	10.6±0.64	9.7±0.46	10.6±0.82
	Frequency (Hz)	0.98±0.22	0.90±0.11	1.01±0.22
NMDA mEPSC	Amplitude (pA)	10.0±0.40	6.7±0.29 ***	9.7±0.62 ##
	Frequency (Hz)	0.78±0.23	0.77±0.15	0.79±0.13

* * p<0.01, * * * p<0.001, compare with Sham group.

p<0.01, compare with SNI, 0.8 mM $[Mg^{2+}]_o$ group.

by either pre- or post-treatment with MgT ($P < 0.05$, Fig. 5Aa and Ba). The similar effects of MgT were also observed in the CA1 and CA3 regions in the hippocampus by immunohistochemistry (Fig. 5Ab and Bb).

Double immunofluorescence staining showed that in the CA1 and CA3 regions TNF-α was co-localized mainly with NeuN, a marker for neuron (Fig. 5C, a and d), to a less extent with GFAP, a marker for astrocyte (Fig. 5C, b and e), and OX-42, a marker for microglia (Fig. 5C, c and f).

Effect of Elevating $[Mg^{2+}]o$ on the Reduction of NMDAR Current Induced by rrTNF-α in Cultured Hippocampal Slices

Having demonstrated that the up-regulation of TNF-α and the dysfunction of NMDAR induced by SNI was dramatically inhibited by oral administration of MgT in vivo or by elevation of $[Mg^{2+}]_o$ in vitro, we hypothesized that over-production of TNF-α may lead to the dysfunction of NMDAR and elevation of $[Mg^{2+}]_o$ may antagonize the effect. To test this, we recorded the NMDA/AMPA ratio at CA3-CA1 synapses in cultured hippocampal slices treated with 0 ng/ml (control), one ng/ml or 10 ng/ml rrTNF-α for 3 days, as the over-expression of TNF-α induced by SNI persisted for weeks. As shown in Fig. 6A, rrTNF-α reduced the synaptic NMDA/AMPA ratio in a concentration-dependent manner, when the culture medium contained 0.8 mM $[Mg^{2+}]_o$, and the effect of 10 ng/ml rrTNF-α was dramatically reversed by elevating $[Mg^{2+}]_o$ to 1.2 mM (Table 2). Furthermore, we found that rrTNF-α also dose dependently reduced the amplitude of NMDA mediated mEPSCs and the effect was again abolished by elevation of $[Mg^{2+}]_o$, while the frequency of NMDA mediated mEPSCs was not affected by the rrTNF-α and the change of $[Mg^{2+}]_o$ (Fig. 6C, Table 2). In contrast, the amplitude and frequency of AMPA receptor mediated mEPSCs were not affected by the changes in both concentration of rrTNF-α and $[Mg^{2+}]_o$ (Fig. 6 C, Table 2). The results indicated that elevation of $[Mg^{2+}]_o$ may attenuate the dysfunction of the NMDAR produced by over-production of TNF-α.

Discussion

In the present study, we reported for the first time that chronic oral application of MgT was able to prevent and restore the STM deficits in an animal model of neuropathic pain (Fig. 2). Mechanistically, both preventative and therapeutic application of MgT abolished the impairment of LTP in the hippocampus (Fig. 3), an important brain sub-region for STM formation (28-30), and normalized the up-regulation of TNF-α in the hippocampus by SNI (Fig. 5). Also, SNI reduced the NMDAR current at CA3-CA1 synapses and the effect was dramatically attenuated by elevation of $[Mg^{2+}]_o$ (Fig. 4). In cultured hippocampal slices, application of rrTNF-α for 3 days reduced NMDAR current in a concentration-dependent manner and the effect was blocked by elevation of $[Mg^{2+}]_o$ (Fig. 6). Taken together, oral application of MgT may prevent and rescue the STM deficits in the condition of neuropathic pain by protecting the function of NMDARs, and normalization of TNF-α expression may play a role in the effect.

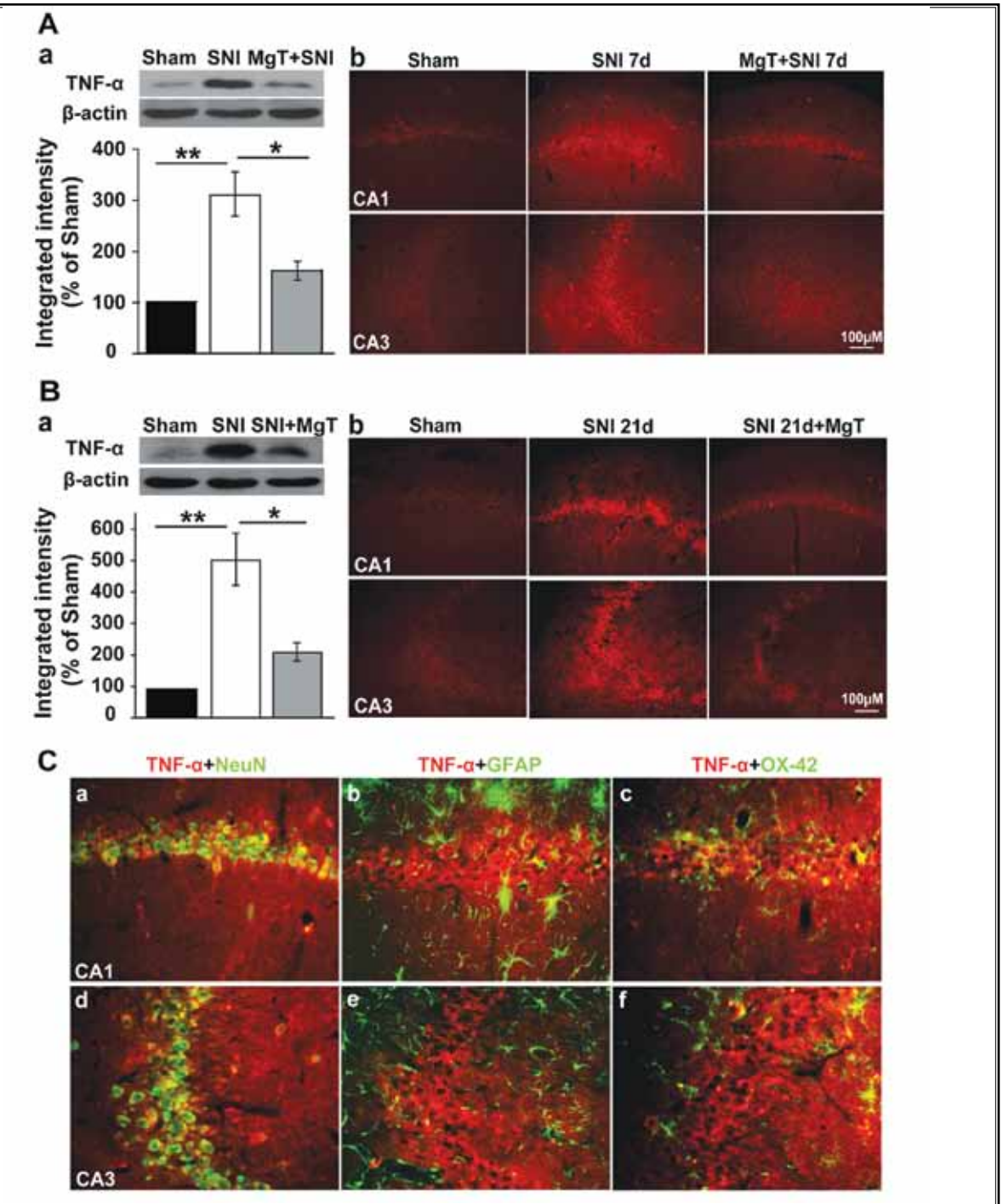

*Fig. 5. Effect of chronic oral administration of MgT on the up-regulation of TNF-α in the hippocampus induced by SNI. A. Western blot shows that oral administration of MgT one week before SNI blocks the up-regulation of TNF-α in the hippocampus produced by SNI (a) and similar change in TNF-α expression is detected by immunohistochemistry staining (b). Scale bar = 100 um. n = 3 in each group. B. Same as A, but MgT was applied one week after SNI. C. Double immunofluorescence staining shows that TNF-α-IR (red, all) was mainly co-localized with NeuN (green, a and d), but to a less extent with GFAP (green, b and e) and OX-42 (green, c and f). Scale bar = 50 um. *P < 0.05. Data are presented as mean ± SEM.*

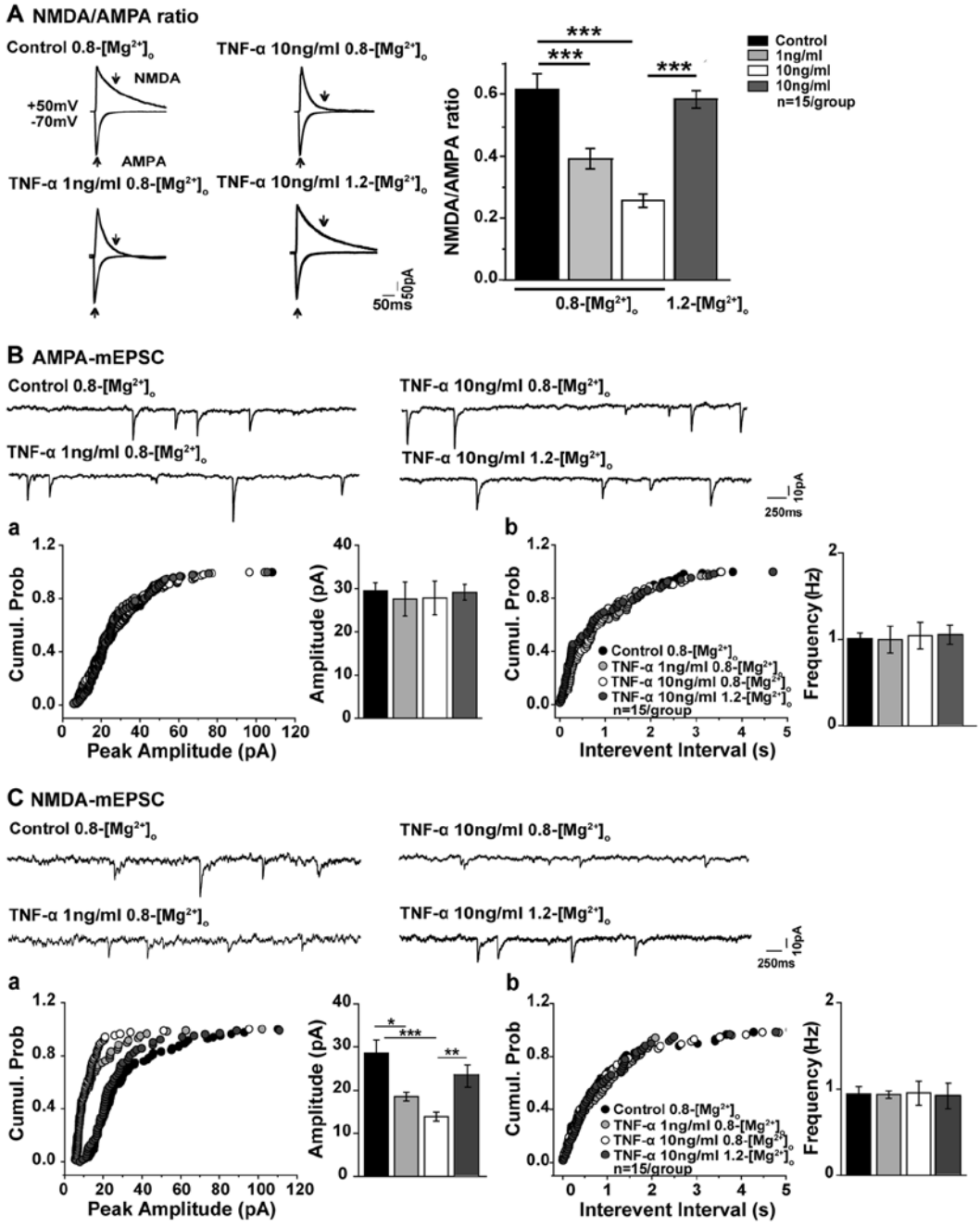

Fig. 6. *Effect of [Mg²⁺]o on the dysfunction of NMDAR induced by rrTNF-α. A. The NMDA/AMPA current ratio recorded in the slices incubated with different concentrations of TNF-α and magnesium as indicated are shown. Inset, Representative traces of evoked AMPA receptor EPSCs (low traces) and evoked NMDAR EPSCs (up traces). The arrow indicates where the peak amplitudes of AMPA or NMDAR currents were measured. B. The amplitude and frequency of AMPA mediated mEPSCs were not affected by the change in concentration of magnesium and rrTNF-α. Inset, Representative traces of AMPA mediated mEPSCs. a. Cumulative probability and average amplitude of AMPA mediated mEPSC. b. Cumulative probability and average frequency of AMPA mediated mEPSCs. C. Same as B, but NMDA mediated mEPSCs were recorded. Elevating [Mg2+]o restored the reduction of amplitude of NMDA mediated mEPSCs produced by rrTNF-α, but did not influence its frequency. N = 15 in each group. *P < 0.05, **P < 0.01, ***P < 0.001. Data are presented as mean ± SEM*

Table 2 - Elevating $[Mg^{2+}]_o$ revised the decrease of NMDAR current induced by rr-TNF-α

$[Mg^{2+}]_o$		0.8 mM			1.2 mM
rr-TNF-α		Control	1 ng/ml	10 ng/ml	10 ng/ml
NMDA/AMPA ratio		0.61±0.05	0.39±0.03***	0.26±0.02***	0.58±0.03###
AMPA mEPSC	Amplitude (pA)	29.5±1.85	27.6±3.92	27.8±3.90	29.2±1.81
	Frequency (Hz)	1.06±0.06	0.99±0.16	1.04±0.16	1.05±0.11
NMDA mEPSC	Amplitude (pA)	28.6±3.12	18.5±1.02*	13.8±1.04***	23.5±2.56##
	Frequency (Hz)	0.94±0.09	0.93±0.05	0.95±0.13	0.90±0.15

* $p<0.05$, * * * $p<0.001$, compare with control group.

$p<0.01$, # # # $p<0.001$, compare with 10 ng/ml rrTNF-α, 0.8 mM $[Mg^{2+}]_o$ group.

Mg is extremely unevenly distributed in the body: around 65% in bone, ~1% in extracellular space, and 34% in intracellular space (31). Previous works have shown that Mg deficiency induces hyperalgesia (32) and memory deficits (33). However, Mg^{2+} in serum and CSF does not change in neuropathic rats (34), so it has been suggested that the Mg-deficiency in extracellular fluid or at the effector site may be responsible for the neurological disorder. MgT has been proven to elevate Mg^{2+} in CSF by 15% and improve the memory function significantly in naive animals (18). It is speculated that MgT may also elevate Mg^{2+} in extracellular fluid or at the effector site with unknown mechanisms.

Preventing and Rescuing the Dysfunction of the NMDAR Contributes to the Beneficial Effect of MgT on Short-Term Memory and Hippocampal LTP

It is well-known that NMDAR-dependent signaling plays a critical role in synaptic plasticity and memory (35). In many neurological disorders the impairment of synaptic plasticity and memory are attributed to the dysfunction of NMDAR, and increasing NMDAR function can reduce such deficits (36,37). Our recent work demonstrated that LTP in the hippocampus was impaired in SNI models (7), and in the present work we confirmed the finding and further demonstrated that SNI led to the dysfunction of NMDARs at CA3-CA1 synapses (Fig. 5). What's more, we found that chronic oral application of MgT for 2 weeks was capable of preventing and reversing the impairment of hippocampal LTP induced by SNI in vivo (Fig. 3), and elevation of $[Mg^{2+}]_o$ could effectively reduce the dysfunction of NMDARs in vitro (Fig. 5).

Several studies have shown the strong relationship between $[Mg^{2+}]_o$ and NMDAR function in normal and aging animals. It has been primarily reported that elevating $[Mg^{2+}]_o$ enhances synaptic plasticity by reduction of Ca^{2+} flux (17). Also, chronic MgT treatment up-regulates NR2B-containing NMDAR and increases activation/expression of downstream signaling molecules not only in the hippocampus (18) but also in the infralimbic prefrontal cortex (22). The underlying mechanisms, however, are unclear. In the present study we showed that in the neuropathic pain condition, elevation of $[Mg^{2+}]o$ may prevent and reduce the dysfunction of NMDARs by normalizing the expression of TNF-α.

The Role of Pro-inflammatory Cytokines for Cognitive Function

In recent years abundant evidence has demonstrated that pro-inflammatory cytokines, such as TNF-α, are critically involved in memory and synaptic plasticity in the hippocampus (38). Genetic deletion of TNF-α or TNFR1 and TNFR2 impairs memory (39). TNF-α at physiological levels promotes insertion of AMPA receptors into the cell membrane that is critical for synaptic scaling (40), a form of synaptic plasticity that stabilizes the neuronal excitability by adjusting the strength of all of the excitatory synapses of an individual neuron (41). At pathological concentration, however, TNF-α is detrimental to memory and synaptic plasticity. For example, deficits of memory and synaptic plasticity in Alzheimer's

disease are associated with up-regulation of TNF-α, and inhibition of TNF-α is effective for treating the disease (42-45). However, how inflammatory cytokines impair synaptic plasticity and memory is largely unknown. In the present work we showed that SNI up-regulated TNF-α and decreased NMDAR current in the hippocampus, suggesting that over-production of TNF-α may lead to dysfunction of NMDARs. Indeed, we found that in cultured hippocampal slices, chronic application of rrTNF-α for 3d reduced NMDAR currents dosedependently (Fig. 6). We focused on TNF-α, because it plays a leading role in activation of a cascade of other cytokines, notably IL-1β, IL-6, and IL-8 in the animal model of neuropathic pain (46). Therefore, other inflammatory cytokines may also contribute to the dysfunction of NMDARs in the condition of neuropathic pain. As TNF-α exerts a bidirectional effect on memory and synaptic plasticity, normalizing TNF-α production, but not simply inhibiting its expression and function, is a better stratagem to treat neurological disorders. Fortunately, in the present study we found that oral application of MgT for 2 weeks reduced over-expression of TNF-α in SNI rats to control level. Therefore, oral application of MgT may be a simple and effective means for treating memory deficits associated with neuroinflammation.

Conclusion

Oral application of MgT was able to prevent and restore the STM deficits in an animal model of chronic neuropathic pain by reversing the dysfunction of NMDAR, and normalization of TNF-α expression may play a role in the effect.

Competing Interest Statement and Acknowledgments

There was no financial relationship with any organization that might have an interest in the submitted work during the previous 3 years, and there are no other relationships or activities that could appear to have influenced the submitted work.

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