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Glutamate Transporters Prevent Excessive Activation of NMDA Receptors and Extrasynaptic Glutamate Spillover in the Spinal Dorsal Horn

Hui Nie and Han-Rong Weng
Department of Anesthesiology and Pain Medicine, Division of Anesthesiology and Critical Care, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

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Nie H, Weng HR. Glutamate transporters prevent excessive activation of NMDA receptors and extrasynaptic glutamate spillover in the spinal dorsal horn. *J Neurophysiol* 101: 2041–2051, 2009. First published February 11, 2009; doi:10.1152/jn.91138.2008. Activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn neurons is a key process related to sensory transmission, neural plasticity, and pathogenesis of pain. In this study, we investigated how activation of NMDA receptors in spinal substantia gelatinosa neurons is regulated by glutamate re-uptake through glutamate transporters located in the astrocytic and neuronal plasma membranes. Using visualized whole cell patch recording techniques, NMDA excitatory postsynaptic currents evoked by graded peripheral inputs in spinal substantia gelatinosa neurons of spinal slices from young adult rats were analyzed before and after combined inhibition of glial and neuronal glutamate transporters by D-threo-β-benzyloxyaspartate (TBOA). Blockade of glutamate transporters increased the number and duration of NMDA receptors activated by weak and by strong primary afferent inputs as well as by exogenous glutamate. The enhancement in activation of NMDA receptors induced by TBOA was greater in neurons that have weaker synaptic input at baseline. Impaired glutamate uptake increased the open probability of NMDA channels and caused glutamate spillover outside the active synapses, leading to activation of extrasynaptic NMDA receptors and/or receptors located in neighboring synapses. Finally, blockade of glutamate transporters resulted in an increased proportion of NR2B subunit activation induced by peripheral input, and this increase was further augmented by stronger afferent input. These data indicate that glutamate transporters regulate spatiotemporal and intensity coding for sensory input and prevent excessive activation of glutamate receptors in the spinal dorsal horn. It is suggested that remedying dysfunctional glutamate transporters may be a potential new avenue to prevent the pathogenesis of pain.

INTRODUCTION

Activation of *N*-methyl-D-aspartate (NMDA) receptors in the spinal dorsal horn neurons is a key process related to sensory transmission, neural plasticity, and pathogenesis of pain. For example, spinal topical application of NMDA elicits nociceptive behaviors in awake animals (Aanonsen and Wilcox 1987; Kontinen and Meert 2002) and activation of dorsal horn neurons in intact anesthetized animals (Davies and Watkins 1983). More importantly, activation of NMDA receptors in the spinal dorsal horn is related to almost all forms of pathological pain induced by tissue injury or inflammation (Ren and Dubner 2007). For decades, researchers have gone to great lengths to investigate the mechanisms related to modulation and activation of NMDA receptors and their intracellular signaling pathways. In comparison, little is known about how the glutamate clearance process regulates activation of NMDA receptors in the spinal dorsal horn.

Glutamate released from presynaptic neurons is not metabolized extracellularly; rather, clearance of glutamate in the synaptic cleft and homeostasis of extracellular glutamate are ensured by a family of glutamate transporters located in the plasma membranes of both glial cells and neurons that re-uptake glutamate from the extracellular space into the cells. Glutamate uptake by glutamate transporters is driven by the electrochemical gradients of Na^+ and K^+ across the plasma membrane (Danbolt 2001; Gegelashvili et al. 2001). Three subtypes of glutamate transporters exist in the spinal cord, including two glial glutamate transporters (glutamate/aspartate transporter, GLAST; glutamate transporter 1, GLT-1) and one neuronal glutamate transporter, excitatory amino acid carrier 1 (EAAC1). GLAST is highly concentrated in lamina I and II, whereas GLT-1 and EAAC1 are expressed diffusely throughout the dorsal and ventral horns (Furuta et al. 1997; Mao et al. 2002; Seal and Amara 1999; Sung et al. 2003; Tao et al. 2004; Vera-Portocarrero et al. 2002; Weng et al. 2005). Recent studies show that hyperalgesia induced by nerve injury or chemotherapy (paclitaxel) is associated with deficiencies in glutamate transporter protein expression (Sung et al. 2003; Weng et al. 2005) and glutamate uptake function (Binns et al. 2005).

The role of glutamate transporters in pain signaling was further validated in behavioral and electrophysiological experiments when deficiency in glutamate re-uptake was induced by selective glutamate transporter blockers. Pharmacological inhibition of glutamate transporters in the spinal cord elevates spinal extracellular glutamate concentrations (Liaw et al. 2005) and produces spontaneous nociceptive behaviors and hypersensitivity to mechanical and thermal stimuli (Liaw et al. 2005; Weng et al. 2006). Such nocifensive behaviors could be significantly blocked by NMDA or non-NMDA receptor antagonists (Liaw et al. 2005). In addition, spinal wide dynamic range neurons exhibit increased spontaneous activities, innocuous and noxious stimulus-evoked responses, and prolonged afterdischarges following inhibition of glutamate transporters (Weng et al. 2006). Spinal synaptic mechanisms underlying the functional alterations induced by deficient glutamate uptake were revealed by our recent study showing that blockade of glial GLT-1 results in enhanced activation of α-amino-3-

Address for reprint requests and other correspondence: H.-R. Weng, Dept. of Anesthesiology and Pain Medicine, Div. of Anesthesiology and Critical Care, The University of Texas M. D. Anderson Cancer Center, 1400 Holcombe Blvd., Unit 409, Houston, TX 77030-4009 (E-mail: hrweng@mdanderson.org).

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hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors in the spinal lamina II neurons, which is due at least in part to glutamate spillover and activation of AMPA receptors in neighboring synapses (Weng et al. 2007).

In this study, we investigated the role of glutamate transporters in regulation of NMDA receptor activation in spinal substantia gelatinosa (SG) neurons by functional blocking all three glutamate transporters in the spinal cord with a transporter blocker, D-*threo*-β-benzyloxyaspartate (TBOA). We found that glutamate transporters regulate the period and number of NMDA receptors activated by primary afferent input in the spinal SG neurons and prevent glutamate spillover to activate NMDA receptors outside the active synapses.

METHODS

All experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center and were fully compliant with the National Institutes of Health Guidelines for Use and Care of Laboratory Animals.

Spinal slice preparation

Young adult male Sprague Dawley rats (140–170 g) were deeply anesthetized with halothane inhalation, and a laminectomy was made for removal of the lumbar spinal cord. The lumbar spinal cord section was placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF) presaturated with 95% O_2-5% CO_2. The sucrose ACSF contained (in mM) 234 sucrose, 3.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 12.0 glucose, and 25.0 $NaHCO_3$. The pia-arachnoid membrane was removed from each section. The L_{4-5} spinal segment, identified by the lumber enlargement and large dorsal roots, was attached with cyanoacrylate glue to a cutting support, which was then glued onto the stage of a vibratome (Series 1000, Technical Products International, St. Louis, MO). Transverse spinal cord slices (400 μm) were cut in the ice-cold sucrose ACSF and then preincubated in Krebs solution oxygenated with 95% O_2-5% CO_2 at 35°C for ≥2 h before they were transferred to the recording chamber. The Krebs solution contained (in mM) 117.0 NaCl, 3.6 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 11.0 glucose, and 25.0 $NaHCO_3$.

Whole cell voltage-clamp recordings

Following preincubation, a single slice was placed in the recording chamber (1.5 ml in volume), perfused with Krebs solution at 35°C and saturated with 95% O_2-5% CO_2. Borosilicate glass recording electrodes (resistance, 3–5 MΩ) were pulled and filled with internal solution containing (in mM) 110 Cs_2SO_4, 2.0 $MgCl_2$, 0.5 $CaCl_2$, 5.0 HEPES, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, and 10 lidocaine *N*-ethyl bromide (QX314), adjusted to pH 7.2 to 7.4 with 1 M CsOH (290–300 mosM). QX314 was added to the internal solution to suppress the action potential generation from the recorded cell. The recording electrodes were directed to the spinal lamina II (SG) region, readily identified by its distinct translucent appearance under a microscope. Live dorsal horn neurons were visualized with an infrared Nomarski microscope system (Leads Instruments) and approached with a three-dimensional motorized manipulator (Sutter Instrument), and whole cell configurations were established by applying moderate negative pressure after electrode contact (Nakatsuka et al. 2003). A seal resistance of ≥2 GΩ and an access resistance of 20–35 MΩ were considered acceptable (Weng et al. 2006; Wu et al. 2005). The series resistance was optimally compensated by ≥70% and constantly monitored throughout the experiments. Experiments showing any evidence of loss of voltage control were discarded. Signals were amplified with an amplifier (Axopatch 700B, Molecular Devices) and displayed and stored in computer.

Excitatory postsynaptic currents (EPSCs) were evoked by constant-current electrical stimuli (0.2 ms duration, repeated every 45 s) applied through a concentric bipolar stimulating electrode placed at the attached dorsal root or root entry zone (Weng et al. 2006; Yoshimura and Nishi 1993). NMDA receptor currents were isolated by including 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM), bicuculline (10 μM), and strychnine (5 μM) in the external solution to block non-NMDA glutamate receptors (AMPA and kainate receptors), $GABA_A$ receptors and glycine receptors, and holding membrane potential at +40 mV to remove the voltage-dependent Mg^{2+} block at NMDA receptors. In a subset of experiments, NMDA EPSCs were evoked by 50 μM L-glutamate injected onto the recorded neuron by puff-application (pressure: 3 psi; duration: 20 ms, repeated every 60 s) through a glass pipette with opening tip size of 8–12 μm. One neuron was recorded per each spinal slice, and two to three slices from each rat were used for recording.

Materials

DNQX, bicuculline, strychnine, (+)-MK-801 maleate (MK-801), L-glutamate, and tetrodotoxin were obtained from Sigma (St. Louis, MO); TBOA, Ro 25–6981 and D-aminophosphonovaleric acid (D-AP5) were obtained from Tocris Bioscience (Park Ellisville, MO). All drugs except DNQX were dissolved in Krebs solution. DNQX was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was always <0.1% in Krebs solution. All pharmacologic agents were applied by perfusion into the recording chamber.

Data analysis

Data were recorded with Axopatch 700B amplifiers, digitized at 10 kHz and analyzed off-line. Three to four EPSCs evoked by electrical stimulation or by puffed glutamate, at baseline and in the presence of tested drugs, were averaged. To measure time constants for the decay phase of NMDA EPSCs, the decay phase was fitted with monoexponential function, and time constants were measured (Weng et al. 2007). Clampfit 10.2 software (Molecular Devices) was used to detect and measure the amplitude, duration and time constants of averaged EPSCs. Data are presented as means ± SE. Student's *t*-test was used to determine statistical differences between measurements before and after tested drugs (paired *t*-test) or between groups (nonpaired *t*-test). Correlation tests and ANOVA with repeated measurements followed by Tukey post hoc tests were used when suitable. A P value <0.05 was considered statistically significant.

RESULTS

Combined inhibition of neuronal and glial glutamate transporters increased the amplitude and duration of NMDA EPSCs in synapses responding to weak or strong primary afferent inputs

To investigate the impact of impaired glutamate uptake on activation of NMDA receptors in synapses that transmit weak primary afferent input and in synapses that respond to strong primary afferent input, responses of NMDA receptors in the spinal SG neurons to graded primary afferent inputs were evoked by stimulating the dorsal root (or root entry zone) at two different stimulating intensities: two times activation threshold (2T) and maximum intensity. The threshold was defined as the minimum intensity required for evoking a visible NMDA EPSC, whereas maximum intensity was a stimulating intensity that evoked a maximum NMDA EPSC response in the recorded neuron. We recorded evoked NMDA EPSCs prior to and after combined blockade of glial and neuronal glutamate transporters. The transporters were blocked by bath-perfusing a

glutamate transporter blocker TBOA (concentration in the recording chamber: 50 μM). TBOA specifically blocks glutamate transporters without any effects on glutamate receptors or presynaptic release of glutamate (Demarque et al. 2004; Jabaudon et al. 1999). TBOA is a nontransportable competitive blocker that nonselectively blocks all subtypes of glutamate transporters with 50% inhibitory concentrations (IC_{50}) of 19 μM for EAAC1 (Waagepetersen et al. 2001), 68 μM for GLAST, and 6 μM for GLT-1 (Shimamoto et al. 1998).

In synapses responding to weak primary afferent inputs (2T stimulation), the blockade of glutamate transporters with TBOA significantly increased the NMDA EPSC amplitude from 46.20 ± 7.06 to 83.85 ± 13.21 pA ($n = 15$, $P < 0.01$) and the duration from 486.63 ± 70.80 to 1380.14 ± 128.05 ms ($n = 15$, $P < 0.01$). Similarly, application of TBOA (50 μM) to the bath increased activation of NMDA receptors in the synapses transmitting strong afferent inputs elicited by maximum stimulation, changing the amplitude from 118.10 ± 27.57 to 240.17 ± 42.90 pA ($n = 15$, $P < 0.01$) and the duration from 650.92 ± 81.86 to 2250.68 ± 163.97 ms ($n = 15$, $P < 0.01$). The kinetics of NMDA EPSCs evoked by 2T and maximum stimulation were altered in the presence of TBOA. Bath perfusion of TBOA delayed the peak latency from 18.37 ± 1.10 to 45.04 ± 5.15 ms ($n = 15$, $P < 0.001$) for NMDA EPSCs evoked by 2T stimulation and from 19.97 ± 1.61 to 57.09 ± 8.82 ms ($n = 15$, $P < 0.001$) for NMDA EPSCs evoked by maximum stimulation. The decay of NMDA EPSCs slowed in the presence of TBOA as the time constants for the decay phase of NMDA EPSCs significantly increased, from 98.75 ± 15.81 to 252.83 ± 34.93 ms for 2T stimulation ($n = 15$, $P < 0.001$) and from 134.86 ± 17.27 to 369.82 ± 45.84 ms ($n = 15$, $P < 0.01$) for maximum stimulation (Fig. 1, *A* and *B*). The EPSCs recorded in the presence of TBOA were caused entirely by activation of NMDA channels because they were completely abolished by a selective NMDA receptor antagonist, D-AP5 (100 μM; tested in 5 neurons).

The percentage increases induced by TBOA in the amplitude of NMDA EPSC evoked by 2T stimuli were inversely correlated with their baseline amplitudes but positively correlated with the baseline ratio between the amplitudes evoked by maximum stimulation and the amplitudes evoked by 2T stimulation (Fig. 1*C*). No clear correlation was found between the percentage changes in the amplitude induced by TBOA and the baseline amplitude evoked by maximum stimulation. Regardless of the baseline EPSC amplitudes evoked by 2T or maximum stimulation, the duration of NMDA EPSCs for all neurons recorded were increased by ≥48.37% in the presence of TBOA. The overall changes (% of baseline) induced by TBOA in EPSC amplitude (209.53 ± 36.10%), duration (355.25 ± 66.57%), decay time constant (365.89 ± 70.79%), and peak latency (258.21 ± 35.84%) evoked by 2T stimulation were not significantly different from those evoked by maximum stimulation (amplitude: 282.51 ± 65.09%; duration: 398.69 ± 40.51%; decay time constant: 331.30 ± 49.55%; peak latency: 300.04 ± 44.80%). These results indicate that glutamate transporters regulate the NMDA EPSC amplitude and the duration of NMDA EPSCs in synapses activated by weak or strong primary afferent inputs and that the net gain in the amplitude of NMDA EPSCs induced by impaired glutamate transporters is much higher in neurons receiving weak synaptic input than in neurons receiving strong synaptic input at baseline.

Combined inhibition of glial and neuronal glutamate transporters enhanced activation of NMDA receptors evoked by exogenous glutamate application

The role of glutamate transporters in synaptic transmission depends on individual synaptic structures (Danbolt 2001). NMDA receptors in spinal SG neurons are located not only in synapses responding to primary afferent input but also in synapses activated by spinal intrinsic inputs (Baba et al. 2000) and in extrasynaptic sites. To study the overall role of glutamate transporters in regulation of NMDA receptor activation, we investigated the effect of TBOA on NMDA EPSCs evoked by exogenous glutamate application. Exogenous glutamate application nonspecifically activates intrasynaptic and extrasynaptic NMDA receptors (Tovar and Westbrook 2002). Because NMDA is not a substrate for glutamate transporters (Danbolt 2001), we used exogenous L-glutamate to better mimic glutamate released from neurons. NMDA receptor currents were pharmacologically isolated and recorded at a holding membrane potential of +40 mV. Tetrodotoxin (TTX, 1 μM) was added to the bath to block voltage-gated Na^+ channels and action potentials to avoid glutamate release from neurons activated by the puffed glutamate. Maximum NMDA EPSCs at baseline were evoked by adjusting the pipette angle and distance (ranging from 20 to 30 μm) between the tip of the puff electrode and the recorded neuron. Blockade of glutamate transporters by perfusion of 50 μM TBOA significantly ($P < 0.05$) increased the NMDA current amplitude from 91.87 ± 21.76 to 143.68 ± 38.76 pA, peak latency from 47.00 ± 3.00 to 89.50 ± 9.76 ms, duration from 953.13 ± 314.45 to 2780.84 ± 941.59 ms, and decay time constant from 192.67 ± 53.78 to 788.10 ± 276.94 ms ($n = 6$; Fig. 2). The EPSC currents evoked by puffed glutamate in the presence of TBOA were due to activation of NMDA receptors as these EPSC currents were completely blocked by D-AP5 (100 μM) (tested in 2 neurons). Thus currents generated by activation of intrasynaptic NMDA receptors in functionally nonspecific synapses and by activation of extrasynaptic NMDA receptors in spinal SG neurons are regulated by glutamate transporters. Moreover, these results demonstrate that impaired glutamate uptake can enhance the response of NMDA receptors on postsynaptic neurons to the same amount of glutamate released presynaptically because, during recording, no presynaptic neurons were activated in the presence of tetrodotoxin. It is worth mentioning that these experiments do not rule out that the amount of glutamate released from the presynaptic neurons could be altered by the impairment of glutamate transporters.

Combined inhibition of glial and neuronal glutamate transporters resulted in glutamate spillover

The delayed peak latency of afferent-evoked NMDA EPSCs induced by TBOA prompted us to hypothesize that on blockade of glutamate transporters, synaptically released glutamate spills over from the synaptic cleft to the extrasynaptic space, leading to activation of distant NMDA receptors located outside the active synapses, i.e., extrasynaptic NMDA receptors and/or receptors in neighboring synapses, which are not acti-

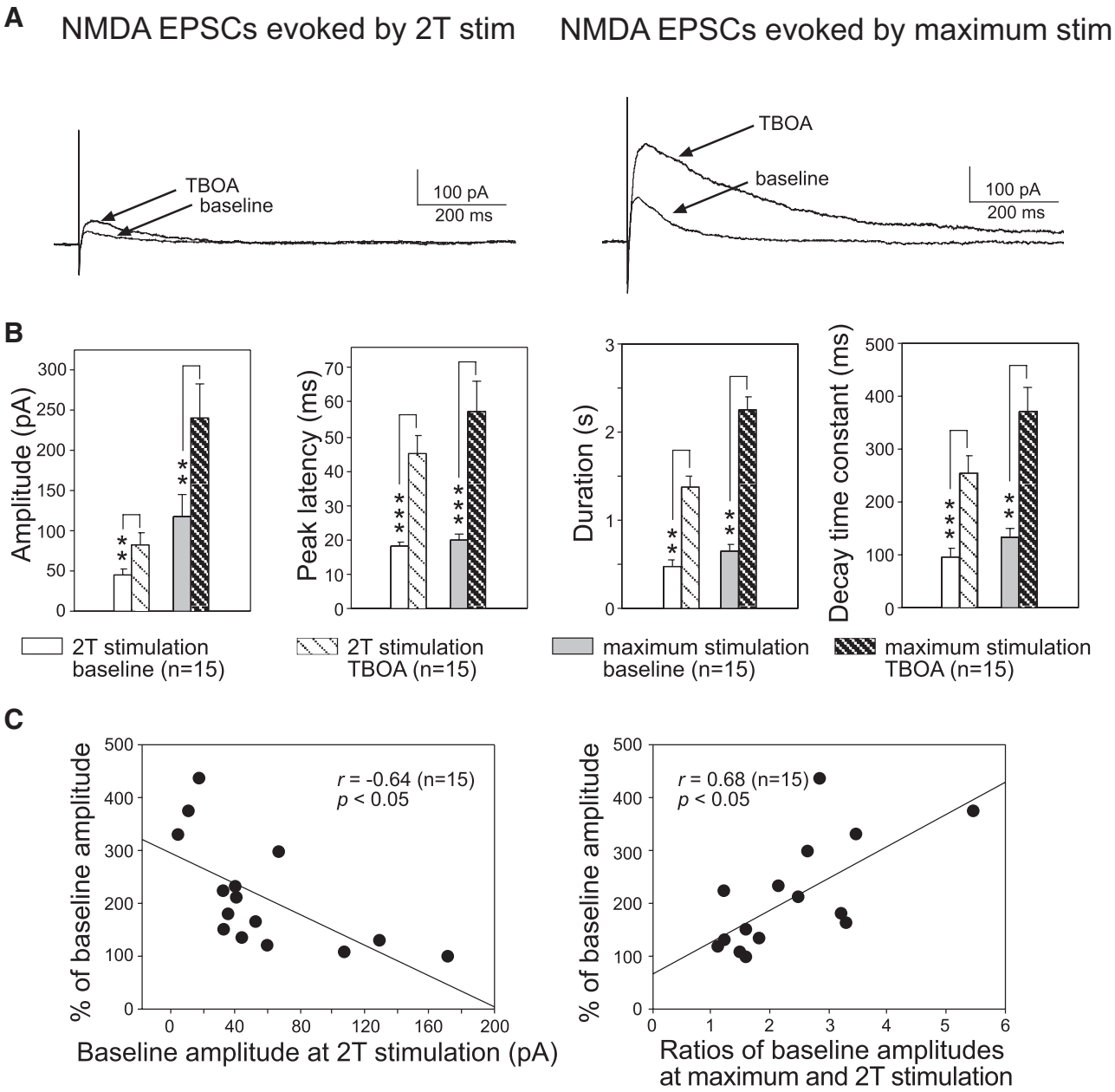

FIG. 1. Combined inhibition of neuronal and glial glutamate transporters increased the amplitude and duration of *N*-methyl-D-aspartate (NMDA) excitatory postsynaptic currents (EPSCs) in synapses responding to weak and strong primary afferent inputs. NMDA receptor currents were pharmacologically isolated and recorded at a holding membrane potential of +40 mV to remove the voltage-dependent Mg^{2+} block at NMDA receptors. Original recording traces (*A*) show samples of NMDA EPSCs evoked by 2T and maximum stimulation recorded at baseline and during blockade of glutamate transporters [(in the presence of 50 μM D-threo-β-benzyloxyaspartate (TBOA)]. Bar graphs (*B*) show the mean ± SE amplitude, peak latency, duration, and decay time constant of the NMDA EPSCs before and after application of TBOA. Scatter plots and regression lines (*C*) show that the percent increases induced by TBOA in the amplitude of NMDA EPSCs evoked by 2T stimuli were inversely related to its baseline amplitude (*left*) but positively related to the baseline ratios between the amplitudes evoked by maximum stimulation and the amplitudes evoked by 2T stimulation (*right*). *r* represents correlation coefficient. $^{**}P < 0.01$; $^{***}P < 0.001$.

vated at baseline. To test this hypothesis, we first used MK-801 to functionally remove all intrasynaptic NMDA receptors in the active synapses at baseline and then observe whether a new EPSC could be evoked when glutamate transporters were blocked with TBOA. MK-801 is a use-dependent NMDA channel blocker, which blocks NMDA receptors irreversibly when NMDA receptors are activated (open) (Huettner and Bean 1988) and is widely used as a tool to isolated active NMDA receptors from inactive NMDA receptors (Massey et al. 2004; Tovar and Westbrook 2002).

Baseline NMDA EPSCs were first pharmacologically isolated and evoked by dorsal root (or root entry zone) stimulation at maximum intensity. Intrasynaptic NMDA receptors are functionally defined as the receptors that respond to glutamate released during low-frequency stimulation (0.1 Hz) (Chen and Diamond 2002; Tovar and Westbrook 2002). To completely block all NMDA receptors inside the active synapses, NMDA EPSCs were evoked by maximum stimulation at 0.1 Hz in the presence of MK-801 (40 μM). Synaptically activated NMDA EPSCs were gradually blocked following each stimulus in the

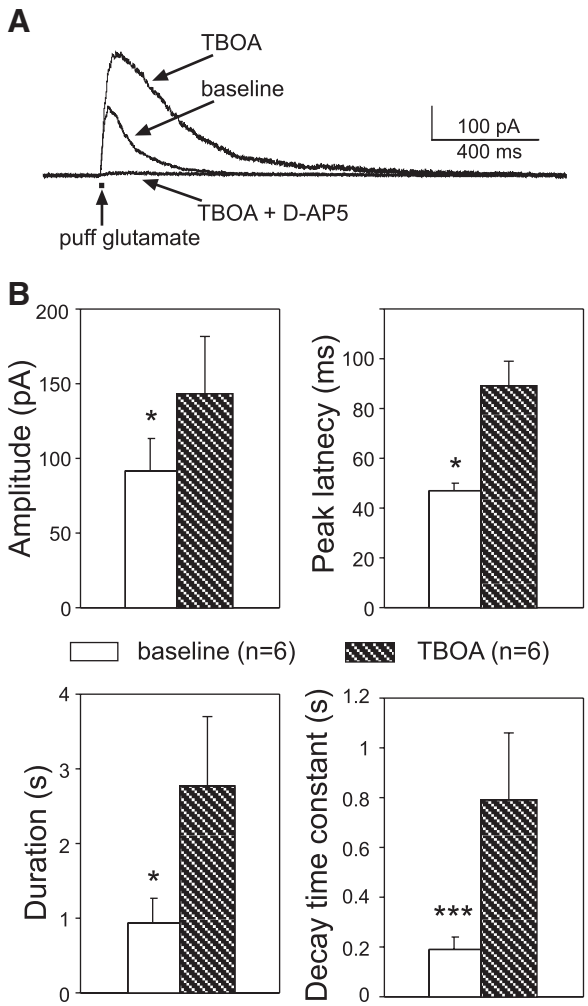

FIG. 2. Inhibition of glutamate transporters enhanced activation of NMDA receptors evoked by exogenous glutamate application. NMDA receptor currents were pharmacologically isolated and recorded at a holding membrane potential of +40 mV in the presence of TTX (1 μM). Original recording traces (*A*) show NMDA EPSCs evoked by puff-application of 50 μM glutamate (20 ms at 3 psi) taken from an substantia gelatinosa (SG) neuron at baseline and, during perfusion of 50 μM TBOA or 50 μM TBOA plus 100 μM D-aminophosphonovaleric acid (D-AP5). Bar graphs (*B*) show the mean ± SE amplitude, peak latency, duration, and decay time constant of the NMDA EPSC before and after application of TBOA. *$P < 0.05$; ***$P < 0.001$.

presence of MK-801 (Fig. 3*A*). After MK-801 blocked the synaptically activated NMDA EPSCs, MK-801 was completely washed out for 10 min. Stimulation at the same intensity after washout did not evoke any new response, indicating that NMDA receptors located inside the active synapse remained effectively blocked by MK-801 (Fig. 3, *A, C,* and *D*). After bath-application of TBOA (50 μM), stimulation at the same intensity evoked a new EPSC. The new EPSCs were characterized by their low amplitudes (24.93 ± 4.81 pA, 26.23 ± 4.82% of baseline), delayed peak latency (52.84 ± 6.21 ms, 202.59 ± 21.79% of baseline), prolonged duration (1784.73 ± 387.77 ms, 213.10 ± 34.53% of baseline), and slow decay time constant (520.55 ± 94.12 ms, 298.30 ± 57.28% of baseline; Fig. 3, *B, C,* and *D*). This new EPSC was completely blocked by bath-application of a specific NMDA receptor antagonist, D-AP5 (100 μM) (tested in 4 neurons; Fig. 3, *A* and *C*), indicating that the new EPSC was due to activation of NMDA receptors. Because NMDA receptors located inside the active synapse were already blocked by the NMDA receptor blocker MK-801, the new EPSC must have come from activation of NMDA receptors located outside the active synapses, i.e., extrasynaptic NMDA receptors and/or receptors located in neighboring synapses, which were not activated at baseline.

The new EPSCs evoked under these conditions were not due to MK-801 dissociating from the previous NMDA binding sites located inside the synapse or to insertion of new NMDA receptors or lateral diffusion of peripheral unblocked NMDA receptors to the active postsynaptic domain. This argument is based on the following facts. First, the peak latency of this new EPSC (52.84 ± 6.21 ms) was 202.59 ± 21.79% of the baseline EPSC peak latency, reflecting a longer distance between the site of glutamate release and the site of NMDA receptors responsible for the new EPSC. Second, in another set of experiments, we found that the newly evoked EPSC disappeared after TBOA was washed out ($n = 4$; Fig. 3*D*), indicating that intrasynaptic NMDA receptors remained effectively blocked by MK-801 even after TBOA application, and no new NMDA receptors appeared in the active postsynaptic domain after MK-801. Taken together, these results indicate that inhibition of glutamate transporters causes glutamate spillover and activation of NMDA receptors located outside the active synapses, i.e., extrasynaptic NMDA receptors and/or receptors sitting in the neighboring synapses.

Blockade of glutamate transporters resulted in increases in the open probability in NMDA channels in postsynaptic neurons

We then asked whether changes in the open probability of NMDA channels occurred in the presence of TBOA. MK-801, an irreversible open channel blocker of the NMDA receptors, has been routinely used to detect changes in open probability of NMDA channels (Chen et al. 1999; Hessler et al. 1993; Rossi et al. 2002). In this regard, the rate of inhibition on NMDA receptors by MK-801 is faster when the open probability of NMDA channels is increased. Thus we compared the rate of inhibition by MK-801 (40 μM) on NMDA EPSCs evoked by maximum stimulation repeated at 0.1 Hz under normal conditions in one group (11 neurons) and when glutamate transporters were blocked by TBOA (50 μM) in another group (9 neurons; Fig. 4). Although the amplitude of NMDA EPSCs prior to MK-801 application in the presence of TBOA (198.52 ± 33.67 pA, $n = 9$) was significantly ($P < 0.05$) higher than that in the absence of TBOA (107.77 ± 25.47 pA, $n = 11$), the rate of blocking NMDA EPSCs by MK-801 was significantly faster in the presence of TBOA ($P < 0.001$, repeated-measures ANOVA) than that in the absence of TBOA. Accordingly, when the progressive reduction of NMDA EPSC amplitudes over repeated stimulation in the presence of MK-801 was fitted by a single-exponential function for each group (Chen et al. 1999; Hessler et al. 1993; Rossi et al. 2002), the mean decay constant (in number of stimulation) of 6.42 in the presence of TBOA was faster by 19.95% than that of 8.02 in the normal conditions. These data indicate that the NMDA channel open probability in spinal SG neurons is increased when glutamate transporters are impaired.

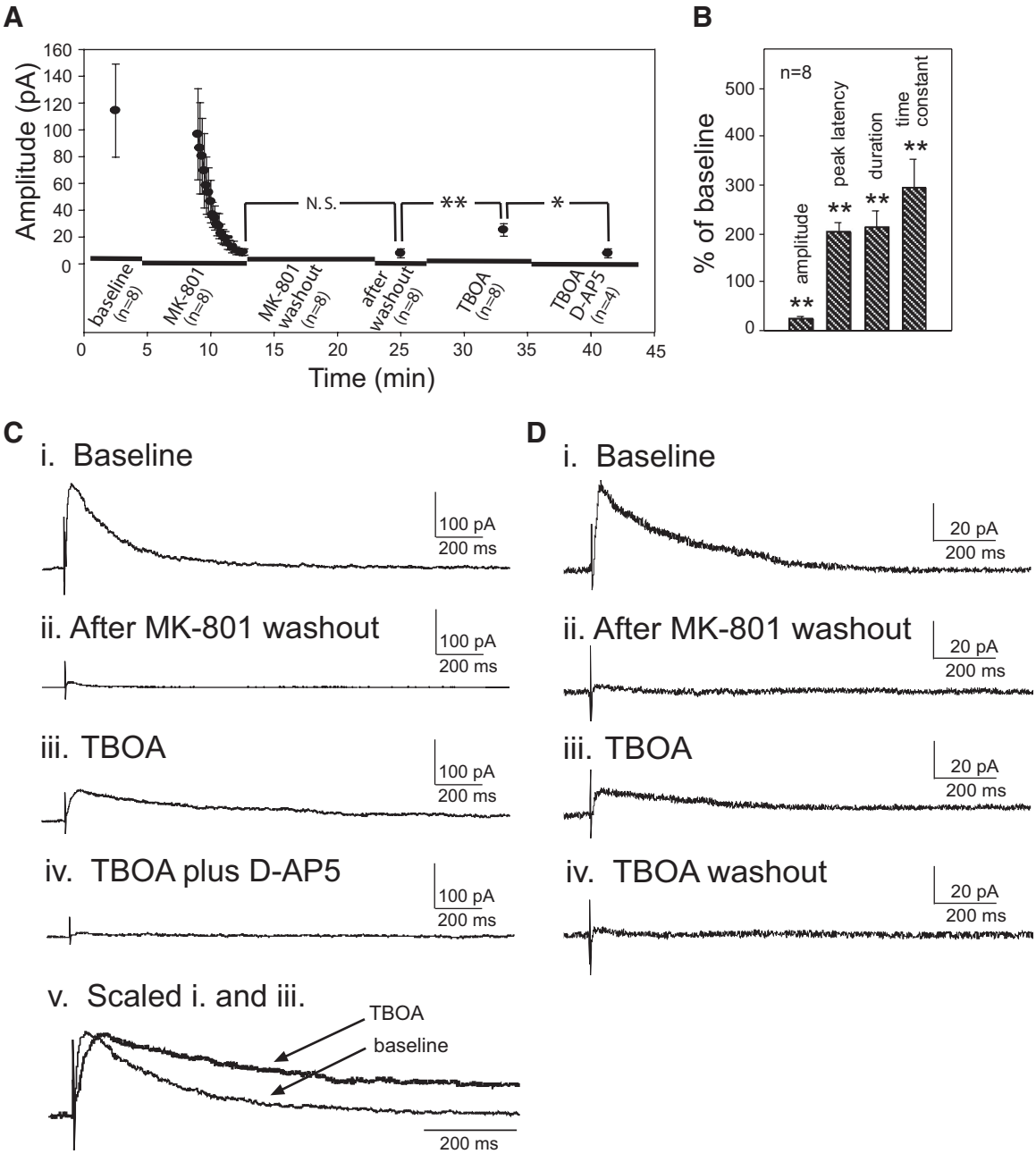

FIG. 3. Blockade of glutamate transporters resulted in extrasynaptic glutamate spillover. The mean amplitudes (±SE) of EPSCs evoked by maximum stimulation at baseline, during blockade of synaptic NMDA channels with MK-801 (40 μM), after MK-801 washout, and then during TBOA (50 μM) and TBOA (50 μM) plus D-AP5 (100 μM) are plotted against time (*A*). Three EPSCs elicited every 45 s before and after MK-801 perfusion were averaged for each cell. Synaptic NMDA currents (channels) were blocked by stimulation at the same intensity (0.1 Hz) in the presence of MK-801, and responses to each stimulation were averaged cross all recorded cells and plotted against time. Stimulation began 4–5 min after bath perfusion of each drug. Samples of recordings from one neuron recorded at different time points are shown in (*C*). The NMDA EPSC (*A*) and (*Ci*) generated by activation of synaptic NMDA receptors at the baseline disappeared after synaptic NMDA channels were blocked by MK-801 (*A*) and (*Cii*). A new EPSC was elicited by the same stimulation in the presence of TBOA (*A*) and (*Ciii*), and D-AP5 completely blocked the new EPSC (*A*) and (*Civ*). Overlay of peak scaled traces from panels *C, i* and *iii,* on an enlarged time scale shows different kinetics between the baseline EPSC and the newly appearing EPSC (*Cv*). The newly D-AP5–sensitive EPSC must have resulted from activation of NMDA receptors located outside the original synapses because NMDA receptors located inside the original active synapses were blocked by MK-801. The new EPSCs evoked under these conditions were not due to MK-801 dissociating from the previous NMDA binding sites inside the synapse, insertion of new NMDA receptors, or lateral diffusion of peripheral unblocked NMDA receptors to the active postsynaptic domain. As shown in *D*, the newly evoked EPSC disappeared after TBOA was washed out in another set of experiments. The average ± SE amplitude, peak latency, duration, and the decay time constant of the newly appearing EPSC were normalized and compared with those at baseline prior to MK-801 perfusion (*B*). NS, no statistical significance. $*P < 0.05$; $**P < 0.01$.

Blockade of glutamate transporters resulted in an increased proportion of NR2B receptor activation in NMDA EPSCs

As functional properties of NMDA receptors are determined by their subunit composition (Cull-Candy and Leszkiewicz 2004; Paoletti and Neyton 2007) and NR2B receptors play a key role in the functional plasticity of the spinal dorsal horn (Chizh and Headley 2005; Guo et al. 2002), we next asked whether activation of NR2B receptors was regulated by gluta-

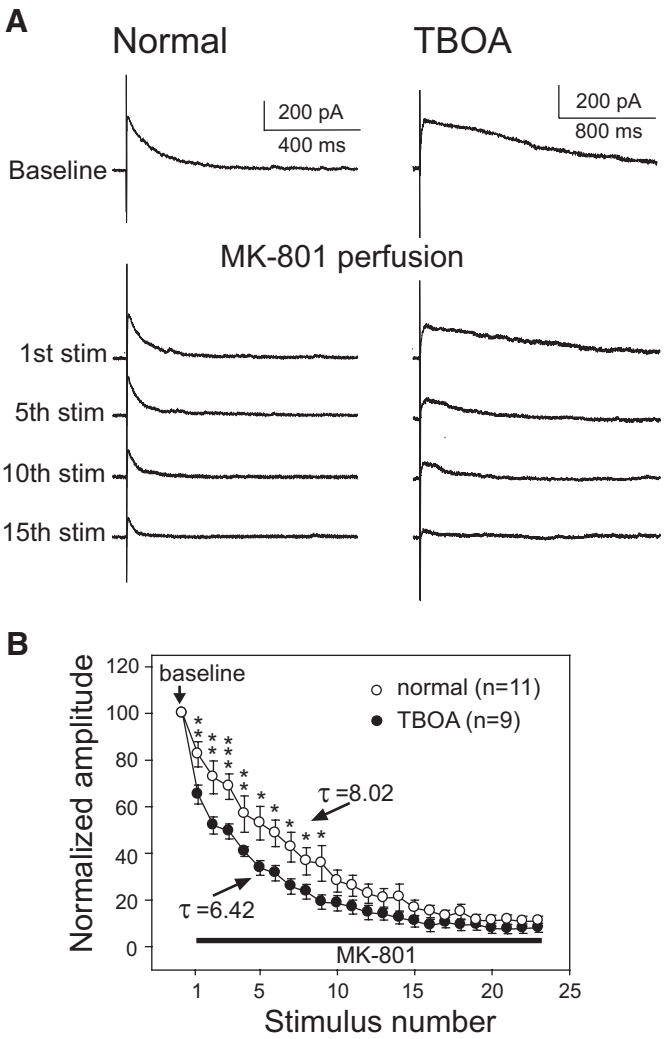

FIG. 4. Blockade of glutamate transporters resulted in increased open probability in NMDA channels in postsynaptic neurons. MK-801, an open channel blocker for NMDA receptor, was used to compare the open probability of NMDA channels between normal conditions and when glutamate transporters were blocked with 50 μM TBOA. Original recording traces (*A*) show responses to the maximum stimulation at baseline and during perfusion of MK-801 taken from an SG neuron in normal conditions and another SG neuron in the presence of TBOA. Three EPSCs elicited by maximum stimulation every 45 s at baseline were averaged in each cell. NMDA currents (channels) were blocked by stimulation at the same intensity (0.1 Hz) in the presence of MK-801 (40 μM). Responses to the 1st, 5th, 10th, and 15th stimulus in the presence of MK-801 are shown. Average ± SE NMDA amplitudes at baseline and to each stimulus during MK-801 perfusion across 11 cells in normal conditions and 9 cells in the presence of TBOA are plotted (*B*). Amplitudes were normalized to the baseline amplitude. The rate of inhibition by MK- 801 on NMDA EPSCs in the presence of 50 μM TBOA was faster than in normal conditions as shown by ANOVA with repeated measurements followed by Tukey post hoc tests. * above the curves indicate *P* values at each stimulation obtained by the post hoc tests. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Mean decay constants (τ) (in number of stimulation) for the progressive reduction of NMDA EPSC amplitudes over repeated stimulation in the presence of MK-801 for each group are shown.

mate transporters. We compared the effect of inhibiting NR2B subunits on the NMDA EPSC evoked by graded stimulation of primary afferents under normal conditions in one group and, in another group, when glutamate transporters were blocked by TBOA. Under normal conditions, bath-application of a selective NR2B receptor antagonist, Ro 25–6981 (1 μM) (Fischer et al. 1997; Mutel et al. 1998) significantly reduced the amplitude of NMDA EPSCs elicited at 2T stimuli by 36.07 ± 6.63% ($n = 5$, $P < 0.05$) and the duration by 12.24 ± 4.76% ($n = 5$, $P > 0.05$) without significant changes in the decay time constant ($n = 5$; Fig. 5, *A* and *C*). In normal conditions, inhibition induced by Ro 25–6981 (1 μM) on the NMDA EPSCs evoked by maximum stimuli was slightly stronger than that evoked by 2T stimuli although the difference did not reach statistical significance (Fig. 5*C*).

The NR2B component of the entire NMDA EPSC was significantly increased on blockade of glutamate transporters. In comparison with its inhibitory effects in normal conditions, Ro 25–6981 (1 μM) produced significantly stronger inhibition on the NMDA EPSC amplitudes and duration evoked by 2T (amplitude reduced by 54.44 ± 7.08%, duration by 31.39 ± 6.51%, $n = 7$) and maximum stimuli (amplitude reduced by 69.40 ± 6.63%, duration by 50.05 ± 4.76%) in the presence of TBOA (50 μM; Fig. 5). Ro 25–6981 also induced significantly stronger inhibition on the decay time constant of NMDA EPSCs elicited at 2T stimulation in the presence of TBOA (34.53 ± 5.63%) than in normal conditions. In the presence of TBOA, furthermore, the inhibition induced by Ro 25–6981 (1 μM) on the NMDA EPSC amplitude and duration evoked by maximum stimuli was significantly stronger than the Ro 25–6981 (1 μM)–induced inhibition on those evoked by 2T stimuli (Fig. 5). These data indicate that in normal synaptic transmission, the majority of NMDA subunits activated by primary afferents are not NR2B subunits (most likely NR2A subunits) but with deficient glutamate uptake the balance between activation of NR2B subunits and other subunits tilted toward NR2B subunits, making NR2B a major NMDA subunit activated by glutamate released from primary afferents.

DISCUSSION

The present study reveals how impaired glutamate uptake by glutamate transporters affects activation of NMDA receptors in spinal SG neurons. First, we demonstrated that activation of NMDA receptors by primary input on the spinal SG neurons is restricted by glutamate uptake carried out by glutamate transporters and that impairment of glutamate transporters leads to an increased duration and amplitude of NMDA EPSCs elicited by primary afferent inputs or exogenous glutamate. Second, we provided evidence that such increased activation of NMDA receptors is due to glutamate spillover that activates NMDA receptors located outside the active synapses and increases open probability of NMDA channels. Third, we showed that deficiency in glutamate uptake leads to an increased proportion of NR2B subunit activation in NMDA EPSCs and that this increase is further augmented by stronger afferent input. These data strongly indicate that an intact glutamate uptake transport system is critical in keeping accurate spatiotemporal and intensity coding in glutamatergic synaptic transmission and preventing abnormal activation of NMDA receptors in the spinal dorsal horn. These findings also suggest that reversing impaired glutamate uptake may be a potential new avenue to relieve the pathological pain related to abnormal activation of NMDA receptors.

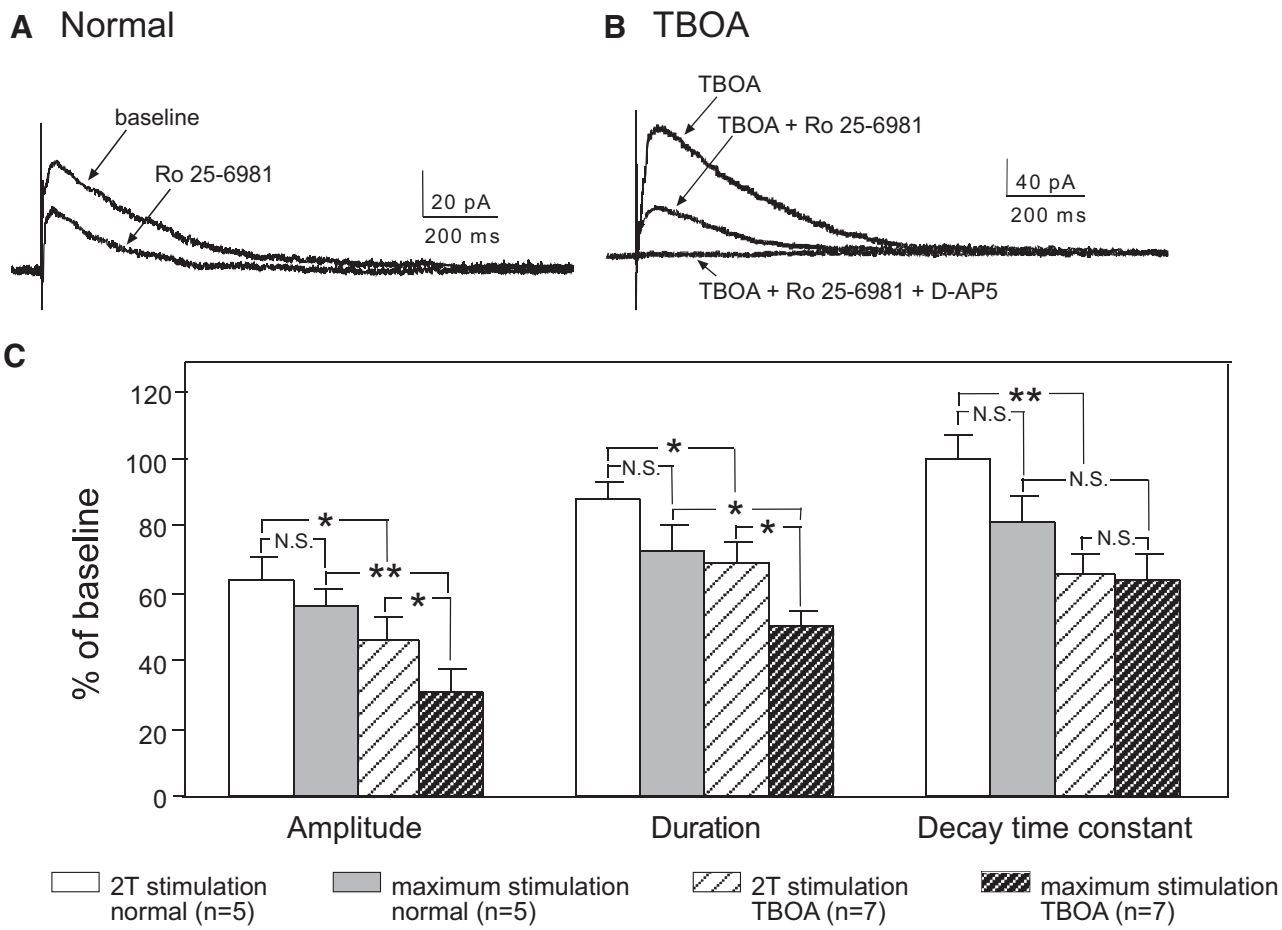

FIG. 5. Blockade of glutamate transporters resulted in an increased proportion of NR2B receptors activation in NMDA EPSCs. Original recordings show samples of NMDA EPSCs evoked by maximum stimulation at baseline and in the presence of a NR2B receptors-specific antagonist (Ro 25–6981, 1 μM) in a neuron under normal conditions (*A*) and another neuron in the presence of TBOA (*B*). The evoked EPSC in the presence of TBOA was completely blocked by a specific NMDA receptor antagonist D-AP5 (100 μM; *B*). Under normal conditions, bath-application of Ro 25–6981 significantly reduced the amplitude and the duration of NMDA EPSCs elicited by 2T or maximum stimuli without significant changes in the decay time constant. In the presence of TBOA, Ro 25–6981 (1 μM) produced significantly stronger inhibition on the NMDA EPSC amplitudes, durations evoked by 2T and maximum stimuli. In the presence of TBOA, the inhibition induced by Ro 25–6981 on the NMDA EPSC amplitude and duration evoked by maximum stimuli was significantly stronger than the Ro 25–6981–induced inhibition on those evoked by 2T stimuli. Average ($\pm$SE) amplitudes, durations, and decay time constants of the NMDA EPSCs evoked by 2T and maximum stimulation in the presence of Ro 25–6981 in normal conditions and in the presence of 50 μM TBOA are presented in bargraphs (*C*). All values were normalized to the baseline values prior to application of Ro 25–6981. A 2 $\times$ 2 ANOVA followed by Tukey post hoc tests was used to determine differences between different groups. $*P < 0.05$; $**P < 0.01$.

Regulation of NMDA receptor activation by glutamate transporters

Three major factors determine the kinetics of EPSCs at glutamatergic synapses: the amount of glutamate released from presynaptic terminals, the number and properties of postsynaptic glutamate receptors. and the process of glutamate clearance. Because glutamate cannot be metabolized extracellularly, the homeostasis of extracellular glutamate ultimately is maintained by the glutamate transporter system. The effect of impaired glutamate uptake on activation of postsynaptic ionotropic glutamate receptors depends on the density, location, and affinity of the glutamate transporters, and the sites and amount of glutamate release, as well as synaptic microstructures in different regions of the CNS (Anderson and Swanson 2000; Danbolt 2001). In small-diameter synapses without glial cell covering, like those between hippocampal Schaffer collaterals and hippocampal pyramidal cells (Isaacson and Nicoll 1993; Ventura and Harris 1999), impairment of glutamate uptake does not alter the decay of afferent-evoked EPSCs, suggesting that diffusion of glutamate is a key process in terminating glutamate action on glutamate receptors. In most synapses, however, glutamate diffusion is restricted by glial cells wrapping the synapse (Danbolt 2001; Halassa et al. 2007; Panatier et al. 2006). In such synapses, glutamate transporters play a critical role in regulating activation of glutamate receptors as demonstrated by the fact that blockade of glutamate uptake increases both the amplitude and duration of AMPA EPSCs in postsynaptic neurons (Overstreet et al. 1999; Takayasu et al. 2004).

The regulation by glutamate transporters of activation of AMPA receptors (Weng et al. 2007) and NMDA receptors in the spinal SG neurons are consistent with the anatomical synaptic structures in the spinal SG neurons the synapses of which are reportedly wrapped by glial cells (Kerr 1975). Interestingly, further analysis in the present study showed that the degree of enhancement induced by TBOA in activation of

NMDA receptors was greater in neurons that had weak synaptic input at baseline. This indicates that the enhanced synaptic inputs caused by impaired glutamate uptake were greater for weak synaptic connections than for strong synaptic connections, consistent with a widely held notion that the effect of uptake block on synaptic transmission is related to the strength of synaptic connections (Marcaggi and Attwell 2004). In our recent report, we also noted that on inhibition of glutamate transporters, the AMPA EPSC amplitudes recorded from spinal SG neurons were increased in neurons with weak synaptic input but decreased in neurons with strong synaptic input (Weng et al. 2007). We found in the present study, on the other hand, that blockade of glutamate transporters increased NMDA EPSC amplitude in neurons receiving either weak or strong synaptic input. These data reinforce our recent conclusion that desensitization of AMPA receptors contributes to reduction of AMPA EPSC amplitude (Weng et al. 2007) during blockade of glutamate transporters and are consistent with a general notion that NMDA receptors are much less vulnerable to desensitization induced by glutamate than AMPA receptors (Dingledine et al. 1999).

Mechanisms underlying enhanced activation of NMDA receptors induced by blockade of glutamate transporters

The macroscopic NMDA receptor-mediated peak current amplitude (I_{peak}) recorded from a cell is determined by the following equation: $I_{peak} = i_* N_* P_o$ where i represents the unitary current passing though each ion channel, N represents number of channels (i.e., number of functional surface NMDA receptors), and P_o represents open probability of ion channels on binding of glutamate to NMDA receptors. The increased peak amplitude of NMDA EPSCs induced by TBOA in the present study may reflect an increase in either N or P_o or both assuming i remains unchanged. Using the NMDA open channel blocker MK-801, we demonstrated that blockade of glutamate transporters increases the open probability (P_o) of NMDA channels in spinal SG neurons. This is consistent with previous findings that TBOA causes elevation of glutamate concentrations (Liaw et al. 2005) and is supported by the present findings showing that TBOA prolongs the transience of synaptically released glutamate (i.e., prolonged NMDA EPSCs) because the open probability of NMDA channels is known to be increased as glutamate concentrations or dwelling times increase (Banke and Traynelis 2003; Popescu et al. 2004). Furthermore, increased glutamate spillover and ambient concentrations induced by the blockade of glutamate uptake may activate metabotropic glutamate receptors, leading to an increase in NMDA receptor open probability (Kalia et al. 2004).

The increased number (N) of NMDA receptors bound to glutamate in the presence of TBOA could come in two ways. In one way, extrasynaptic glutamate spillover induced by TBOA would recruit additional number (N) of NMDA receptors outside the active synapse as demonstrated in this study. The receptors activated by the spilled glutamate may be located in extrasynaptic sites and/or inside neighboring synapses. The activation of extrasynaptic NMDA receptors in the presence of TBOA is supported by another set of experiments in this study in which bath-application of TBOA led to a greater contribution of NR2B receptor activation to the entire NMDA EPSC, i.e., recruitment of additional activated NR2B receptors. Because NR2B receptors in the extrasynaptic site are generally believed to be more dominant than those inside the synaptic cleft (Lozovaya et al. 2004; Momiyama 2000), an increased proportion of NR2B receptor activation to composition of NMDA EPSCs suggests glutamate spillover to the extrasynaptic site. As we have recently shown that blockade of glial GLT-1 transporters in the spinal dorsal horn results in synaptically released glutamate spillover to AMPA receptors at neighboring synapses (Weng et al. 2007), it is conceivable that glutamate spillovers induced by TBOA lead to activation of NMDA receptors at the neighboring inactive synapses. Furthermore, activation of NMDA receptors outside the active synapse by glutamate spillover also contributes importantly to the prolonged duration of NMDA EPSCs, as the duration of NMDA EPSCs after removal of the active synaptic NMDA receptors in the presence of TBOA was more than twice the baseline, which is comparable to the prolonged NMDA EPSC duration induced by TBOA with intact synaptic NMDA receptors.

In another way, the NMDA receptors recruited additionally should be located inside the synapse activated at baseline. This notion is based on the following facts. Of the 182.51% increase in the NMDA EPSC peak amplitude in the presence of TBOA, 26.23% came from activation of NMDA receptors outside the original active synapses, and 19.95% were caused by an increase in the channel open probability (P_o). The remaining difference must come from activation of NMDA receptors inside the active synapse at baseline. Hence it is conceivable that not all the NMDA receptors located inside the synapse responding to afferent input in spinal SG neurons are occupied fully by glutamate released from presynaptic sites at the baseline (i.e., unsaturated synaptic NMDA receptors) and that impaired glutamate uptake leads to accumulation and dwelling of glutamate in the synaptic cleft that in turn leads to glutamate binding of the unsaturated synaptic NMDA receptors. This is in line with recent studies showing that NMDA receptors at hippocampal synapses are not generally saturated by synaptically released glutamate (Ishikawa et al. 2002; McAllister and Stevens 2000).

Functional considerations

The SG (lamina II) (Molander et al. 1984; Rexed 1952) in the spinal dorsal horn is the first station for sensory (particularly nociceptive) processing in the CNS. It receives and processes extrinsic nociceptive inputs from Aδ and C fiber primary afferents (Kumazawa and Perl 1977; Kumazawa and Perl 1978) as well as intrinsic inputs from spinal interneurons and supraspinal centers (Furue et al. 2004; Willis and Coggeshall 2004). Fast synaptic transmission in the spinal dorsal horn is conducted mainly by activation of AMPA and NMDA receptors. The regulation by glutamate transporters of activation of AMPA receptors found in our previous study (Weng et al. 2007) and of NMDA receptors found in this study strongly indicate the key role of glutamate transporters in spinal sensory transmission. Physiologically, prompt clearance of synaptically released glutamate by glutamate transporters prevents excessive activation of glutamate receptors and limits the receptor activation duration, thus warranting accurate signal coding for intensity and timing. Furthermore, glutamate uptake also prevents extrasynaptic glutamate spillover. Gluta-

mate, which spilled over to NMDA receptors on the recorded neurons, may come from presynaptic terminals that do not form synaptic contact with the recorded neuron or from presynaptic terminals that form direct synapses onto the recorded cell. Restriction of glutamate spillover by glutamate transporters renders independent signal transmission in individual synapses and neurons, thus minimizing "cross-talk" between neighboring synapses or between neurons and securing accuracy in spatial coding and modality specificity.

Recent studies from our laboratory and others have strongly suggested that impairment of glutamate uptake is one of key factors leading to excessive activation of glutamate receptors, a crucial process related to the initiation and maintenance of pathologic pain. For example, functional blockade of glutamate transporters in the spinal cord induces nociceptive behaviors in awake animals (Liaw et al. 2005; Weng et al. 2006). Blockade of glutamate transporters increases response amplitude and duration of spinal wide dynamic range neurons to peripheral stimuli (Weng et al. 2006). Downregulation of glutamate transporter protein expression in the spinal dorsal horn is associated with hyperalgesia induced by chronic constrictive nerve injury (Sung et al. 2003), chemotherapy (e.g., paclitaxel) (Weng et al. 2005), and opioids (Mao et al. 2002; Thomson et al. 2006). Glutamate homeostasis is altered in the spinal dorsal horn when peripheral tissue is injured or inflamed (Sluka and Willis 1998; Sung et al. 2007). The abnormal activation induced by blockade of glutamate transporters in NMDA receptors in this study and AMPA receptors in our previous study (Weng et al. 2007) may elucidate in part the synaptic mechanisms underlying the pathological pain seen in these models. Furthermore, because >90% of glutamate uptake in the CNS is carried out by glutamate transporters located in glial cells (Danbolt 2001), the dysfunctional glutamate transporters in glial cells may well be a key player, causing excessive excitation in the spinal dorsal horn neurons under pathological conditions. In other words, glutamate transporters in glial cells may be a key mediator for the interactions between glial cells and neurons in normal spinal sensory processing and pathogenesis of pain.

Taken together, our present and previous studies indicate that spinal synaptic independence and temporal and intensity coding for sensory input rely on an intact glutamate uptake system and suggest remedying the impaired glutamate uptake may be a potential new avenue toward treatment of pathogenesis of pain.

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