Involvement of Reactive Oxygen Species in Long-Term Potentiation in the Spinal Cord Dorsal Horn

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Lee KY, Chung K, Chung JM. Involvement of reactive oxygen species in long-term potentiation in the spinal cord dorsal horn. J Neurophysiol 103: 382–391, 2010. First published November 11, 2009; doi:10.1152/jn.90906.2008. Recent studies suggest that reactive oxygen species (ROS) are functional messenger molecules in central sensitization, an underlying mechanism of persistent pain. Because spinal cord long-term potentiation (LTP) is the electrophysiological basis of central sensitization, this study investigates the effects of the increased or decreased spinal ROS levels on spinal cord LTP. Spinal cord LTP is induced by either brief, high-frequency stimulation (HFS) of a dorsal root at C-fiber intensity or superfusion of a ROS donor, tert-butyl hydroperoxide (t-BOOH), onto rat spinal cord slice preparations. Field excitatory postsynaptic potentials (fEPSPs) evoked by dorsal root stimulations with either Aβ- or C-fiber intensity are recorded from the superficial dorsal horn. HFS significantly increases the slope of both Aβ- and C-fiber evoked fEPSPs, thus suggesting LTP development. The induction, not the maintenance, of HFS-induced LTP is blocked by a N-methyl-d-aspartate (NMDA) receptor antagonist, d-2-amino-5-phosphonopentanoic acid (D-AP5). Both the induction and maintenance of LTP of Aβ-fiber-evoked fEPSPs are inhibited by a ROS scavenger, either N-tert-buty1-α-phenylisctrone or 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl. A ROS donor, t-BOOH-induced LTP is inhibited by N-tert-buty1-α-phenylisctrone but not by D-AP5. Furthermore, HFS-induced LTP and t-BOOH-induced LTP occlude each other. The data suggest that elevated ROS is a downstream event of NMDA receptor activation and an essential step for potentiation of synaptic excitability in the spinal dorsal horn.

INTRODUCTION

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism. An excessive level of ROS, however, is involved in many degenerative diseases in the CNS (Contestabile 2001; Jenner 1994) due to a prominent role of ROS in cell damage and death, including that of neurons. Recent evidence suggests that ROS also play a critical role in persistent neuropathic (Kim et al. 2004) and inflammatory (Salvemini et al. 2006) pain. Several lines of indirect evidence suggest that ROS serve as functional messenger molecules in central sensitization, an underlying mechanism for persistent pain (Gao et al. 2007; Lee et al. 2007; Schwartz et al. 2008). To investigate ROS involvement in central sensitization in detail, we examined the effects of ROS donors and ROS scavengers on spinal cord long-term potentiation (spinal cord LTP), which is considered to be the electrophysiological basis of central sensitization.

As a model of activity-dependent synaptic plasticity, LTP has been studied extensively in the hippocampus and is thought to be a cellular basis for learning and memory (Bliss and Collingridge 1993). LTP-like phenomena also have been observed in the spinal cord in both in vitro and in vivo experiments and have been termed spinal cord LTP (Ikeda et al. 2003; Liu and Sandkuhler 1995; Randic et al. 1993). Spinal cord LTP is in many ways similar to the better-studied hippocampal LTP because both types show an increased responsiveness of the affected neurons with a prolonged time course and also show a dependency on N-methyl-d-aspartate acid (NMDA) receptors for the triggering process (Liu and Sandkuhler 1995, 1998; Randic et al. 1993; Svendsen et al. 1998). Spinal cord LTP is thought to be a major mechanism of central sensitization, a nociceceptor-dependent increase in neuronal excitability in the spinal cord dorsal horn (Cook et al. 1987; Ji et al. 2003; Woolf 1983), which is characterized by a reduction in the activation threshold, an increase in the responsiveness of dorsal horn neurons, and an enlargement of their receptive fields (Cook et al. 1987). Maintained central sensitization is an important mechanism underlying persistent neuropathic and inflammatory pain (Ji et al. 2003; Sandkuhler 2000), and spinal cord LTP is the physiological representation of central sensitization (Ji et al. 2003; Sandkuhler 2000; Willis 2002). Thus investigating the involvement of ROS in spinal cord LTP may provide insights into the role of ROS in central sensitization and persistent pain.

To determine the role of ROS in central sensitization, the present study examines the effects of ROS scavengers and donors on the development and maintenance of spinal cord LTP by recording field excitatory synaptic potentials (fEPSPs) from the spinal cord dorsal horn using an in vitro spinal cord slice preparation. The results show that ROS are essential not only for the induction but also for the maintenance of spinal cord LTP as manifested by increased Aβ-fiber-evoked fEPSPs, which might be the underlying mechanism of Aβ-fiber-mediated pain (touch-evoked pain, allodynia).

METHODS

Experimental animal preparation

Young male Sprague-Dawley rats (18-22 days old) were purchased from Harlan Sprague-Dawley in Houston, TX. Protocols for animal care and use are approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and comply with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Rats were anesthetized with isoflurane in oxygen and maintained on a 12:12-h light-dark cycle. The animal was placed in a stereotaxic instrument and the scalp was incised. After exposure of the spinal cord, the surgery was performed. The spinal cord was fixed in 4% paraformaldehyde or 4% formaldehyde for 12 h, then washed in PBS for 12 h. The samples were then embedded in paraffin and 5-μm-thick cryostat sections were cut. Then, the samples were stained with hematoxylin and eosin (H&E) and examined by light microscopy.
gas of 95% $O_2$-5% $CO_2$. After being trimmed and embedded in an agar block, the Lc-L6 spinal cord segments were then sliced transversely at a thickness of 450–500 µm, with the corresponding dorsal roots attached using a vibratome (Leica, VT1000S). Spinal cord slices with the attached dorsal roots were incubated in ACSF at 30°C for 1 h.

Determination of stimulation paradigms

To determine the type of afferent fibers activated by electrical stimulation, compound action potentials were recorded from the dorsal root ($n=5$) while stimulating the peripheral nerve in an in vitro peripheral nerve preparation (Fig. 1A). The isolated in vitro peripheral nerve was prepared by dissecting out the dorsal root and dorsal root ganglion along with the attached either L4 or L5 spinal nerve. After placing the peripheral nerve preparation in a recording chamber, the cut proximal end of the spinal nerve was placed in a suction electrode for electrical stimulation. Extracellular compound action potentials were recorded from the dorsal root using a silver wire hook electrode while stimulating the spinal nerve with various stimulus parameters.

Field potential recordings

After incubation in ACSF, the spinal cord slices were placed in a recording chamber equipped with an ACSF superfusion system. Recordings were made with a glass micropipette electrode (ACSF internal solution, 2 MΩ), and electrical stimulation was applied through a suction electrode attached to the cut end of the dorsal root (Fig. 1D). fEPSPs in response to dorsal root stimulation (test stimulus) were recorded and amplified using the Multiclamp 700B (Axon Instruments). Data were collected and analyzed using pCLAMP (Axon Instruments). Recording temperature was kept at 30°C. The parameter of test stimuli for $A\beta$-fibers was 30–50 µA (0.5 ms) and that for C-fibers was 1–1.2 mA (0.5 ms). Test stimuli were delivered once every 30 s (4 times every 2 min). From each recorded tracing, the slope of fEPSP was measured between 10 and 90% of the fEPSP peak amplitude. Each presented fEPSP slope value is an average of four individual fEPSP recordings obtained during the designated 2-min period. Baseline responses were recorded for 20 min before any experimental manipulations.

To avoid contamination from $A\delta$- and C-fiber-evoked events, $A\beta$-fiber-evoked fEPSPs were identified based on the following two criteria: the fEPSPs with a clear first peak and a constant latency. The latency between the stimulus artifact and the beginning of fEPSPs was measured and used for conduction velocity calculation. The fEPSPs that were evoked by stimuli of $A\beta$-fiber intensities and had $A\beta$-fiber latencies were considered as $A\beta$-fiber evoked fEPSPs.

To find the most suitable location for recording of $A\beta$-fiber-evoked fEPSPs, we initially recorded fEPSPs from four different locations in the spinal dorsal horn while stimulating the dorsal root with $A\beta$ fiber intensities (Fig. 1E). These four areas include superficial medial (SM; medial part of the substantia gelatinosa (SG)), superficial lateral (SL; lateral part of SG), deep medial (DM: medial part of lamina IV-V), and deep lateral (DL: lateral part of lamina IV-V) dorsal horn. $A\beta$-fiber-evoked fEPSPs were recorded routinely from the medial (SM and DM) region but rarely from the lateral (SL and DL) region. The fEPSPs recorded from the SM usually showed a monophasic wave form with a single peak. The fEPSPs recorded from the DM,

![FIG. 1.](http://jn.physiology.org/)

- A: compound action potentials were recorded with a hook electrode from the isolated dorsal rootlet while stimulating the cut proximal end of the dorsal root by a suction electrode. B and C: examples of extracellular compound action potential recordings evoked at 2 different stimulus intensities (B, 30–60 µA; C, 1 mA). The threshold intensities for $A\beta$, $A\delta$, and C-fiber activation were 40, 60, and 700 µA, respectively. The stimulus duration was 0.5 ms. Calculated conduction velocities were 17.6, 6.1, and 1.5 m/s for $A\beta$, $A\delta$, and C-fibers, respectively. D: field excitatory postsynaptic potential (fEPSP) recording setup and examples of $A\beta$-fiber-evoked fEPSPs. fEPSPs recorded from the superficial medial dorsal horn while stimulating the cut proximal end of the attached dorsal root with a suction electrode. The fEPSPs evoked by $A\beta$-strength stimulus was completely blocked by bath application of 20 µM 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), suggesting that fEPSP is mediated by glutamate AMPA/kinate receptors. E: an outline of the dorsal quadrant of a transverse spinal cord slice with recorded fEPSPs at 4 locations: superficial medial (SM), superficial lateral (SL), deep medial (DM), and deep lateral (DL) dorsal horn. The probabilities of recording fEPSPs with a clear 1st peak were signified by symbols ( granite, 6–8 of 10 samples; 1–3 of 10 samples; 0 of 10 samples). Typical examples of raw traces of fEPSP from each location (SM, SL, DM, and DL) are shown. Calibration bars are 3 ms and 0.1 mV. Note that most of fEPSPs (6 of 10) from DM have multiple peaks.
However, frequently showed a complex wave form with multiple peaks, which made it difficult to analyze. From the recordings made in 10 preparations, a single peak wave form was observed in 8 of 10 from SM but 2 of 8 from DM. No single peak wave form was recorded from the SL and DL. Based on these results, all fEPSP recordings in this study were made from the SM region of the dorsal horn. The superficial dorsal horn encompasses substantia gelatinosa and can be identified as a distinctive translucent band across the dorsal part of the dorsal horn as shown in Fig. 1D.

To induce spinal cord LTP, the cut end of the dorsal root was stimulated with high-frequency stimulation (HFS) with a suction electrode. The parameters of HFS are 1-s-long trains of pulses (100 Hz, 1.2 mA, 0.5 ms) repeated five times at 10-s intervals that activate C-fibers. The HFS used in this study consistently induced a prolonged increase of fEPSP slope values >20% compared with the pre-HFS control levels, which condition was defined as LTP.

**Pharmacological compounds**

For pharmacological studies, compounds were dissolved in an ACSF superfusion solution at various concentrations immediately before use. N-N-tert-butylnaphthyridine (PBN), 4-hydroxy-2,2,6,6-tetramethylpiperidine-oxyl (TEMPOL), and tert-butyl hydroperoxide (t-BOOH) were purchased from Sigma (St. Louis, MO). D(−)-2-amino-5-phosphopentanoic acid (D-AP5) and 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) were purchased from Tocris (Ellisville, MO). PBN and TEMPOL were used as ROS scavengers and t-BOOH as a nonspecific ROS scavenger while CNQX, were prepared as a 1,000 μM stock solution and then diluted in ACSF before use.

**Statistical analysis**

The slope values of four consecutive fEPSPs evoked every 30 s were averaged to produce one slope value for each 2-min interval, and data are presented as single slope values. The average and variability (SE) of the slope value data from multiple preparations (spinal cord slices) were plotted at 2-min intervals in the figures. The values at the mid time points during control period (e.g., 10–12 min of 20 min of control) as well as after certain manipulations (drug application or HFS) were used for statistical comparisons of means. The statistical evaluations were made by using one-way ANOVA, followed by the Bonferroni post hoc test. P < 0.05 was considered to be significant.

**RESULTS**

**Test stimulus parameters for A- and C-fibers**

The ranges of stimulus intensities required to activate Aβ-, Aδ-, and C-fibers were 32 ± 6, 68 ± 18, and 850 ± 67 μA with 0.5-ms stimulus pulse duration, respectively (Fig. 1, B and C, and Table 1, n = 5). No Aβ- and C-fiber-evoked response was detected with stimulus intensities <25 and 700 μA (0.5 ms), respectively. The conduction velocities of Aβ-, Aδ-, and C-fibers were 15.7 ± 3.8, 8.6 ± 2, and 1.1 ± 0.2 m/s, respectively. Based on these results, the stimulus intensities of 30–50 μA (0.5 ms) and 1–1.2 mA (0.5 ms) were selected to evoke Aβ- and C-fiber-mediated fEPSPs, respectively, in spinal cord slice preparation. These stimulus parameters are similar to those used in other studies of spinal cord LTP (Ikeda et al. 1998; Sandkuhler et al. 1997; Schneider and Perl 1988).

Examples of Aβ- and C-fiber-evoked fEPSPs are shown in Fig. 2, B and C, respectively. Treatment with an AMPA/kainate receptor antagonist, CNQX (20 μM), completely blocked both Aβ- and C-fibers-evoked fEPSPs within 3–5 min after the drug treatment (n = 5). An example of CNQX effect on Aβ-fibers-evoked fEPSPs is shown in Fig. 1D. Thus the data indicate that the recorded fEPSPs are mediated by postsynaptic glutamate AMPA and kainate receptors.

**TABLE 1. Compound action potential**

<table>
<thead>
<tr>
<th>Threshold, μA</th>
<th>Aβ</th>
<th>Aδ</th>
<th>C</th>
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<tr>
<td>Means ± SE</td>
<td>32 ± 8</td>
<td>68 ± 18</td>
<td>850 ± 67</td>
</tr>
<tr>
<td>Conduction Velocity, m/s</td>
<td>Aβ</td>
<td>Aδ</td>
<td>C</td>
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<td></td>
<td>15.7 ± 3.8</td>
<td>8.6 ± 2</td>
<td>1.1 ± 0.2</td>
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**FIG. 2.** Recordings of Aβ- and C-fiber-evoked fEPSPs before and after LTP induction in spinal cord slices. A: each fEPSP slope value is the average of 4 recordings made every 2 min in each specimen and then averaged again from the recordings of 6 different specimens. Slope values were plotted as percent of control against time (n = 6). The intensities of test stimuli to elicit Aβ- and C-fiber-evoked fEPSPs were 30–50 μA (0.5-ms duration) and 1–1.2 mA (0.5-ms duration), respectively. Baseline fEPSPs in response to the test stimuli were recorded for 20 min. The conditioning high-frequency stimuli (HFS), which consisted of 5 1-s trains of 100-Hz pulses (1.2 mA, 0.5 ms) given at 10-s intervals, were delivered at 20 min (†). After HFS, recording was paused for 10 min for stabilization of preparation. Responses to test stimuli were then recorded for an additional 40 min. The slopes of fEPSPs were significantly increased after HFS, indicating the induction of LTP. B and C: examples of Aβ- and C-fiber-evoked fEPSP recordings before (a) and after (b) HFS. ▲, stimulus artifacts.

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HFS induces LTP of Aβ- and C-fiber-evoked fEPSPs through NMDA receptor activation

The Aβ- and C-fiber evoked fEPSPs were recorded for 20 min before LTP induction by high-frequency stimulation and used as baseline responses. The fEPSPs were also recorded for 40 min starting 10 min after HFS, and the summary data are shown in Fig. 2A (n = 6). Examples of Aβ- and C-fiber-evoked fEPSP recordings at baseline (a) and after LTP induction (b) are shown in Fig. 2, B and C, respectively. The slope values of four fEPSP recordings (30-s intervals) made over a 2-min period were averaged and the summary data of relative averaged slope values are plotted in Fig. 2A. After conditioning HFS with C-fiber intensity [1-s-long trains of pulses (100 Hz, 1.2 mA, 0.5 ms) repeated five times at 10-s intervals], ~71% of spinal cord slices (41/58) showed a >20% enhancement of the slopes of the Aβ-fiber-evoked fEPSPs and C-fiber-evoked fEPSPs (Fig. 2, A–C). On average, the slopes of Aβ-fiber-evoked fEPSPs at 30 min after HFS showed an increase to 140 ± 9% (mean ± SE; n = 6) and C-fiber-evoked fEPSPs showed an increase to 144 ± 8% (n = 6) after HFS compared with pre-HFS control levels (Fig. 2A).

On the other hand, when HFS (100 Hz) was delivered with Aβ-fiber intensity (30–50 μA, 0.5 ms), spinal cord LTP was induced in 2 of 10 tested spinal cord slices (20%). Low-frequency stimulation (20 Hz) with C-fiber intensity yielded LTP induction in 3 of 10 slices (30%). Thus the data indicate that although it is possible to induce LTP with either HFS with Aβ-fiber intensities or low-frequency stimulation with C-fiber intensities, the probability of LTP induction was too low to be useful. Thus HFS with C-fiber intensity, although parameters are artificial and nonphysiological, was used to induce LTP in this study. The data also suggest that the enhanced fEPSPs by Aβ-fiber stimuli, suggesting LTP at Aβ-fiber synapses, may be heterosynaptic LTP because it requires C-fiber activation.

To determine whether NMDA receptors are involved in LTP of Aβ-fiber-evoked fEPSPs, we examined the effects of an NMDA receptor antagonist, d-AP5, on the induction and maintenance phases of the LTP. As shown in Fig. 3A (n = 5), application of 50 μM of d-AP5 alone did not affect the baseline slopes of Aβ-fiber-evoked fEPSPs. When conditioning HFS was delivered during the period of d-AP5 superfusion (30 min), the magnitudes of the fEPSPs at 20 min after HFS were not significantly changed (98 ± 7%) from the pre-HFS control values (100 ± 2%). When the same conditioning HFS was delivered after d-AP5 was washed out (indicated by the 2nd ↑ in Fig. 3A); however, the slopes of the fEPSPs at 20 min after HFS were significantly increased (154 ± 12% of control, P < 0.05, n = 5), showing the development of LTP in the absence of the NMDA receptor antagonist (Fig. 3A). Thus the data indicate that the induction of LTP by HFS is NMDA receptor dependent. On the other hand, when d-AP5 was applied (50 μM, for 20 min) after LTP was fully established, the magnitudes of fEPSPs were not changed compared with the pre-d-AP5 levels (P > 0.05, n = 6, Fig. 3B). These data indicate that NMDA receptor activation is necessary for the induction but not the maintenance of LTP of Aβ-fiber-evoked fEPSPs when it is induced by HFS.

ROS scavengers block the induction of spinal cord LTP

First, we tested whether ROS are involved in the generation of Aβ-fiber-evoked fEPSPs. After 20 min of control baseline Aβ-fiber-evoked fEPSP recordings, the recording chamber was superfused with 1 mM PBN for 30 min, and Aβ-fiber-evoked fEPSPs were recorded during the entire PBN superfusion period. The magnitude of fEPSP slopes during PBN treatment was not significantly different from that of the pretreatment baseline values (P > 0.05, n = 6, Fig. 4A), thus indicating that ROS are not involved in baseline responses. To determine whether ROS play a role in the induction of LTP, the same conditioning HFS was delivered twice: once during the PBN infusion and then 30 min after PBN was washed out with

\[ \text{ROS} \quad \text{in} \quad \text{spinal cord LTP} \]
As a mimic of superoxide dismutase. The mean slope value of Aβ-fiber-evoked fEPSPs during 30 min TEMPOL treatment (5 mM) did not change compared with the baseline (P > 0.05, n = 3). Thus TEMPOL alone has no effect on fEPSPs under normal conditions. When HFS was delivered during TEMPOL (5 mM) treatment, the fEPSPs were not increased compared with the baseline (P > 0.05, n = 3). On the other hand, when the second HFS was delivered after TEMPOL was washed out (HFS without TEMPOL), the slope magnitudes of fEPSPs were increased significantly (P < 0.05, n = 3). These data indicate that ROS play a critical role for induction of spinal cord LTP.

ROS are involved on the maintenance phase of spinal cord LTP

To test the role of ROS in the maintenance of spinal cord LTP, the effects of a ROS scavenger, PBN, on Aβ-fiber-evoked fEPSPs were examined after LTP induction by HFS. After confirming the induction of LTP following HFS, the recording chamber was superfused with 1 mM of PBN for 30 min and then flushed with ACSF. The data obtained from six slice preparations are shown in Fig. 5A. The fEPSP slopes were increased significantly after HFS (n = 6), thus confirming LTP development. Ten minutes after PBN application, the slopes of fEPSPs fell to 114 ± 12%, which is significantly lower than the levels in LTP state (at 10 min after HFS, 152 ± 18%), thus showing a reversal of LTP by PBN (P < 0.05; n = 6; Fig. 5A). When PBN was washed out, the slopes of the fEPSPs increased back to 158 ± 15% (at 10 min after washing out), indicating that LTP is reestablished.

To confirm the role of ROS in the enhancement of Aβ-fiber-evoked fEPSPs, we tested another ROS scavenger TEMPOL. When TEMPOL (5 mM) was applied to spinal cord slices for 20 min, it greatly reduced LTP. The fEPSPs recorded 10 min after TEMPOL treatment were significantly decreased compared with the potentiated fEPSPs at 20 min after HFS (from 148 ± 6 to 110 ± 5%, P < 0.001, n = 5), as shown in Fig. 5B. Thus both ROS scavengers, PBN and TEMPOL, reduce LTP, suggesting the involvement of ROS in the maintenance phase of spinal cord LTP.

ROS donor, t-BOOH, induces spinal cord LTP independent from NMDA receptor activation

Thus far, this study shows that ROS are critical for the development and maintenance of spinal cord LTP of Aβ-fiber-evoked fEPSPs following HFS of primary afferents. The question then arises whether ROS alone is sufficient to induce spinal cord LTP. To test this, we examined the effect of a ROS donor, t-BOOH, on fEPSPs. After 20-min recordings of baseline Aβ-fiber-evoked fEPSPs, 5 mM of t-BOOH was superfused for 30 min and fEPSPs were recorded during t-BOOH superfusion, as shown in Fig. 6A. There was a gradual potentiation of fEPSPs during the t-BOOH superfusion and a further significant increase for another 30 min compared with control, reaching 138 ± 5% at 30 min after washing out t-BOOH (P < 0.05, n = 6). This indicates that t-BOOH alone can induce and maintain LTP-like potentiation of fEPSPs. The potentiated fEPSPs were subsequently attenuated to 119 ± 8% of control at 10 min after 1 mM PBN treatment and then returned to high.
LTP levels (135 ± 6%) at 10 min after PBN was removed. The data show that exogenously supplied ROS can produce an enhancement of Aβ-fiber-evoked fEPSPs in the spinal cord, thus mimicking the HFS-induced spinal cord LTP. The data suggest that ROS are both necessary and sufficient for the induction of spinal cord LTP.

Because both NMDA receptors and ROS are involved in the induction of spinal cord LTP (Figs. 3A and 4), it became important to investigate the relationship of these two factors. To test this, we examined the effect of NMDA receptor antagonist d-AP5 on t-BOOH-induced LTP. As shown in Fig. 6B, d-AP5 failed to block the potentiation of Aβ-fiber-evoked fEPSPs ($P < 0.05$, $n = 6$) in 30 min t-BOOH (5 mM) superfusion, although it did block the LTP induced by HFS (Fig. 3A). This experiment was repeated in six different preparations with the same result, thus showing that NMDA receptors are necessary when spinal LTP is induced by HFS but not by a ROS donor, suggesting that the involvement of ROS in spinal cord LTP may be a downstream event following NMDA receptor activation.

Spinal cord LTP induced by a ROS donor occludes HFS-induced LTP

Based on the preceding data, we deduce that HFS activates the NMDA receptors on spinal dorsal horn neurons, which leads to endogenous ROS generation, and then ROS leads to

\[ \text{t-BOOH (5 mM, 30 min)} \]

\[ \text{PBN (1 mM, 30 min)} \]

\[ \text{D-AP5 (50 μM, 20 min)} \]

\[ \text{t-BOOH (5 mM, 30 min)} \]

\[ \text{D-AP5 (50 μM, 20 min)} \]

\[ \text{PBN (1 mM, 30 min)} \]

\[ \text{D-AP5 (50 μM, 20 min)} \]

\[ \text{t-BOOH (5 mM, 30 min)} \]

\[ \text{D-AP5 (50 μM, 20 min)} \]

\[ \text{PBN (1 mM, 30 min)} \]
the development and maintenance of spinal cord LTP. This hypothesis thus predicts that LTP induced by HFS will occlude with that induced by a ROS donor or vice versa. To test this hypothesis, we performed occlusion experiments, and results are shown in Fig. 7. In one preparation, LTP was induced by a ROS donor, t-BOOH (5 mM), and then HFS was delivered after LTP is clearly established. As shown in Fig. 7A, the slopes of fEPSPs at 20 min after t-BOOH treatment were significantly increased compared with the pre-t-BOOH levels (from 100 ± 0.5 to 124 ± 3%, P < 0.05, n = 6), thus showing induction of LTP. When HFS was delivered to this LTP condition (†), the slopes of fEPSPs were slightly attenuated (114 ± 1%, P < 0.05, n = 6) rather than enhanced at 20 min after HFS. When the sequence of LTP induction methods were reversed (Fig. 7B), the LTP established by HFS was significantly reduced at 10 min after t-BOOH treatment (from 126 ± 5 to 103 ± 3%, P < 0.05, n = 6). The data thus suggest that either manipulation can generate ROS that are sufficient for LTP induction and both manipulations share the same downstream mechanism in that overproduction of ROS beyond the optimal level by both manipulations may be detrimental to LTP.

**DISCUSSION**

The present study shows that HFS of the dorsal root induces an enhancement of both Aβ- and C-fiber-evoked fEPSPs of the spinal dorsal horn, thus indicating the development of spinal cord LTP. The ROS scavengers reduce the induction and maintenance of spinal cord LTP. Spinal cord LTP is mimicked by a ROS donor, t-BOOH, and this is reduced by a ROS scavenger. These data suggest that ROS are both essential and sufficient for induction and maintenance of spinal cord LTP. The induction, but not the maintenance, of spinal cord LTP is dependent on NMDA receptors when induced by HFS. The induction of spinal cord LTP by t-BOOH, however, is not dependent on NMDA receptor activation. Thus it seems reasonable to speculate that NMDA receptor activation following HFS leads to spinal ROS elevation, which in turn induces spinal cord LTP. The data suggest that the involvement of ROS in spinal cord LTP may be a downstream event following NMDA receptor activation.

The CNS has multiple possible sources of ROS. They include various enzyme activities, such as monoamine oxidase, cyclooxygenase, nitric oxide synthase, and NADPH oxidase, as well as mitochondrial oxidative phosphorylation (Chetkovich et al. 1993; Kukreja et al. 1986; Pou et al. 1992). Biologically important ROS are also multiples: such as superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxinitrite, etc. Among these, three different types of ROS have been proposed to be involved in the LTP process: 1) superoxide generated from mitochondrial oxidative phosphorylation (Jenner 1994) and cyclooxygenase reaction, 2) hydrogen peroxide (H₂O₂) produced through enzymatic or chemical dismutation (Dringen et al. 2005), and 3) nitric oxide (NO) generated from L-arginine by NO synthase (Garthwaite and Boulton 1995). Of these, mitochondria are considered the major ROS source in LTP because superoxide is a natural metabolic byproduct of the mitochondrial oxidative phosphorylation process that increases during enhanced Ca²⁺ signaling (Dugan et al. 1995; Dykens 1994; Reynolds and Hastings 1995). Studies on isolated mitochondria indicate that Ca²⁺ accumulation causes leakage of electrons from the respiratory chain and increases the production of superoxide (Castilho et al. 1995). In cultured cortical neurons, Ca²⁺-dependent mitochondrial superoxide production is also observed after NMDA receptor activation (Dugan et al. 1995; Gunasekar et al. 1995; Reynolds and Hastings 1995). Furthermore, superoxide generation during hippocampal LTP induction (Bindokas et al. 1996) and inhibition of hippocampal LTP by superoxide dismutase (Klann 1998) strongly support the involvement of superoxide in LTP.
induction in the hippocampus. Superoxide accumulation due to MnSOD (SOD2) inactivation by nitration is also observed in the spinal dorsal horn neurons in capsaicin-induced persistent pain (Schwartz et al. 2009). A great reduction of spinal cord LTP by a superoxide dismutase mimetic, TEMPOL, shown in this study, further supports that superoxide may be a critical type of ROS in spinal cord LTP. Another type of ROS that has shown to be involved in synaptic plasticity is NO generated by activation of NO synthase (NOS). NOS activity is increased not only in hippocampal synaptic plasticity (Schuman and Madison 1991; Zorumski and Izumi 1993) but also in capsaicin-induced central sensitization in the spinal cord (Meller and Gebhart 1993; Wu et al. 1998, 2001). In addition, NO, produced from postsynaptic neurons as a consequence of NMDA receptor activation, is shown to serve as a retrograde messenger to enhance glutamate release from presynaptic terminals (Arancio et al. 1996; Schuman and Madison 1991; Xu et al. 2007). While it is shown that the induction of LTP is initiated postsynaptically by Ca$^{2+}$ influx through NMDA receptors, the maintenance of LTP can be greatly influenced by presynaptic transmitter release. Thus NO as a retrograde messenger could be an important contributor for spinal cord LTP. Because either a NOS inhibitor (Osborne and Codere 1999) or a SOD mimetic (Tal 1996; Wang et al. 2004) interfere with the development of pain, both NO and superoxide are likely ROS involved in pain and central sensitization. The role of hydrogen peroxide (H$_2$O$_2$), however, is open to dispute. A brief exposure of brain slices to H$_2$O$_2$ induces LTP in the hippocampus (Katsuki et al. 1997; Thiel et al. 2000b). However, extended exposure of hippocampal slices to either a low or a high concentration of H$_2$O$_2$ reduces or completely blocks LTP (Auerbach and Segal 1997; Pellmar et al. 1991). One explanation for this apparent contradiction is that H$_2$O$_2$ generation may be a necessary step for the full expression of LTP, but too high a concentration of H$_2$O$_2$ or prolonged exposure to exogenous H$_2$O$_2$ may interfere with LTP. The significant reduction of fEPSPs after t-BOOH application on already established spinal cord LTP may also represent the case of too high a level of ROS having a negative impact on LTP, as shown in this study (Fig. 7). Taking all these together, we speculate that superoxide, hydrogen peroxide, and NO are all likely candidate ROS contributing to LTP of the spinal cord. The relationships among these different types of ROS and their exact mechanism in spinal cord LTP and central sensitization need to be further investigated in future studies.

The present data show that ROS scavengers do not depress fEPSPs in the control state but transiently depress fEPSPs after LTP, suggesting that elevated ROS levels in the spinal cord are linked to the maintenance of spinal cord LTP. There is a basal level of ROS production in a normal physiological condition, and multiple endogenous antioxidant mechanisms (such as MnSOD in mitochondria and CuZnSOD in the cytosol) maintain the level of ROS very low normally (McCord and Fridovich 1969). That must be the reason why ROS scavengers do not produce any effect, because there are not many ROS to scavenge or influence cell function. On the other hand, data show that ROS scavengers depress fEPSPs after LTP. A possible explanation is that ROS production is somehow greatly increased in LTP condition, thus higher than normal levels of ROS are maintained during LTP. This high level of ROS is an important contributor to LTP, possibly acting as intracellular signaling molecules. Removal of excessive ROS by scavengers consequently reduces the level of LTP transiently until the high level of ROS is restored by increased production.

It is not known how elevated spinal ROS enhance responsiveness of the dorsal horn neurons. In both hippocampal CA1 neurons and spinal dorsal horn neurons, induction of LTP is dependent on postsynaptic Ca$^{2+}$ influx after NMDA receptor activation (Collingridge et al. 1983; Liu and Sandkühler 1995; Malenka et al. 1988; Svendsen et al. 1998), which is triggered by HFS of afferent fibers. Calcium influx, in turn, activates numerous signal transduction cascades, e.g., calcium/calmodulin-dependent protein kinase II (CaMKII) (Xia and Storm 2005; Yang et al. 2004), protein kinase C (PKC) (Klann et al. 1998; Yang et al. 2004), and extracellular signal-regulated kinase (ERK) (Xin et al. 2006), which are known to be essential for LTP. In addition, several studies suggest that ROS also activate PKC and ERK2, both of which are shown to be activated after LTP-inducing stimulation and necessary for the induction of LTP (Kanterewicz et al. 1998; Klann et al. 1998). Thus it is reasonable to speculate that ROS act as intermediate steps between intracellular calcium increases and the activation of various kinases that are essential for LTP. In line with this idea, studies have shown that NMDA receptor activation results in calcium-dependent production of superoxide (Bindokas et al. 1996) and an increased PKC activation during hippocampal LTP (Klann et al. 1998; Knapp and Klann 2002; Thiel et al. 2000a). In addition, increased levels of spinal ROS are correlated with an increased phosphorylation of NMDA receptor subunit 1 (NR1) through PKC activation in neurologic rats (Gao et al. 2007). Thus the data suggest that ROS are acting as signaling molecules to activate PKC during LTP in the spinal cord as well as in the hippocampus. Future study is warranted to identify a definite connection between ROS and signal transduction cascades in spinal cord LTP.

First-order synapses of primary afferent terminals in the spinal cord are heterogeneous and arise from many different specialized afferents that can be selectively excited by specific stimuli and that have specific projection distribution patterns in the spinal cord (Mager et al. 2001; Schmidt et al. 1995). Although the vast majority of SG neurons respond to high-threshold nociceptive inputs (Willis and Coggeshall 1991), an excitation of SG neurons by innocuous mechanical stimuli and A$\beta$-fiber electrical stimulation have been reported in vivo studies (Bennett et al. 1980; Woolf and Fitzgerald 1983). Furthermore, A$\beta$ fiber-mediated excitatory postsynaptic currents detected from SG neurons were facilitated by peripheral inflammation when recorded from spinal cord slice preparations (Baba et al. 1999). Because A$\beta$ fibers do not project directly to SG (Woolf 1987) and the dendrites of many SG neurons do not leave SG (Bennett et al. 1980), it is assumed that the A$\beta$ fiber-evoked fEPSPs in the SG are the results of indirect synaptic relays from deeper laminae. Whether conveyed directly or indirectly, the SG cells seem to receive inputs not only from C-fibers but also from A$\beta$ fibers and modify the output of projection neurons in lamina I as well as deep layers (laminae IV-VI) of the dorsal horn (Willis and Coggeshall 1991). Our fEPSP recordings from spinal cord slices confirm that conditioning HFS, which activates C-fibers, induces LTP of both C- and A$\beta$-fiber-evoked fEPSPs. A$\beta$-fiber inputs that normally carry tactile sensation to the spinal cord have shown to produce pain in the spinal cord sensitized condition—touch-
evoked pain. And ROS scavengers blocked Aβ-fiber-evoked fEPSPs in spinal cord LTP as well as touch-evoked pain in neuropathic animals (Kim et al. 2004). Based on these data, we speculate that ROS may be involved in potentiation of synaptic excitability of dorsal horn neurons receiving low-threshold afferent fiber input, which is considered the basis of allodynia in chronic pain (Schmidt and Willis 2007). However, it is still possible that the present data reflect a potentiation of the activity of purely nonnociceptive spinal neurons because it is not clear if the recorded signals come from the activity of nonnociceptive spinal neurons or spinal neurons receiving input from both nociceptive and nonnociceptive afferents.

As a technical issue, this study used HFS at C-fiber intensity as a conditioning stimulus to induce spinal cord LTP. This is the most frequently used conditioning stimulation to induce LTP in the brain and spinal cord both in vivo and in vitro (Ikeda et al. 2003; Randic et al. 1993; Sandkuhler 2007). HFS produces highly reliable LTP induction in the present study as well. The physiological relevance of this HFS, however, is not clear at this time since primary C-fiber afferents do not normally fire beyond a few spikes per second (Ji et al. 2003). Nevertheless, Duggan et al. (1995) and Liu and Sandkuhler (1997) have shown that bursts of 100-Hz stimulation of C-fibers are more effective than bursts of 20-Hz stimulation or prolonged 2-Hz stimulation. Furthermore, dorsal horn AMPA-receptor-mediated excitatory postsynaptic potentials remain prolonged 2-Hz stimulation. Furthermore, dorsal horn AMPA-receptor-mediated excitatory postsynaptic potentials remain potentiated for tens of minutes following brief HFS (Ji et al. 2003; Randic et al. 1993). Therefore we used this same brief HFS as a convenient experimental tool to elicit potent LTP.

In summary, the results of the present study suggest that ROS play a critical role in both induction and maintenance of spinal cord LTP. The data also suggest that ROS may act as intracellular signaling molecules activating various signaling cascades situated downstream from NMDA receptor activation during the induction of spinal cord LTP following HFS.

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