Mitochondrial Ca^{2+} Uptake Is Essential for Synaptic Plasticity in Pain

Hee Young Kim,¹ Kwan Yeop Lee,¹ Ying Lu,¹ Jigong Wang,¹ Lian Cui,² Sang Jeong Kim,² Jin Mo Chung,¹ and Kyungsoo Chung¹

¹Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-1069, and ²Department of Physiology, Brain and Cognitive Sciences, Seoul National University College of Medicine, Seoul, 110-799, Korea

The increase of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_c$) due to NMDA receptor activation is a key step for spinal cord synaptic plasticity by altering cellular signal transduction pathways. We focus on this plasticity as a cause of persistent pain. To provide a mechanism for these classic findings, we report that $[\text{Ca}^{2+}]_c$ does not trigger synaptic plasticity directly but must first enter into mitochondria. Interfering with mitochondrial Ca^{2+} uptake during a $[\text{Ca}^{2+}]_c$ increase blocks induction of behavioral hyperalgesia and accompanying downstream cell signaling, with reduction of spinal long-term potentiation (LTP). Furthermore, reducing the accompanying mitochondrial superoxide levels lessens hyperalgesia and LTP induction. These results indicate that $[\text{Ca}^{2+}]_c$ requires downstream mitochondrial Ca^{2+} uptake with consequent production of reactive oxygen species (ROS) for synaptic plasticity underlying chronic pain. These results suggest modifying mitochondrial Ca^{2+} uptake and thus ROS as a type of chronic pain therapy that should also have broader biologic significance.

Introduction

Long-term potentiation (LTP), a long-lasting synaptic plasticity, has been recorded in various regions of the brain and considered as an underlying mechanism of learning and memory. An LTP-like phenomenon found in the spinal cord (spinal LTP) is the initiation part of central sensitization that contributes to persistent pain (Woolf, 1983; Randić et al., 1993; Liu and Sandkühler, 1995; Ji et al., 2003). Spinal LTP shares mostly the same mechanisms as the well known LTP of hippocampal CA1 neurons but with minor differences (Ji et al., 2003). Like NMDA receptor (NMDAR)-dependent hippocampal LTP, spinal LTP is initiated by Ca^{2+} influx through activated NMDARs in spinal dorsal horn neurons (Randić et al., 1993; Liu and Sandkühler, 1995, 1998; Woolf and Salter, 2000; Ji et al., 2003). The increased cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_c$) then triggers intracellular signaling cascades causing post-translational, translational and transcriptional changes that lead to central sensitization (Ghosh and Greenberg, 1995; Woolf and Salter, 2000). One intriguing fact is that $[\text{Ca}^{2+}]_c$ is quickly sequestered by adjacent mitochondria (Peng and Greenamyre, 1998), to maintain intracellular Ca^{2+} homeostasis

thus preventing a sustained $[\text{Ca}^{2+}]_c$ increase. Furthermore, accumulating evidence suggests that mitochondria modulate neuronal activity, intracellular signaling and synaptic plasticity (Kann and Kovács, 2007).

Mitochondrial Ca^{2+} sequestration causes increased production of mitochondrial reactive oxygen species (ROS), particularly superoxide. ROS seem to be critically involved in synaptic plasticity in the hippocampus and spinal cord. ROS scavengers block LTP in both the hippocampus and spinal cord, and ROS donors produce LTP in both regions (Knapp and Klann, 2002; Lee et al., 2010). Similarly, persistent pain behaviors can be either reduced by decreasing spinal ROS levels (Kim et al., 2004; Schwartz et al., 2008) or generated by increasing spinal ROS levels (Kim et al., 2008; Schwartz et al., 2009). Furthermore, the levels of persistent pain are influenced by the levels of mitochondrial superoxide dismutase in the spinal cord (Schwartz et al., 2009). Increased mitochondrial superoxide can activate Ca^{2+} -dependent protein kinases, including protein kinase C (PKC), Ca^{2+} /calmodulin-dependent kinase II (CaMKII), protein kinase A (PKA), and extracellular signal-related kinase (ERK) that are critical for synaptic plasticity (Hongpaisan et al., 2004; Li et al., 2011). These facts suggest that mitochondria may be an unidentified link between $[\text{Ca}^{2+}]_c$ increase and the signaling processes for synaptic plasticity in the spinal cord.

While the current hypothesis emphasizes the direct effect of increased $[\text{Ca}^{2+}]_c$ to synaptic plasticity (Malenka et al., 1988; Woolf and Salter, 2000), our study indicates that $[\text{Ca}^{2+}]_c$ increase alone does not trigger spinal synaptic plasticity without entering into mitochondria. Supporting evidence includes that an inhibition of mitochondrial Ca^{2+} uptake completely blocks: (1) NMDA-induced hyperalgesia; (2) spinal LTP without affecting $[\text{Ca}^{2+}]_c$ increase; and (3) NMDA-induced activation of protein kinases. Furthermore, reduction of mitochondrial super-

Received June 17, 2011; accepted July 11, 2011.

Author contributions: J.M.C. and K.C. designed research; H.Y.K., K.Y.L., Y.L., J.W., and L.C. performed research; S.J.K. contributed unpublished reagents/analytic tools; H.Y.K. and J.M.C. analyzed data; H.Y.K. and K.C. wrote the paper.

This study was supported by NIH Grants R01 NS031680 and P01 NS11255, and by the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology [No. 2009-0080939 and WCU (World Class University) program R32-10142]. We express our gratitude to Dr. Richard E. Coggeshall for his critical reading and inputs to this manuscript.

Correspondence should be addressed to Dr. Kyungsoo Chung, Department of Neuroscience and Cell Biology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1069. E-mail: kchung@utmb.edu.

H. Y. Kim's present address: College of Oriental Medicine, Daegu Haany University, 165 Sang-dong, Suseong-gu, Daegu 706-060, Republic of Korea.

DOI:10.1523/JNEUROSCI.3093-11.2011

Copyright © 2011 the authors 0270-6474/11/3112982-10\$15.00/0

Figure 1. Effects of mitochondrial Ca^{2+} uptake inhibition on NMDA- and capsaicin-induced pain behaviors (**a–d**) and mitochondrial Ca^{2+} levels (**e–k**). Intrathecal injection of Ru360, RuR, or FCCP/oligomycin (Oli) reduced intrathecal NMDA- or intradermal capsaicin (Cap)-induced mechanical hyperalgesia when pretreated (**a, c, d**) but not post-treated (**b**). The effect of Ru360 was dose dependent (**a**). $^*p < 0.05$ vs vehicle in **a–d**. **f–i**, Representative mitochondrial Ca^{2+} images in the spinal deep dorsal horn (laminae IV–V) visualized by Rhod2/AM (33 μM , 10 μl , i.t. injection). $[\text{Ca}^{2+}]_m$ levels were quantified from both superficial (laminae I–II) and deep (laminae IV–V) dorsal horns indicated by red rectangles in the schematic drawing (**e**). $[\text{Ca}^{2+}]_m$ levels were increased significantly after NMDA (55 μM , 5 μl , i.t.), and this increase was blocked by pretreatment (**h**) but not post-treatment (**i**) with Ru360 (50 μM). Scale bar, 20 μm . **j, k**, The average of total Rhod2/AM-positive profile areas in the superficial (I–II; **j**) and deep (IV–V; **k**) dorsal horn (5 mice per group). $^{\dagger}p < 0.05$ vs Vehicle, $^*p < 0.05$ vs NMDA in **j, k**.

oxide levels also blocks both the NMDA- or capsaicin-induced hyperalgesia and spinal LTP. These results indicate that mitochondrial Ca^{2+} uptake and consequent superoxide generation are essential downstream steps for the synaptic plasticity of spinal dorsal horn neurons after intracellular Ca^{2+} increase.

Materials and Methods

Behavioral testing. Young male C57BL/6J mice (3 weeks old for patch recordings and 8–10 weeks old for behavioral studies, Jackson Laboratory) were used. Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch. Mechanical sensitivity of the hindpaw was assessed by measuring foot withdrawal frequencies to 10 repeated von Frey stimuli (vF #3.61, 0.52 g force, Stoelting Co.) (Kim et al., 2008; Schwartz et al., 2008).

Intrathecal injections were performed at the L5–L6 intervertebral space by a transcutaneous intrathecal injection method (Lee et al., 2007; Schwartz et al., 2009) modified from the original (Hylden and Wilcox, 1980). For NMDA-induced pain, 5 μl of NMDA (8 $\mu\text{g}/100 \mu\text{l}$ saline) was injected intrathecally. Mechanical sensitivity of the hindfoot was measured up to 120 min after NMDA injection. For capsaicin-induced pain, 20 μl of capsaicin (0.001% capsaicin/13.5% dimethyl sulfoxide in saline) was injected intradermally into the left hindpaw by using a 30 gauge needle attached to a Hamilton syringe. Mechanical sensitivity of the hindfoot was measured before and several times after capsaicin treatment up to 120 min, at the region of secondary hyperalgesia, 5–7 mm away from the capsaicin injection, as described before (Schwartz et al., 2008, 2009). Five to eight animals per group were used.

Drugs. The following drugs were used: Ru360 (a specific inhibitor of mitochondrial Ca^{2+} uniporter; 10, 20 and 50 μM in saline, 5 μl for intrathecal injection or 25 μM in artificial CSF (ACSF) for *in vitro* superfusion; Calbiochem); Ruthenium Red (RuR; a nonspecific inhibitor of mitochondrial Ca^{2+} uniporter; 50 μM in saline, 5 μl , i.t.); FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; a mitochondrial uncoupler; 50 μM in saline, 5 μl , i.t. or 1 μM in ACSF); oligomycin (an ATP-synthase inhibitor; 20 μM in saline or ACSF); rotenone [a mitochondrial electron transport complex (ETC) I inhibitor; 10, 20 and 50 μM in olive oil, 5 μl , i.t.]; PBN (phenyl *N*-tert-butyl-nitrone; a nonspecific ROS scavenger; 50 mg/kg, 5 ml/kg, i.p.); TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; a superoxide scavenger; 1 mM in ACSF). All chemicals were purchased from Sigma Chemical Co., unless otherwise specified.

Determination of mitochondrial calcium or superoxide levels in fixed spinal cord. To measure mitochondrial free calcium and superoxide in the spinal dorsal horn, Rhod2/AM (a mitochondrial Ca^{2+} marker, Invitrogen) and

MitoSox Red (a mitochondrial superoxide marker, Invitrogen) were used, respectively. They were dissolved in 2% dimethyl sulfoxide in saline at 33 μM . Ten microliters of Rhod2/AM or MitoSox Red with or without Ru360 (50 μM , 5 μl) was injected intrathecally 30 min before intrathecal NMDA. Forty minutes after NMDA, mice were perfused with saline followed by a fixative. Four percent EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; 40 mg/ml saline) (Tymianski et al., 1997) and 4% paraformaldehyde were used for fixatives for Rhod2/AM and MitoSox Red imaging, respectively. The L4/5 spinal cords were removed, postfixed, and cryoprotected in 30% sucrose. Tissues were cryosectioned in cross at 30 μm and mounted on gelatin-coated slides. Ten sections were randomly sampled from each animal and the superficial (laminae I–II) and deep (laminae IV–V) dorsal horns were photographed with a 60 $\times$ objective under Olympus BX50 epifluorescent microscope with a rhodamine filter and a Retiga 2000R digital camera. The Rhod2/AM and MitoSox Red-positive profiles were visible as red punctuates scattered in the sampled field. To quantify these profiles, the total area of red punctuates (μm^2) per each sample field ($6.5 \times 10^6 \mu\text{m}^2$) was measured by using the Image-Pro Plus (Media Cybernetics) densitometric image analysis system (Oberholzer et al., 1996; Kim et al., 2008). In brief, each image was converted to grayscale picture with 8-bit codification (1280×1024 pixels), giving a signal intensity from 0 (black) to 255 (white). The background intensity was determined from 20 random samples and then subtracted from all images. Then, the areas (in pixels) with intensity greater than the background value were counted as Rhod2/AM or MitoSox Red-positive profiles. Representative fluorescent images were acquired by laser-scanning confocal microscopy (Olympus).

Whole-cell patch-clamp recordings. Evoked EPSCs, evoked by an electrical stimulation of the dorsal root entry zone, were recorded from substantia gelatinosa (SG) neurons in cord slices using a whole-cell patch-clamp recording system. Under isoflurane anesthesia, the lumbar spinal cord was quickly removed and transferred into cold ACSF (containing, in mM: 117 NaCl, 3.6 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 1.2 NaH_2PO_4 , 25 NaHCO_3 , 11 glucose) aerated with 95% O_2 –5% CO_2 . The spinal cord was sliced transversely at 350 μm thickness using a Vibratome (Leica, VT1000S). The slices were incubated for 1 h in ACSF at 30°C, transferred to the recording chamber and perfused with ACSF (2 ml/min) at room temperature. The patch pipette (4–6 M Ω) was filled with internal solution (containing, in mM: 120 K-glucuronate, 10 KCl, 2 Mg-ATP, 0.5 Na-GTP, 0.5 EGTA, 20 HEPES, 10 phosphocreatine). The electrophysiological properties of the neurons were investigated in a voltage-clamp mode using a Multiclamp 700B amplifier (Molecular Devices) and pCLAMP 9 data acquisition software (Molecular Devices). The recordings were made from the SG neurons with the resting membrane potentials < -55 mV. EPSC was induced by an electrical stimulation of the dorsal root entry zone with a theta glass electrode. At the

Figure 2. Inhibition of mitochondrial Ca^{2+} uptake blocked spinal LTP while permitting a cytosolic Ca^{2+} increase. **a–d**, Effect of mitochondrial Ca^{2+} uptake inhibition on NMDA-induced spinal LTP. Examples of evoked EPSC (eEPSC) tracings (**a**, **c**) and summary data of eEPSC amplitudes (**b**, **d**) before and after application of a LTP protocol (blue bar; a perfusion of NMDA/glycine with depolarization for 5 min) with and without mitochondrial inhibition by inclusion of Ru360 (25 μM) in the patch pipette (**a**, **b**) or preincubation with FCCP (1 μM)/oligomycin (Oli, 20 μM) (**c**, **d**) ($n = 7$ –8 per group). Both manipulations blocked the NMDA-induced LTP induction. **e**, Confocal images of cytosolic Ca^{2+} in the dorsal horn neuron before and 150 s after the initiation of an LTP protocol using Oregon Green BAPTA-1 injected into the neuron through a patch pipette. **f**, Summary of results of such experiments measured from dendrites (one example is shown as white squares in **e**) with or without Ru360. Ru360 did not decrease cytosolic Ca^{2+} levels during LTP induction compared with the vehicle. **g**, Epifluorescence images of mitochondrial Ca^{2+} by Rhod2/AM in a dorsal horn neuron soma (indicated by arrows) before and 150 s after the initiation of the LTP induction without Ru360. **h**, Summary of mitochondrial Ca^{2+} changes in the soma during LTP induction with or without Ru360. Ru360 blocked the increase of $[\text{Ca}^{2+}]_m$ during LTP protocol. Scale bar, 20 μm . Each trace shown in **f** and **h** is the average of 6 and 8 cells per group, respectively.

holding potential of -70 mV, test pulses (0.5 ms, 30–70 μA) were given at 30 s intervals. Currents were filtered at 5 kHz, sampled at 10 kHz and analyzed using pCLAMP 9. Only monosynaptic EPSCs were studied. To elicit LTP, cord slices were subjected to a LTP induction protocol (3 min perfusion with an ACSF containing 50 μM NMDA, 50 μM glycine, 0.1 mM Mg^{2+} and 3.6 mM Ca^{2+} during depolarization of the postsynaptic neuron to -30 mV). Synaptic strength was assessed by measuring the peak

Figure 3. Effects of mitochondrial ROS reductions either by an ETC I inhibitor, rotenone, or a ROS scavenger, PBN, on NMDA- or capsaicin-induced hyperalgesia (*a–d*) and mitochondria superoxide levels (*e–j*). *a–d*, Rotenone and PBN were injected 30 min and 10 min before NMDA injection, respectively. Both rotenone (20 and 50 but not 10 μ M, i.t.) and PBN (50 mg/kg, i.p.) significantly reduced NMDA (*a, b*) or capsaicin (*c, d*)-induced hyperalgesia. $^*p < 0.05$ vs vehicle in *a–d*. *e–h*, Images of mitochondrial superoxide (visualized by MitoSox Red) in the spinal dorsal horn (lamina IV) (scale bar, 20 μ m) and the average mitochondrial superoxide levels in the superficial (I–II; *i*) and deep (IV–V; *j*) dorsal horn. The superoxide levels were quantified 30 min after intrathecal NMDA injection. $^{\dagger}p < 0.05$ vs Vehicle, $^*p < 0.05$ vs NMDA in *i, j*.

amplitude of EPSCs. To block mitochondrial Ca^{2+} uptake, Ru360 (25 μ M) was applied through the patch pipette or FCCP (1 μ M) in combination with oligomycin (20 μ M) was perfused for 5 min before LTP induction.

Measurement of intracellular or mitochondrial Ca^{2+} levels in live cells. Oregon Green 488 BAPTA-1 (OGB-1, 0.2 mM; excitation at 488 nm; Invitrogen) or Rhod2/AM (5 μ M; excitation at 510 nm; Invitrogen) was added to the internal solution of patch pipette to visualize intracellular or mitochondrial calcium, respectively. Imagings were done by either a confocal (FV300, Olympus) or an epifluorescence microscope (BX51WI, Olympus) equipped with a 40 $\times$ water immersion objective (LUMPlanFL/IL 40 $\times$, 0.8 NA, Olympus). The images were acquired using a U-N4001-HQ set (excitation, 480 nm; emission, 535 nm; Chroma) and a U-41006-HQ (excitation, 510 nm, emission, 580 nm), respectively. After

establishment of a patch, the cell was allowed to fill with the dyes for 5–10 min. The fluorescent images were taken before (for 20–60 s) and during (up to 200 s) LTP-inducing conditional stimuli and analyzed on dendrites for intracellular calcium and in soma for mitochondrial calcium by using ImageJ program (NIH). Background corrections were performed by subtracting the fluorescence intensity value of a background region far from the cell soma. Changes in background-adjusted fluorescence (ΔF) were expressed as percent ratios ($\Delta F/F_0\%$ = $[(F - F_0)/F_0] \times 100$), where F is the fluorescence intensity at a specific time, and F_0 is the mean resting fluorescence before stimulation.

Immunohistochemistry for PKC, PKA, or ERK. Forty minutes after intrathecal NMDA, L4/5 spinal cord segments were removed, post-fixed, cryoprotected in 30% sucrose, cryosectioned in cross at 30 μ m in thickness and mounted on slides. The sections were incubated with various combinations of the following primary antibodies and then followed by secondary antibodies conjugated with either Alexa Fluor 568 (red) or 488 (green). The primary antibodies used include anti-phospho-PKC α (pPKC α at Ser657; anti-rabbit; 1:500; Millipore), anti-phospho-PKA (pPKAII α at Ser96; anti-goat; 1:500; Santa Cruz Biotechnology), anti-phospho-ERK (pERK at Tyr204; anti-rabbit; 1:50; Santa Cruz Biotechnology), anti-NeuN (neuronal marker; anti-mouse; 1:2000; Millipore Bioscience Research Reagents), anti-GFAP (astrocytic marker; anti-mouse; 1:500; Millipore) and anti-OX-42 (microglial marker; anti-mouse; 1:50; Abcam). The immunostained dorsal horn sections were examined and photographed on an epifluorescent microscope (Olympus BX50, with 60 $\times$ oil immersion objective) for data analysis purpose. Photographs were taken from the lateral half of the superficial (laminae I–II) and deep (laminae IV–V) dorsal horn as shown in Figure 1*e*. Representative sections were also photographed on a laser-scanning Olympus confocal microscope for figures in the paper. The changes in immunostaining were analyzed either by counting the number of immunostained neurons (for pPKC and pPKA, with double labeling with NeuN) or the total immunostaining-positive area was quantified (for pERK, laminae I–II; see outlined region in Fig. 6*k–m*, below) as the same way as Rod2/AM or MitoSox Red by using ImagePro Plus image analysis system. The change in immunostaining was thus presented either as a percent ratio

of the number of immunostained neurons to the total number neurons or an immunostaining-positive area in each sample area of the dorsal horn region for each group (e.g., Normal, NMDA).

Statistics. Data are presented as mean $\pm$ SEM and analyzed by one- or two-way repeated-measurement ANOVA, followed by *post hoc* testing using the Holm–Sidak method or, when appropriate, Student's *t* test. $p \leq 0.05$ was considered statistically significant.

Results

First, to test the role of mitochondrial Ca^{2+} uptake (MCU) in pain, the effect of MCU inhibition on pain development was examined in 2 different pain models in mice: intrathecal NMDA- and intradermal capsaicin-induced pain. An NMDA (55 μ M, 5

μl , i.t.) injection produced long-lasting mechanical hyperalgesia (>120 min) on both hindpaws, indicating the development of spinal synaptic plasticity. Inhibition of MCU by a uniporter inhibitor, Ru360, or RuR (i.t., pretreatment; Fig. 1*a,c*), but not post-treatment (Fig. 1*b*), inhibited the NMDA-induced mechanical hyperalgesia in a dose-dependent manner ($p < 0.05$; Fig. 1*a–c*). In our preliminary study, Ru360, even with high doses (100 or 200 μM), did not change nociceptive thresholds nor induce motor impairment in normal mice, indicating that Ru360 does not affect normal sensory and motor processing.

In physiological conditions, synaptic plasticity is mediated mainly by activation of postsynaptic NMDA receptors (Woolf and Salter, 2000). To eliminate possible presynaptic and non-synaptic NMDAR activation by intrathecal NMDA, the same experiments as above were repeated using the intradermal capsaicin-induced animal pain model. Capsaicin injection evokes an acute nociceptive pain behavior and subsequent long-lasting secondary hyperalgesia in the adjacent region, mainly through a central mechanism of postsynaptic NMDA receptor activation (Sakurada et al., 1998; Zou et al., 2000). To investigate spinal dorsal horn neuron plasticity, the long-lasting mechanical hyperalgesia (up to 120 min) was measured in the region of secondary hyperalgesia, away from the injection site as described before (Schwartz et al., 2008, 2009). Blocking mitochondrial Ca^{2+} uptake by an intrathecal pretreatment with either Ru360 (50 μM) or FCCP (50 μM) significantly reduced the long-lasting mechanical hyperalgesia after capsaicin treatment ($p < 0.05$; Fig. 1*d*), without affecting sensory behaviors in naive mice (data not shown; preliminary study). The results show that MCU inhibition either by a uniporter inhibitor or by a depolarizing mitochondrial membrane potential in spinal dorsal horn neurons blocks long-lasting hyperalgesia and thus presumably similar synaptic mechanisms take place during postsynaptic NMDA receptor activation.

To check whether Ru360 blocked MCU, mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_m$) levels were examined by using a $[\text{Ca}^{2+}]_m$ dye, Rhod2/AM (Peng et al., 1998), with carbodiimide tissue fixation (Tymianski et al., 1997). Forty minutes after intrathecal NMDA, the spinal cord showed about a 2.5-fold increase in the total Rhod2/AM stained area both in the superficial ($p < 0.05$; laminae I–II) and deep ($p < 0.01$; laminae IV–V) dorsal horn, compared with the vehicle group (Normal). This increased Rhod2/AM staining was significantly inhibited by pretreatment with Ru360 before NMDA injection [NMDA + Ru360 (Pre); Fig. 1*h*], but not by post-treatment [NMDA + Ru360 (Post); Fig. 1*i*]. These data show that NMDA receptor activation increases $[\text{Ca}^{2+}]_m$ levels in the spinal dorsal horn, which is blocked by the pretreatment with the mitochondrial Ca^{2+} uniporter inhibitor, Ru360 (Fig. 1*f–k*).

Blocking mitochondrial Ca^{2+} uptake during NMDA receptor activation should prolong increased $[\text{Ca}^{2+}]_c$ levels. To determine that spinal synaptic plasticity does not occur with increased $[\text{Ca}^{2+}]_c$ when mitochondrial Ca^{2+} uptake is blocked, spinal LTP was measured in combination with $[\text{Ca}^{2+}]_c$ imaging in spinal cord slices. EPSCs were measured to determine LTP development by using whole-cell patch-clamp recordings from superficial dorsal horn neurons (laminae II). Application of an LTP induction protocol

Figure 4. Effects of a superoxide scavenger, TEMPOL, on spinal LTP. Examples of eEPSC tracings before and after LTP induction with or without TEMPOL are shown in *a*. TEMPOL superfusion (1 mM, 10 min; red bar), which blocked induction of spinal LTP by a LTP protocol (blue bar), is shown in *b* ($n = 8$ per group).

increased EPSCs by $76 \pm 13\%$ above the baseline. This EPSC potentiation persisted for 1–2 h, thus indicating an induction of LTP. When mitochondrial Ca^{2+} uptake was inhibited by either adding Ru360 (25 μM) in the patch pipette (Fig. 2*a,b*) or superfusing with FCCP (1 μM) with oligomycin (Oli, 20 μM) (Duan et al., 2007) (Fig. 2*c,d*), the induction of spinal LTP was blocked. To verify that Ru360 blocked mitochondrial Ca^{2+} uptake without affecting cellular Ca^{2+} influx, the cytosolic and mitochondrial calcium levels ($[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$) were measured during LTP induction by a patch-clamp/fluorescence imaging system with and without Ru360. For measurement of $[\text{Ca}^{2+}]_c$ levels, Oregon Green BAPTA-1 was loaded into the cell through a patch pipette and the fluorescent intensity changes were measured on dendrites (Fig. 2*e*). For measurement of $[\text{Ca}^{2+}]_m$, Rhod2/AM was loaded into the cell and the fluorescent intensity changes were measured in somata (Fig. 2*g*). A dramatic increase in $[\text{Ca}^{2+}]_c$ was observed during LTP induction and this increase was not affected by the presence of Ru360 (Fig. 2*f*), while the increase of mitochondrial Ca^{2+} during LTP induction was almost completely blocked by Ru360 (Fig. 2*g,h*), thus indicating that Ru360 blocks mitochondrial Ca^{2+} uptake without interfering cellular Ca^{2+} influx during LTP induction. Therefore the results show that an inhibition of mitochondrial Ca^{2+} uptake blocked spinal LTP despite a cytosolic Ca^{2+} increase. These data strongly suggest that high levels of $[\text{Ca}^{2+}]_c$ do not directly trigger LTP unless Ca^{2+} is transported into the mitochondria.

The next question was how mitochondrial Ca^{2+} uptake led to LTP and thus pain. One well known consequence of a $[\text{Ca}^{2+}]_m$ increase is increased production of mitochondrial ATP and ROS (Brookes et al., 2004). The primary source of mitochondrial ROS is superoxide (O_2^-) released from the electron transport complex (ETC) I and III during oxidative phosphorylation. To test whether increased mitochondrial superoxide production leads to dorsal horn (DH) neuron sensitization, mitochondrial superoxide generation was blocked by an ETC I inhibitor, rotenone, or superoxide was removed by a ROS scavenger, PBN (Kotake, 1999; Votyakova and Reynolds, 2001). The effects of rotenone or PBN were examined in NMDA- and capsaicin-induced hyperalgesia. Pretreatment with rotenone (20 or 50 μM , i.t., 5 μl) or PBN (50 mg/kg, i.p., 5 ml/kg) significantly reduced NMDA-induced hyperalgesia (Fig. 3*a,b*) and capsaicin-induced hyperalgesia (Fig. 3*c,d*) ($p < 0.05$). When mitochondrial superoxide is visualized by a superoxide-sensitive dye, MitoSox Red, the NMDA-treated group showed a significant increase of MitoSox-positive profiles in the dorsal horn, compared with the vehicle (normal) group. Such an increase was not observed in groups pretreated either with a MCU inhibitor, Ru360, or an ETC I inhibitor, rotenone,

Figure 5. The effect of mitochondrial Ca^{2+} uptake inhibition on intrathecal NMDA-induced protein kinase C activation. *a–l*, Images of the spinal dorsal horn (laminae I–II) immunostained for phosphorylated PKC α (pPKC α Ser-657) in 4 groups of mice: normal (*a–c*), with intrathecal NMDA (*d–f*), with intrathecal NMDA + Ru360 (*g–i*), and with intrathecal NMDA + FCCP/oligomycin (*j–l*). Intense pPKC α staining (red circles) in neurons (identified by NeuN immunoreactivity, green) in the NMDA group (*d–f*) indicates the PKC translocation/activation by NMDA receptor activation. The PKC translocation was prevented by pretreatment with Ru360 (*g–i*) or FCCP/oligomycin (*j–l*). *m, n*, Percentages of neurons that displayed pPKC α immunoreactivity, presumably translocation to the plasma membrane, in the superficial (I–II; *m*) and deep dorsal horn (IV–V; *n*). [†] $p < 0.05$ vs

before NMDA treatment ($p < 0.01$; Fig. 3*e–j*). Furthermore, spinal LTP induction was completely blocked by a superoxide scavenger TEMPOL (1 mM) (Fig. 4). The results indicate that mitochondrial superoxide generation is an essential step for the development of spinal DH neuron sensitization after NMDA receptor activation. And the mitochondrial superoxide generation is prevented by blocking either the mitochondrial Ca^{2+} uptake or the oxidative phosphorylation process. These data suggest that mitochondrial superoxide generation is a link between mitochondrial Ca^{2+} uptake and spinal synaptic plasticity.

Since this study suggests that mitochondrial Ca^{2+} uptake is an essential step between a $[\text{Ca}^{2+}]_c$ increase and spinal synaptic plasticity, activation of protein kinases that are critical for synaptic plasticity should be inhibited by blocking mitochondrial Ca^{2+} uptake. The protein kinases that have been identified to be critical for spinal synaptic plasticity include PKC, PKA, and ERK (Ji and Woolf, 2001; Hu and Gereau, 2003). By using an immunohistochemical staining method, we tested whether NMDA induces the activation of these kinases and this activation is inhibited by blocking mitochondrial Ca^{2+} uptake. For PKC activation assay, the translocation of phospho-PKC α (pPKC α -Ser657) to the plasma membrane was used as an indicator. The results showed that the number of neurons showing PKC translocation was significantly increased in the spinal dorsal horn after intrathecal NMDA, compared with the vehicle (normal) group. Preventing mitochondrial Ca^{2+} uptake by Ru360 or FCCP blocked the NMDA-induced enhancement of PKC activation ($p < 0.05$; Fig. 5*a–n*). To test whether this PKC activation by NMDA also occurs in other cell types, the spinal cords from NMDA-treated mice ($n = 3$) were immunostained for an astrocyte marker, GFAP, or a microglial marker, OX-42, in combination with pPKC α . Representative images of the GFAP/pPKC α and OX-42/pPKC α double immunostained dorsal horns are

Vehicle, $*p < 0.05$ vs NMDA. The data from 5–7 animals 30 min after NMDA treatment were averaged for each group. *o–t*, Examples of superficial dorsal horn (laminae I–II) double immunostained for pPKC α (red; *o, r*) and for either an astrocyte marker, GFAP (green; *p*) or a microglial marker, OX-42 (green; *s*), in intrathecal NMDA-treated mice. pPKC α -positive staining was not colocalized with either astrocytic or microglial staining (*q, t*), thus indicating that PKC α activation is primarily in neurons. Scale bar, 20 μm .

shown in Figure 5*o–t*. As shown in the images, none of pPKC α -positive profiles (ring-shape) were observed colocalized with either astrocytes or microglial cells. The data thus suggest that the NMDA-mediated PKC α activation during spinal synaptic plasticity occurs predominantly in neurons. For PKA and ERK activation assay, percentages of pPKA (pPKAII α -Ser96)-positive neurons or positive profile areas of pERK (pERK-Tyr204) were determined in the superficial spinal dorsal horn (laminae I–II) 40 min after NMDA with or without R360 pretreatment. Significant increases in pPKA/NeuN double-labeled cells (Fig. 6*a–i*) and pERK-positive areas (Fig. 6*k–m*) were observed in NMDA-treated mice, compared with the controls. These increases were blocked by a Ru360 pretreatment. The data for pPKA and pERK immunostaining are summarized in Figure 6, *j* and *n*, respectively. The data suggest that the involvement of PKA, ERK, and PKC as signaling molecules in NMDA-mediated synaptic plasticity is a downstream event of mitochondrial Ca^{2+} uptake and consequent ROS generation. Taken all together, the results suggest that mitochondrial Ca^{2+} uptake provides a link from cellular Ca^{2+} influx to kinase activation in spinal dorsal horn neurons (Figs. 5–7).

Discussion

Mitochondrial Ca^{2+} uptake is essential for the development of spinal cord synaptic plasticity and pain

A cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) increase during NMDAR activation is considered as an initial key event for synaptic plasticity in the spinal cord (Woolf and Salter, 2000). The present study, however, suggests that mitochondrial Ca^{2+} uptake and superoxide generation are necessary downstream events of this $[\text{Ca}^{2+}]_c$ increase for spinal synaptic plasticity and pain. Supporting evidence is that an inhibition of mitochondrial Ca^{2+} uptake blocks: intrathecal NMDA- or intradermal capsaicin-induced hyperalgesia; spinal LTP without affecting the $[\text{Ca}^{2+}]_c$ increase; and NMDA-induced protein kinase activation.

A few previous studies indicate mitochondrial involvement during synaptic plasticity. MCU is increased during hippocampal LTP (Stanton and Schanne, 1986; Kann and Kovács, 2007). Blocking MCU inhibits post-tetanic potentiation (Tang and Zucker, 1997). Sustained rises in $[\text{Ca}^{2+}]_c$ are prevented by quick Ca^{2+} uptake by mitochondria (Brookes et al., 2004). Together, the evidence supports the critical role of mitochondria during NMDAR-mediated synaptic plasticity in the CNS.

One intriguing fact is that spinal LTP or hyperalgesia is not developed despite a significant $[\text{Ca}^{2+}]_c$ increase when MCU is

Figure 6. The effect of mitochondrial Ca^{2+} uptake inhibition on intrathecal NMDA-induced PKA or ERK activation in spinal dorsal horn neurons. *a–i*, Images of superficial dorsal horn (laminae I–II) immunostained for pPKA in 3 groups of mice: normal (*a–c*), with intrathecal NMDA (*d–f*), and with intrathecal NMDA + Ru360 (*g–i*). Double-labeled cells with pPKA (red) and NeuN (green; neuronal marker) appear in yellow in merged panels (*c, f, i*). *j*, The averaged percentages of pPKA-positive neurons (I–II). *k–m*, Images of superficial dorsal horn immunostained for pERK in normal (*k*), intrathecal NMDA (*l*)-treated, and intrathecal NMDA + Ru360 (*m*)-treated mice. The areas showing immunopositivity to pERK were quantified by densitometry in laminae I–II (within the dashed line area), and the data are shown in the graph in *n*. Both PKA and ERK were activated by intrathecal NMDA, but this activation was significantly reduced by blocking mitochondrial Ca^{2+} uptake. [†] $p < 0.05$ vs Vehicle, ^{*} $p < 0.05$ vs NMDA. The data from 5–7 animals were averaged for each group. Scale bar, 20 μm .

blocked. This suggests a modification of the current hypothesis that a $[\text{Ca}^{2+}]_c$ increase triggers synaptic plasticity, which has long been supported by several lines of evidence. Blocking Ca^{2+} influx by NMDAR antagonists decreases pain behaviors and hyperexcitability of spinal nociceptive neurons (Cox, 2000; Petrenko et al., 2003). An intracellular Ca^{2+} increase is observed in spinal dorsal horn neurons after peripheral tissue or nerve injury

Figure 7. A diagrammatic representation of the proposed hypothesis that mitochondrial Ca^{2+} uptake is an essential downstream event of $[\text{Ca}^{2+}]_c$ influx for synaptic plasticity in the spinal cord. During NMDA receptor activation, there is a massive Ca^{2+} influx into spinal dorsal horn neurons. Most of the Ca^{2+} is rapidly sequestered by mitochondria. This consequently increases ROS production, which leads to activation of intracellular signaling cascades (i.e., PKC, PKA, and ERK), which in turn results in synaptic plasticity of the dorsal horn neurons. Glu, glutamate; AMPAR, AMPA receptor; circled P, phosphorylation.

(Kawamata and Omote, 1996). Pain behaviors are either enhanced by increased intracellular Ca^{2+} levels or reduced by decreasing it (Coderre and Melzack, 1992). LTP induction in spinal dorsal horn neurons is blocked by intracellular Ca^{2+} chelation (Ikeda et al., 2003). An increase in $[\text{Ca}^{2+}]_c$ initiates Ca^{2+} -sensitive signaling pathways, such as activation of PKC, PKA, and CaMKII, that are important in spinal synaptic plasticity and pain (Woolf and Salter, 2000). Thus these findings support that an intracellular Ca^{2+} increase is essential to induce synaptic plasticity (Malenka et al., 1988).

Most cytosolic free Ca^{2+} influxed through NMDAR, however, is rapidly taken up by adjacent mitochondria (Brookes et al., 2004). This takes place because mitochondria maintain $[\text{Ca}^{2+}]_c$ homeostasis, have an enormous capacity of Ca^{2+} storage and are closely apposed to a cluster of NMDARs (Peng and Greenamyre, 1998). Considering that spinal LTP or hyperalgesia is blocked by MCU inhibition, it is possible that calcium levels inside the mitochondria ($[\text{Ca}^{2+}]_m$) may be a more direct player than $[\text{Ca}^{2+}]_c$ in synaptic plasticity after NMDAR activation. The present study shows strong evidence for this because high levels of $[\text{Ca}^{2+}]_c$ do not trigger spinal synaptic plasticity unless Ca^{2+} is transported into the mitochondria. Several previous studies suggested the necessity of a $[\text{Ca}^{2+}]_m$ in-

crease for NMDAR-mediated neuronal injury and synaptic transmission. Inhibition of mitochondrial Ca^{2+} uptake blocks glutamate-mediated neuronal death (Stout et al., 1998; Tang et al., 2005) and a short-term post-tetanic potentiation (Tang and Zucker, 1997), and decreases presynaptic release (Billups and Forsythe, 2002), despite high $[\text{Ca}^{2+}]_c$. This suggests that $[\text{Ca}^{2+}]_m$, rather than $[\text{Ca}^{2+}]_c$, is a critical player for the development of spinal synaptic plasticity and pain following NMDAR activation.

To block MCU, three different inhibitors were used: Ru360, RuR, and FCCP with oligomycin. It is known that Ru360 is a specific high affinity blocker for mitochondrial Ca^{2+} uniporter without affecting other Ca^{2+} channels or ATP synthesis, while RuR inhibits various Ca^{2+} channels including MCU (Matlib et al., 1998). Since both Ru360 and RuR bear positively charged ruthenium ion (Matlib et al., 1998), there is a possibility that they may affect intracellular signaling by reacting with negatively charged proteins and lipids in the cell. Therefore, the effect of FCCP (a mitochondria uncoupler) with oligomycin (an ATP-synthase inhibitor) was also tested, because these two chemicals exist in electrically neutral forms and inhibit mitochondrial Ca^{2+} uptake through depolarizing mitochondrial membrane potentials while preventing ATP depletion (Leyssens et al., 1996; Ichas et al., 1997). Thus, we consider the inhibition of LTP or hyperalgesia by the above 3 methods is due to blocking mitochondrial calcium uptake since all 3 different drugs provide the same results.

One concern of using Ru360 or FCCP is a possible energy depletion causing cell death after blocking mitochondrial func-

tions. There are several indications that neither energy depletion nor cell death is a cause of the inhibition of LTP and hyperalgesia. First, Ru360 is a specific inhibitor for mitochondrial uniporter (Matlib et al., 1998; Brookes et al., 2004) and does not produce cell death even with higher doses (Landowski et al., 2005) than used in this study. On the contrary, Ru360 protects neurons from NMDA- or glutamate-induced cell death (Stout et al., 1998; Duan et al., 2007). In our preliminary study, no significant effect of Ru360 on nociceptive behaviors and electrophysiological properties of dorsal horn neurons are observed in normal animals (data not shown). Second, when FCCP/oligomycin are used, ATP depletion is prevented by oligomycin while mitochondrial Ca^{2+} uptake is prevented by reducing the mitochondrial membrane potential by FCCP (Leyssens et al., 1996). Third, ATP was continuously supplied into the cell via the patch pipette. Thus, the inhibition of spinal LTP by Ru360 or FCCP/oligomycin is not likely due to ATP depletion.

Mitochondrial superoxide generation is a link between mitochondrial Ca^{2+} uptake and spinal synaptic plasticity
An important question is how a $[\text{Ca}^{2+}]_m$ increase causes spinal LTP and thus hyperalgesia. One of the consequences of a

$[Ca^{2+}]_m$ increase is an enhancement of mitochondrial oxidative phosphorylation and thus increased production of ATP and superoxide (Brookes et al., 2004). Superoxide is proposed as an intracellular signaling molecule for hippocampal LTP. Scavenging superoxide blocks hippocampal LTP induced by a high-frequency stimulation (Klann, 1998). Incubation of hippocampal slices with xanthine/xanthine oxidase, a superoxide-generating system, induces LTP that can be inhibited by pretreatment with superoxide dismutase (Knapp and Klann, 2000, 2002). The present study also shows that suppressing spinal superoxide levels with Ru360, rotenone, PBN, or TEMPOL, significantly reduces NMDAR-mediated pain behaviors or blocks spinal LTP. These results indicate that mitochondrial superoxide generation is an essential step connecting MCU to spinal synaptic plasticity. The present data are in line with several previous findings that superoxide scavengers reduce persistent pain (Kim et al., 2004; Schwartz et al., 2009), dorsal horn neuron hyperexcitability (Lee et al., 2007) and spinal LTP induction (Lee et al., 2010). In contrast, an artificial elevation of spinal superoxide produces pain behaviors in normal animals (Kim et al., 2008). Thus, it seems reasonable to conclude that mitochondrial superoxide generation induced by calcium uptake acts as a signaling ROS for the induction of spinal synaptic plasticity.

In a normal physiological condition, mitochondrial superoxide is rapidly converted to hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase (SOD2) (Brookes et al., 2004) and H_2O_2 is shown to be involved in hippocampal synaptic plasticity (Kamsler and Segal, 2003). This raises a question as to whether the downstream H_2O_2 is more critical than superoxide in the development of spinal synaptic plasticity. Our recent study, however, showed that the conditions that enhance H_2O_2 production, such as SOD2 overexpressing transgenic mice or administration of SOD mimetics, suppress hyperalgesia. In contrast, SOD2 knockout mice or application of SOD inhibitors, the conditions that increase superoxide levels, enhance hyperalgesia (Schwartz et al., 2009). This suggests that mitochondrial superoxide, rather than H_2O_2 , is the main ROS in the development of spinal synaptic plasticity and persistent pain.

The mechanism of spinal dorsal horn neuron sensitization by increased superoxide is not known. Still the present data suggest that activation of PKC α , PKA, and ERK, the essential part of spinal neuron sensitization (Coderre, 1992), is dependent on superoxide rather than $[Ca^{2+}]_c$ itself. At the present time, conformational changes, translocation to membranes, and phosphorylation of catalytic domains (Siegelbaum et al., 2000; Shirai and Saito, 2002) have been recognized as the mechanisms of kinase activation by $[Ca^{2+}]_c$ increase. In hippocampal or amygdala neurons, however, mitochondrial superoxide generation, induced by strong NMDAR activation, enhances the activation of PKA, CaMKII, PKC, and ERK (Knapp and Klann, 2000; Dröge, 2002; Hongpaisan et al., 2004; Li et al., 2011). Furthermore, superoxide is shown to activate the catalytic domain of PKC through thiol oxidation of autoinhibitory zinc fingers in the regulatory domain of PKC (Knapp and Klann, 2000). It is possible that similar mechanisms may operate in spinal dorsal horn neurons during sensitization. The mechanism of Ca^{2+} -sensitive kinase activation through superoxide in spinal neuron sensitization needs to be further explored by future studies.

In summary, the present study presents evidence that mitochondrial calcium uptake and consequent mitochondrial ROS generation are essential steps for synaptic plasticity in spinal dorsal horn neurons (Fig. 7).

References

- Billups B, Forsythe ID (2002) Presynaptic mitochondrial calcium sequestration influences transmission at mammalian central synapses. *J Neurosci* 22:5840–5847.
- Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 287:C817–C833.
- Coderre TJ (1992) Contribution of protein kinase C to central sensitization and persistent pain following tissue injury. *Neurosci Lett* 140:181–184.
- Coderre TJ, Melzack R (1992) The role of NMDA receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. *J Neurosci* 12:3671–3675.
- Cox B (2000) Calcium channel blockers and pain therapy. *Curr Rev Pain* 4:488–498.
- Dröge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev* 82:47–95.
- Duan Y, Gross RA, Sheu SS (2007) Ca^{2+} -dependent generation of mitochondrial reactive oxygen species serves as a signal for poly (ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. *J Physiol* 585:741–758.
- Ghosh A, Greenberg ME (1995) Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* 268:239–247.
- Hongpaisan J, Winters CA, Andrews SB (2004) Strong calcium entry activates mitochondrial superoxide generation, upregulating kinase signaling in hippocampal neurons. *J Neurosci* 24:10878–10887.
- Hu HJ, Gereau RW 4th (2003) ERK integrates PKA and PKC signaling in superficial dorsal horn neurons. II. Modulation of neuronal excitability. *J Neurophysiol* 90:1680–1688.
- Hylden JL, Wilcox GL (1980) Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 67:313–316.
- Ichase F, Jouaville LS, Mazat JP (1997) Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell* 89:1145–1153.
- Ikeda H, Heinke B, Ruscheweyh R, Sandkühler J (2003) Synaptic plasticity in spinal lamina I projection neurons that mediate hyperalgesia. *Science* 299:1237–1240.
- Ji RR, Woolf CJ (2001) Neuronal plasticity and signal transduction in nociceptive neurons: implications for the initiation and maintenance of pathological pain. *Neurobiol Dis* 8:1–10.
- Ji RR, Kohno T, Moore KA, Woolf CJ (2003) Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci* 26:696–705.
- Kamsler A, Segal M (2003) Hydrogen peroxide modulation of synaptic plasticity. *J Neurosci* 23:269–276.
- Kann O, Kovács R (2007) Mitochondria and neuronal activity. *Am J Physiol Cell Physiol* 292:C641–C657.
- Kawamata M, Omote K (1996) Involvement of increased excitatory amino acids and intracellular Ca^{2+} concentration in the spinal dorsal horn in an animal model of neuropathic pain. *Pain* 68:85–96.
- Kim HK, Park SK, Zhou JL, Tagliatela G, Chung K, Coggeshall RE, Chung JM (2004) Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain* 111:116–124.
- Kim HY, Chung JM, Chung K (2008) Increased production of mitochondrial superoxide in the spinal cord induces pain behaviors in mice: the effect of mitochondrial electron transport complex inhibitors. *Neurosci Lett* 447:87–91.
- Klann E (1998) Cell-permeable scavengers of superoxide prevent long-term potentiation in hippocampal area CA1. *J Neurophysiol* 80:452–457.
- Knapp LT, Klann E (2000) Superoxide-induced stimulation of protein kinase C via thiol modification and modulation of zinc content. *J Biol Chem* 275:24136–24145.
- Knapp LT, Klann E (2002) Potentiation of hippocampal synaptic transmission by superoxide requires the oxidative activation of protein kinase C. *J Neurosci* 22:674–683.
- Kotake Y (1999) Pharmacologic properties of phenyl *N*-tert-butyl nitron. *Antioxid Redox Signal* 1:481–499.
- Landowski TH, Megli CJ, Nullmeyer KD, Lynch RM, Dorr RT (2005) Mitochondrial-mediated dysregulation of Ca^{2+} is a critical determinant of Velcade (PS-341/bortezomib) cytotoxicity in myeloma cell lines. *Cancer Res* 65:3828–3836.
- Lee I, Kim HK, Kim JH, Chung K, Chung JM (2007) The role of reactive

- oxygen species in capsaicin-induced mechanical hyperalgesia and in the activities of dorsal horn neurons. *Pain* 133:9–17.
- Lee KY, Chung K, Chung JM (2010) Involvement of reactive oxygen species in long-term potentiation in the spinal cord dorsal horn. *J Neurophysiol* 103:382–391.
- Leysens A, Nowicky AV, Patterson L, Crompton M, Duchen MR (1996) The relationship between mitochondrial state, ATP hydrolysis, $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ studied in isolated rat cardiomyocytes. *J Physiol* 496:111–128.
- Li Z, Ji G, Neugebauer V (2011) Mitochondrial reactive oxygen species are activated by mGluR5 through IP3 and activate ERK and PKA to increase excitability of amygdala neurons and pain behavior. *J Neurosci* 31:1114–1127.
- Liu XG, Sandkühler J (1995) Long-term potentiation of C-fiber-evoked potentials in the rat spinal dorsal horn is prevented by spinal *N*-methyl-D-aspartic acid receptor blockage. *Neurosci Lett* 191:43–46.
- Liu XG, Sandkühler J (1998) Activation of spinal *N*-methyl-D-aspartate or neurokinin receptors induces long-term potentiation of spinal C-fiber-evoked potentials. *Neuroscience* 86:1209–1216.
- Malenka RC, Kauer JA, Zucker RS, Nicoll RA (1988) Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* 242:81–84.
- Matlib MA, Zhou Z, Knight S, Ahmed S, Choi KM, Krause-Bauer J, Phillips R, Altschuld R, Katsube Y, Sperelakis N, Bers DM (1998) Oxygen-bridged dinuclear ruthenium amine complex specifically inhibits Ca^{2+} uptake into mitochondria in vitro and in situ in single cardiac myocytes. *J Biol Chem* 273:10223–10231.
- Oberholzer M, Östreicher M, Christen H, Brühlmann M (1996) Methods in quantitative image analysis. *Histochem Cell Biol* 105:333–355.
- Peng TI, Greenamyre JT (1998) Privileged access to mitochondria of calcium influx through *N*-methyl-D-aspartate receptors. *Mol Pharmacol* 53:974–980.
- Peng TI, Jou MJ, Sheu SS, Greenamyre JT (1998) Visualization of NMDA receptor-induced mitochondrial calcium accumulation in striatal neurons. *Exp Neurol* 149:1–12.
- Petrenko AB, Yamakura T, Baba H, Shimoji K (2003) The role of *N*-methyl-D-aspartate (NMDA) receptors in pain: a review. *Anesth Analg* 97:1108–1116.
- Randić M, Jiang MC, Cerne R (1993) Long-term potentiation and long-term depression of primary afferent neurotransmission in the rat spinal cord. *J Neurosci* 13:5228–5241.
- Sakurada T, Wako K, Sugiyama A, Sakurada C, Tan-No K, Kisara K (1998) Involvement of spinal NMDA receptors in capsaicin-induced nociception. *Pharmacol Biochem Behav* 59:339–345.
- Schwartz ES, Lee I, Chung K, Chung JM (2008) Oxidative stress in the spinal cord is an important contributor in capsaicin-induced mechanical secondary hyperalgesia in mice. *Pain* 138:514–524.
- Schwartz ES, Kim HY, Wang J, Lee I, Klann E, Chung JM, Chung K (2009) Persistent pain is dependent on spinal mitochondrial antioxidant levels. *J Neurosci* 29:159–168.
- Shirai Y, Saito N (2002) Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. *J Biochem* 132:663–668.
- Siegelbaum SA, Schwartz JH, Kandel ER (2000) Modulation of synaptic transmission: second messengers. In: *Principles of neural science*, Ed 4 (Kandel ER, Schwartz JH, Jessel TM, eds), pp 229–252. New York: McGraw-Hill.
- Stanton PK, Schanne FA (1986) Hippocampal long-term potentiation increases mitochondrial calcium pump activity in rat. *Brain Res* 382:185–188.
- Stout AK, Raphael HM, Kanterewicz BI, Klann E, Reynolds IJ (1998) Glutamate-induced neuron death requires mitochondrial calcium uptake. *Nat Neurosci* 1:366–373.
- Tang TS, Slow E, Lupu V, Stavrovskaya IG, Sugimori M, Llinás R, Kristal BS, Hayden MR, Bezprozvanny I (2005) Disturbed Ca^{2+} signaling and apoptosis of medium spiny neurons in Huntington's disease. *Proc Natl Acad Sci U S A* 102:2602–2607.
- Tang Y, Zucker RS (1997) Mitochondrial involvement in post-tetanic potentiation of synaptic transmission. *Neuron* 18:483–491.
- Tymianski M, Bernstein GM, Abdel-Hamid KM, Sattler R, Velumian A, Carlen PL, Razavi H, Jones OT (1997) A novel use for a carbodiimide compound for the fixation of fluorescent and nonfluorescent calcium indicators in situ following physiological experiments. *Cell Calcium* 21:175–183.
- Votyakova TV, Reynolds IJ (2001) DeltaPsi(m)-dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79:266–277.
- Woolf CJ (1983) Evidence for a central component of post-injury pain hypersensitivity. *Nature* 306:686–688.
- Woolf CJ, Salter MW (2000) Neuronal plasticity: increasing the gain in pain. *Science* 288:1765–1769.
- Zou X, Lin Q, Willis WD (2000) Enhanced phosphorylation of NMDA receptor 1 subunits in spinal cord dorsal horn and spinothalamic tract neurons after intradermal injection of capsaicin in rats. *J Neurosci* 20:6989–6997.