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Induction of long-term potentiation and long-term depression is cell-type specific in the spinal cord

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Abstract

The underlying mechanism of chronic pain is believed to be changes in excitability in spinal dorsal horn (DH) neurons that respond abnormally to peripheral input. Increased excitability in pain transmission neurons, and depression of inhibitory neurons, are widely recognized in the spinal cord of animal models of chronic pain. The possible occurrence of 2 parallel but opposing forms of synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD) was tested in 2 types of identified DH neurons using whole-cell patch-clamp recordings in mouse spinal cord slices. The test stimulus was applied to the sensory fibers to evoke excitatory postsynaptic currents in identified spinothalamic tract neurons (STTn) and GABAergic neurons (GABAn). Afferent conditioning stimulation (ACS) applied to primary afferent fibers with various stimulation parameters induced LTP in STTn but LTD in GABAn, regardless of stimulation parameters. These opposite responses were further confirmed by simultaneous dual patch-clamp recordings of STTn and GABAn from a single spinal cord slice. Both the LTP in STTn and the LTD in GABAn were blocked by an NMDA receptor antagonist, AP5, or an intracellular Ca^{2+} chelator, BAPTA. Both the pattern and magnitude of intracellular Ca^{2+} after ACS were almost identical between STTn and GABAn based on live-cell calcium imaging. The results suggest that the intense sensory input induces an NMDA receptor-dependent intracellular Ca^{2+} increase in both STTn and GABAn, but produces opposing synaptic plasticity. This study shows that there is cell type–specific synaptic plasticity in the spinal DH.

Keywords: Long-term potentiation, Long-term depression, Spinothalamic tract neurons, GABA neurons, Neuropathic pain, Synaptic plasticity, NMDA receptors

1. Introduction

Neuronal plastic changes, such as long-term potentiation (LTP) and long-term depression (LTD) of synaptic connectivity, are considered the bases for many types of normal neural function, such as learning and memory.[31] Furthermore, LTP and LTD are also involved in abnormal neuronal functions in various pathologic conditions, such as drug addiction and Alzheimer disease.[31]

Chronic pain, especially neuropathic pain, is often difficult to treat effectively because the underlying mechanisms are not clearly understood.[54] The afferent inputs due to intense noxious stimuli or tissue injuries often lead to abnormal pain sensation and also trigger plastic changes in the spinal dorsal horn (DH).[20,21] Abnormal pain sensation can manifest as hyperalgesia (elevated nociception to noxious sensory inputs) and/or allodynia (nociception to nonnoxious sensory inputs). Increased excitability of pain transmission neurons with depression of inhibitory functions is also observed in the spinal DH in chronic pain states.[55] The plastic changes in spinal DH neurons are thus considered a fundamentally important mechanism underlying chronic pain.[45,55]

Two forms of synaptic plasticity, LTP and LTD, were recorded in the spinal DH neurons after high-frequency stimulation of primary afferent fibers about 2 decades ago.[44] In this study, LTP was recorded in some spinal DH neurons, whereas LTD was recorded in some others, in response to the same stimulus. This indicates that both LTP and LTD can be induced in the spinal DH neurons in response to the same stimulation. Because the neuronal population in the superficial DH is highly heterogenous and responds differentially to primary afferent stimulation,[39,58] it is difficult to identify the critical factors that determine the direction of plasticity, LTP vs LTD. It is also difficult to identify the overall effects of these changes to pain, without knowing the identity of recorded neurons in the spinal cord.

In this study, the effects of intense afferent inputs on synaptic plasticity were tested in 2 identified groups of neurons of the spinal cord DH: spinothalamic tract neurons (STTn) and GABAergic neurons (GABAn). Spinothalamic tract neurons were identified by retrograde labeling from injected dye (DiI) to the thalamus, whereas GABAn were recognized by using a transgenic mice line of which GAD-67 (a synthetic enzyme of GABA) was tagged with green fluorescent protein (GFP). Evoked excitatory postsynaptic currents (eEPSCs) were recorded from these neurons by using whole-cell patch-clamp techniques in spinal cord slices. The results show that various means of intense peripheral stimulation consistently induces LTP in STTn but LTD in GABAn, with similar induction mechanisms. The data suggest that spinal LTP and LTD are induced in a cell-type dependent manner.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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2. Materials and methods

2.1. Animals

All experiments were conducted using GAD67-GFP mice (FVB-Tg[GadGFP] 45704Swn/J; Jackson Lab, Bar Harbor, ME) expressing GFP in GABAn. Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch.

2.2. Labeling spinothalamic tract neurons

Spinothalamic tract neurons were labeled by a retrogradely transported dye that was injected into the thalamus. Two to 3-week-old mice were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and placed on a rodent stereotactic apparatus fitted with a mouse adapter (Stoelting, Wood Dale, IL). A retrograde tracer, FAST-DiI (1 μL, 5% in ethanol, Invitrogen), was injected into the ventrobasal complex (VB) of the thalamus, using a micropipette attached to a 5 μL Hamilton syringe needle.[24,46] For VB injection, the stereotactic coordinates[41] were calculated and adjusted with respect to the bregma, using the ratio of the bregma-lambda distance of young mice to that of adult mice (4.5 mm). The coordinates of the injection site were 1.48 mm posterior from the bregma, 1.37 mm lateral from the mid-sagittal plane, and 3.3 mm deep from the surface. Three to 4 days were allowed for the dye to be transported to the lumbar spinal cord.

2.3. Whole-cell recordings of labeled spinothalamic tract neurons and GABAergic neurons

Under isoflurane anesthesia, the lumbar spinal cord was quickly removed and transferred into cold high-magnesium (high-Mg) artificial cerebrospinal fluid (ACSF) (in millimolars; 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 7 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose) aerated with 95% O_2–5% CO_2. The spinal cord was then cut transversely at 350-μm thickness for whole-cell recordings using a Vibratome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). The slices were kept for 1 hour in high-Mg ACSF at 34°C, transferred to the recording chamber on the stage of an Olympus BX51W1 microscope (Olympus America Inc., Center Valley, PA), and perfused with standard ACSF for recording (in millimolars; 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose; 2 mL/min) at room temperature. Labeled STT or GABA neurons were identified under a fluorescence microscope and then visualized using infrared differential interference contrast optics for whole-cell configuration.

Recordings were made in a voltage-clamp mode using a MultiClamp 700B amplifier and pCLAMP 9 data acquisition software (Molecular Devices, Sunnyvale, CA). The patch pipette (4-6 MΩ) was backfilled with an internal solution containing the following (in millimolars): 120 K-gluconate, 10 KCl, 2 Mg-ATP, 0.5 Na-GTP, 0.5 EGTA, 20 HEPES, and 10 phosphocreatine. At the holding potential of −70 mV, EPSCs were induced by focal electrical stimulation (0.5 millisecond, 30-70 μA; 0.05 Hz) to the dorsal root entry zone (DREZ) with a glass micropipette electrode or electrical stimulation (0.5 millisecond, 90-210 μA; 0.05 Hz) of the dorsal root through a suction electrode. We studied only monosynaptic EPSCs based on 3 criteria: (1) a constant short latency, (2) a smooth waveform with a single peak (without jitter), and (3) consistent responses without failure.[3] The rate of finding cells showing monosynaptic EPSCs was about 60%.

To examine synaptic plasticity of STTn or GABAn after afferent stimulation, we applied various afferent conditioning stimulation (ACS) protocols that are known to induce LTP or LTD in the hippocampus or spinal cord.[14,16,53] The ACS was applied within 5 minutes after establishing the whole-cell configuration to avoid washout of intracellular contents that are critical for the establishment of synaptic plasticity. Data were discarded if the series resistance varied by >20% during recording. For each cell, the amplitudes of 3 consecutive eEPSCs were averaged and normalized to the baseline recorded before application of the ACS. Long-term potentiation or LTD is defined as at least 20% changes from baseline in amplitude over a period of 15 to 25 minutes after the ACS.[44]

3. Intracellular Ca^{2+} imaging and quantification

Intracellular Ca^{2+} levels were measured from the cells injected intracellularly with Oregon Green 488 BAPTA-1 (OGB-1; 0.2 mM OGB-1; Thermo Fisher Scientific, Waltham, MA) by using a patch-clamp recording setup equipped with an epifluorescent imaging apparatus. For imaging of OGB-1, EGTA in the pipette solution was replaced with OGB-1, and excitation and emission filter sets of 480 and 535 were used. To reduce fluorescence bleaching, light intensity was minimized by adjusting field and aperture stops and using a neutral density filter (ND-25). After establishing whole-cell patch with a pipette (4-6 mΩ), cells were allowed to be filled with the dyes for about 5 minutes. Fluorescence images were captured before and after stimulation at 10 frames per second through the QICAM monochrome camera (QImaging, Surrey, Canada) and a time-lapse capture program. The fluorescent intensity in the somata was analyzed from each captured image using the NIH Image J system. Background correction was done by subtracting the background fluorescence intensity. Changes in fluorescent intensity were expressed as percent ratios of $\Delta F/F_0$, where $\Delta F = F - F_0$; F is the intensity of each image after stimulation; F_0 is the mean intensity of resting state before stimulation $\{\Delta F/F\% = ([F–F_0]/F_0) \times 100\}$.

4. Results

4.1. Identification of spinothalamic tract neurons and GABAergic neurons

In 2- to 3-week-old GAD67-GFP mice that express GFP in GABAn, STT neurons were identified by labeling them with retrogradely transported FAST-DiI, which was injected into the brain that included the ventrobasal complex (VB) of the thalamus (**Fig. 1A**). Three to 4 days after the injection, the L4 and 5 spinal cord was removed and prepared for whole-cell patch recordings. As shown in **Figures 1B and C**, labeled STTn and GFP^+ GABAn in the superficial DH were identified under the fluorescence microscope, and recordings were made. Monosynaptic EPSCs (as judged by a constant short latency, a smooth waveform with a single peak, and consistent responses without failure) evoked by electrical test stimuli applied to the DREZ were recorded for 3 to 5 minutes as a baseline response. As an example, overlapped tracings of 6 consecutively recorded EPSCs before and after high-frequency stimulation are shown on top of the graphs shown in **Figure 1D and E** (a and b). Any recording that showed jitters on the fast initial rising phase (suggesting polysynaptic inputs) were discarded.

4.2. Spinal long-term potentiation and long-term depression are cell-type specific, but not stimulation parameter dependent

The first experiment was to test whether the same ACS produces the same synaptic plastic changes in STTn and GABAn.

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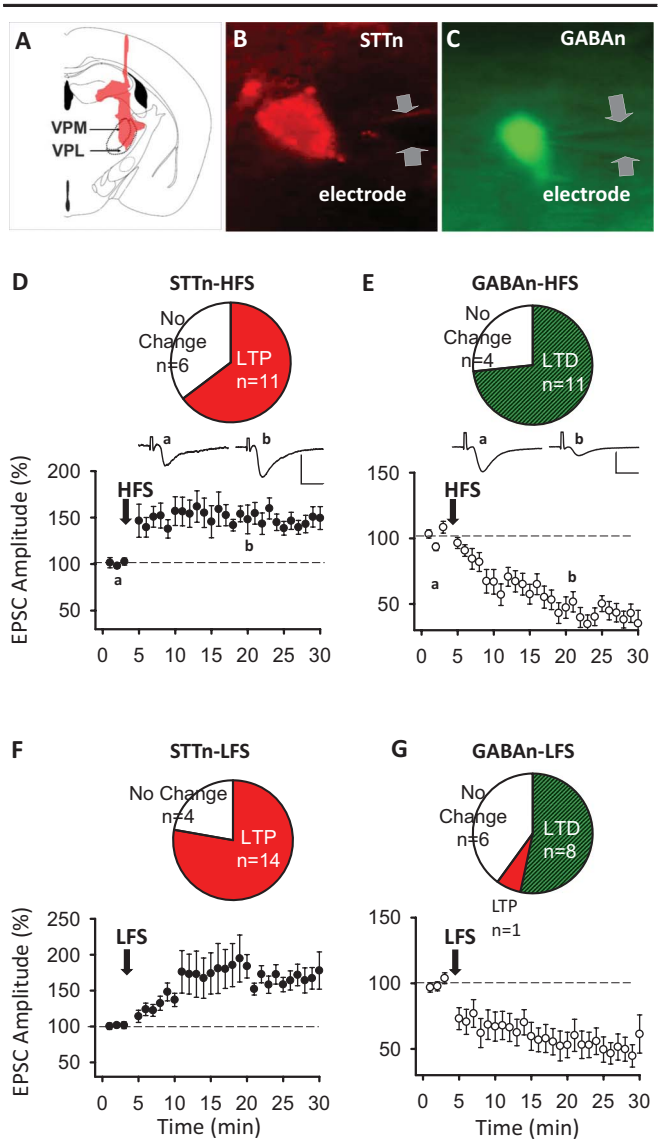

Figure 1. High- or low-frequency afferent conditioning stimulation (ACS) (high-frequency conditioning stimulation [HFS] or low-frequency conditioning stimulation [LFS]) evokes long-term potentiation (LTP) in spinothalamic tract neurons (STTn) but long-term depression (LTD) in GABAn in the mouse spinal cord. (A) Frozen sections of the brain were made from mice injected with a tracer dye (DiI) and a composite drawing of DiI marking was made from 1 mouse as an example. DiI (red) spread in many areas of the brain but included the ventral posterior lateral (VPL) and ventral posterior medial (VPM) nuclei of the thalamus. (B and C) Examples of STTn identified by retrogradely labeled DiI (B) and GFP^+ GABAn (C) that were patch-clamped for excitatory postsynaptic currents (EPSC) recordings (recording electrode edges are indicated by paired enclosing arrows). (D and E) Effects of a HFS (either 100 Hz for 1 second, repeated 3 times at a 10-second interval, holding potential at −50 mV or continuous 10-Hz pulses for 10 seconds with a holding potential of +10 mV) on EPSC amplitudes averaged from 11 STTn (D) and 11GABAn (E). The recording traces (shown above each plot) are the averages of 6 consecutive EPSC recordings before (a) and 15 minutes after (b) HFS. Calibration: 100 pA, 10 milliseconds. (F and G) Effects of an LFS (1 Hz for 100 seconds, holding potential −40 mV or 2-Hz pulses for 40 seconds, holding potential of +30 mV) on EPSC amplitudes averaged from 14 STTn (F) and 8 GABAn (G). Pie charts (shown above the plots in D–G) show the numbers of STT or GABA neurons that showed a significant change (>20%) after ACS.

A high-frequency conditioning stimulation (HFS), which had been commonly used to induce LTP in the spinal cord and in the hippocampus, was applied. Two parameters of HFS used were 1) bursts of 100 Hz (1 second long) repeated 3 times at a 10-second interval (total of 300 pulses for 30 seconds) with a holding potential of −50 mV,[16] and 2) continuous 10-Hz pulses for 10 seconds (100 pulses) with a holding potential of +10 mV.[14] The baseline eEPSC recordings were made for 3 to 5 minutes before delivering an HFS, and then the eEPSC recordings were continuously made for another 30 minutes after the HFS. The peak amplitude of eEPSC was measured and compared before and after the HFS. Induction of LTP (or LTD) was defined when the mean amplitude of eEPSC increases (or decreases) >20% from the baseline over a period of 15 to 25 minutes after the ACS.[44] The HFS induced a rapidly developed and prolonged increase in the amplitude of eEPSCs (>20%) in 11 of 17 STTn tested (4 of 7 with 100 Hz and 7 of 10 with 10 Hz; **Fig. 1D**), whereas no change (<20% increase) was detected in the remaining STTn. The increased responses on those 11 STTn are plotted in **Figure 1D**. The same HFS, however, produced a gradually developed but prolonged decrease (>20% decrease) in the amplitude of eEPSCs in GABAn (7 of 8 with 100 Hz and 4 of 7 with 10 Hz; **Fig. 1E**). The decreased responses on those 11 GABAn are plotted in **Figure 1E**.

To see whether these responses are dependent on cell-type or stimulus-parameter, we repeated the experiment with application of a low-frequency conditioning stimulation (LFS). Two parameters of LFS used were 1) continuous 1 Hz for 100 seconds with a holding potential of −40 mV,[8] and 2) continuous 2-Hz pulses for 40 seconds (80 pulses) with a holding potential of +30 mV.[52] With such LFS, a majority of STTn still showed a gradually developed but prolonged increase in the eEPSC amplitude (>20%) in 14 of 18 STTn tested (4 of 5 with 1 Hz and 10 of 13 with 2 Hz; **Fig. 1F**). Many GABAn (8 of 15), however, exhibited a rapidly developed and sustained decrease (>20%) in eEPSC amplitude (3 of 7 with 1 Hz and 5 of 8 with 2 Hz). One GABAn showed LTP with 1 Hz stimulation and insignificant changes (<20%) were detected from all remaining recorded GABAn (n = 6).

When data are combined with both HFS and LFS, ACS induced LTP in the majority of the STT neurons (28 of 38; 74%), whereas LTD is induced in the majority of GABA neurons (19 of 31; 61%). The present data thus suggest that induction of LTP and LTD in the spinal cord is cell-type specific, but not stimulation parameter dependent.

Among 4 tested electrical conditioning stimulations, a 2-Hz continuous ACS (for 40 seconds with a holding potential of +30 mV) most reliably produced LTP in STTn (10 of 13; 77%) and LTD in GABAn (5 of 8; 63%). Therefore, this ACS protocol was used in all the subsequent experiments.

4.3. Dual patch recordings show the simultaneous induction of long-term potentiation in spinothalamic tract neurons and long-term depression in GABAergic neurons to the same afferent input

Thus far, all recordings were made from individual single cells of either STTn or GABAn (**Fig. 1**). To test a possibility that differential induction of LTP or LTD is due to the difference in stimulation and recording conditions, we performed simultaneous dual patch recordings of STTn and GABAn from a single spinal cord slice. An example of these dual recordings is shown in **Figure 2**. In this sample, a small green fluorescent GABAn (top) and an adjacent large labeled STTn (bottom) were identified in the same spinal cord slice and both were patched with recording electrodes (identified by paired enclosing arrows). When recordings were made simultaneously, 2-Hz ACS induced LTP in STTn but LTD in GABAn, as shown in the recordings in **Figures 2B and C**.

We were able to repeat the dual patch recordings from another pair of STTn and GABAn with the same result. The results of these

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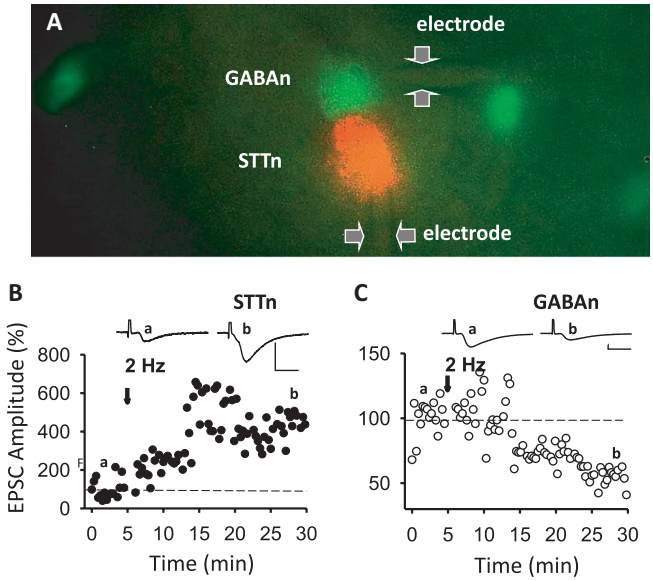

Figure 2. An example of dual patch-clamp recordings of long-term potentiation in spinothalamic tract neurons (STTn) and long-term depression in GABAergic neurons (GABAn) from a single spinal cord slice. (A) A dual patch recording setup showing a DiI (red fluorescent)-labeled STTn (lower neuron) and GFP^+ GABAn (upper neuron) with 2 patched recording electrodes (indicated by paired enclosing arrows) approached at a right angle. (B and C) Effects of 2-Hz afferent conditioning stimulation (ACS) (2 Hz for 40 seconds, holding potential +30 mV) on the amplitudes of excitatory postsynaptic currents (EPSCs) in STTn (B) and GABAn (C). The recording traces shown above the plots are averages of 6 consecutive EPSC recordings before (a) and 25 minutes after (b) the ACS. Calibration: 100 pA, 10 milliseconds.

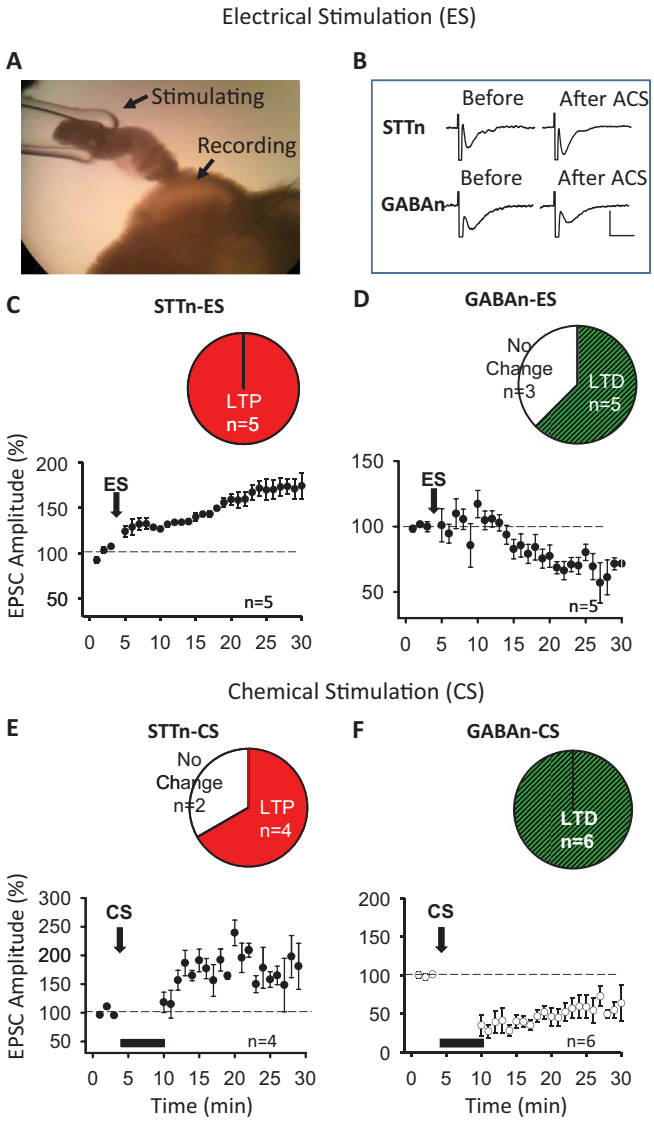

Figure 3. Long-term potentiation (LTP) and long-term depression (LTD) induced by either electrical stimulation (ES) of the dorsal root or chemical stimulation (CS) of the spinal cord. (A–D) Induction of LTP and LTD by an electrical conditioning stimulation applied to the dorsal root. An attached dorsal root was suctioned into a glass stimulating electrode (A). Afferent conditioning stimulation (ACS) was delivered to the dorsal root, and the changes of the evoked excitatory postsynaptic current (EPSC) amplitudes were recorded from spinothalamic tract neurons (STTn) (B and C [n = 5]) and GABAergic neurons (GABAn) (B and D [n = 5]). The recording traces in B are averages of 6 consecutive EPSC recordings before and 15 minutes after ACS (calibration: 100 pA, 10 milliseconds). (E and F) Induction of synaptic plasticity in STTn and GABAn by a chemical conditioning stimulation. Spinal cord slices were treated with a mixture of 50 μM NMDA and 50 μM glycine for 5 minutes (indicated by a thick bar) while holding the membrane potential at −30 mV. The EPSC amplitudes were significantly increased in STTn while decreased in GABA neurons after NMDA/glycine treatment, suggesting the induction of LTP in STTn (E, n = 4) and LTD in GABA neurons (F, n = 6). Pie charts (shown above the plots in C–F) show the numbers of STT or GABA neurons that showed significant changes (>20%) after conditioning stimulations.

dual patch recordings demonstrated that LTP and LTD can be induced in STTn and GABAn, respectively, under the same stimulation and recording conditions.

4.4. Stimulation of dorsal root produces a similar effect to that of the dorsal root entry zone

All recordings shown in **Figures 1** and **2** were made with an electrical stimulation applied to the DREZ. A problem with a focal stimulation of DREZ is that it might have activated other types of fibers, such as descending, ascending, and propriospinal pathways in addition to primary afferent fibers.[40] To minimize this problem, ACS was applied to the dorsal root in 1 set of experiments. Spinal cord slices were prepared with a short segment of dorsal root attached (a length of 3-5 mm), and electrical stimuli were applied to the proximal cut end of the dorsal root with a suction electrode (**Fig. 3A**) with an intensity of 90 to 210 μA, which is known to recruit Aδ and C-fiber.[5] Afferent conditioning stimulation application to the dorsal root with 2 Hz (40 seconds, a holding potential of +30 mV) produced LTP in all 5 tested STTn (**Fig. 3C**). However, electrical stimulation of the dorsal root produced LTD in 5 of 8 GABAn (4 with 2 Hz and 4 with 10 Hz). No significant change was produced in the remaining 3 GABAn. Data obtained from those 5 GABAn with positive LTD are shown in **Figure 3D**. Therefore, ACS applied to the dorsal root also produced LTP in most of the STTn and LTD in the majority of GABAn.

Another technical option of simulating nociceptive inputs is to use chemical stimulation rather than electrical ACS. For this purpose, spinal cord slices were subjected to the chemical LTP induction protocol that was used successfully in our previous study.[25] The protocol involves superfusion of the spinal cord slices with ACSF, which contains 50 μM NMDA, 50 μM glycine, 0.1 mM Mg^{2+}, and 3.6 mM Ca^{2+} while holding the cell membrane potential at −30 mV. The chemical stimulation (chemical activation of NMDAR with NMDA and glycine) was applied for 5 minutes and then patch recordings were resumed and continued another 30 minutes. As shown in **Figures 3E and F**, this chemical stimulation mimicked the electrically induced plasticity in STTn or GABAn (**Figs. 3E and F**). The results suggest that intense afferent inputs (both electrical and chemical) cause long-term changes in

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the synaptic strengths in STTn and GABAn to opposite directions: LTP in STTn and LTD in GABAn.

4.5. Induction of both long-term potentiation in spinothalamic tract neurons and long-term depression in GABAergic neurons, but not the maintenance, is dependent on NMDAR activation

The next step of the experiments was to investigate mechanisms of LTP and LTD in STTn and GABAn, respectively. The 2 commonly involved factors in synaptic plasticity are NMDA receptor (NMDAR) dependency and intracellular Ca^{2+} increase.[12,37] To test the NMDAR dependency of both induction and maintenance phases of synaptic plasticity, the effect of an NMDA receptor blocker, AP5 (100 μM) was examined, with both pretreatment and posttreatment paradigms.

For the pretreatment paradigm, the cord slice was superfused with AP5 for 15 minutes before application of ACS. During superfusion, a whole-cell patch is established, and baseline eEPSCs were recorded (3-5 minutes); and then, the ACS (2 Hz, 40 seconds, +30 mV holding potential) was applied to the DREZ. Immediately after ACS application, the superfusion solution was replaced with normal ACSF and recordings were continued for another 30 minutes. As shown in **Figures 4A and B**, the amplitudes of eEPSCs were not changed after ACS application compared with the baseline levels in both STTn and GABAn. The results show that induction of either LTP in STTn or LTD in GABAn was blocked when D-AP5 was applied before the ACS. The data suggest that induction of both LTP in STTn and LTD in GABAn is NMDAR dependent.

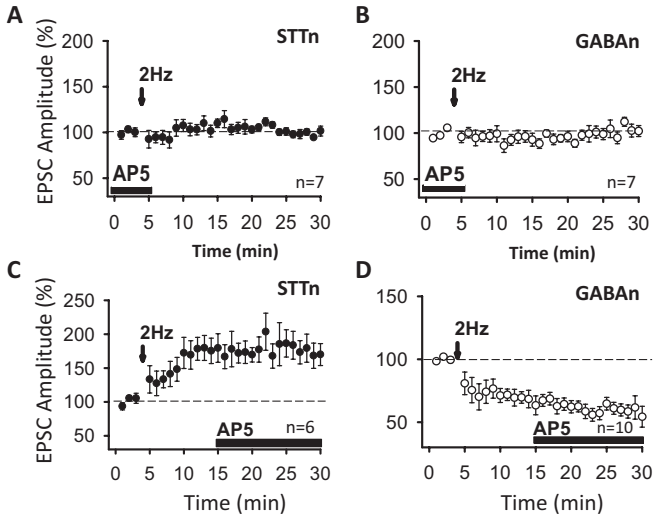

Figure 4. Induction, but not maintenance, of both long-term potentiation (LTP) in spinothalamic tract neurons (STTn) and long-term depression (LTD) in GABAergic neurons (GABAn) is NMDAR dependent. (A and B) The effect of pretreatment with an NMDAR blocker, AP5, on excitatory postsynaptic current (EPSC) amplitude in STTn (A, n = 7) and GABAn (B, n = 7). Spinal cord slices were superfused with AP5 (100 μM in ACSF) for the initial 15 minutes (only last 5 minutes is indicated by thick bars) during which baseline EPSCs were recorded, and then an afferent conditioning stimulation (ACS) was applied. The AP5 application was terminated immediately after the ACS application. Neither LTP in STTn nor LTD in GABAn was elicited by ACS after pretreatment with NMDA receptor antagonist. (C and D) The posttreatment effect of an NMDA receptor blocker, AP5, on EPSC amplitude in STTn (C, n = 6) and GABAn (D, n = 10). Spinal cord slices were superfused with AP5 (100 μM in ACSF) for at least 20 minutes (only first 15 minutes is indicated by thick bars) starting from 10 minutes after the ACS. Superfusion with AP5 produced no effect on already established LTP in STTn or LTD in GABAn.

To test whether NMDAR activation is also necessary for maintenance of LTP in STTn and LTD in GABAn, AP5 was applied for 15 minutes starting 10 minutes after ACS. As shown in **Figure 4C and D**, LTP in STTn or LTD in GABAn was not affected by posttreatment of D-AP5. The data suggest that the maintenance of either LTP in STTn or LTD in GABAn is not NMDAR dependent.

4.6. Blocking metabotropic glutamate receptors groups I and II did not interfere with long-term depression induction in GABA neurons.

Several studies indicated that metabotropic glutamate receptors are critically involved in LTD of cortical and hippocampal neurons.[9,18,23,27,49] Furthermore, it has been suggested that metabotropic glutamate receptors (mGluRs) 1 and 5 are involved in spinal cord synaptic plasticity mediated by increasing intracellular Ca^{2+}.[37] Thus, we tested whether mGluRs are involved in LTD induction in spinal GABA neurons. As shown in **Fig. 5A**, pretreatment with metabotropic glutamate receptors group I and II antagonist (RS)-alpha-methyl-4-carboxyphenylglycine (MCPG) (500 μM) for 5 minutes followed by ACS (2 Hz, 40 seconds, holding potential at +30 mV), did not interfere with the induction of LTD in GABA neurons (n = 5). In addition, pretreatment with 2-amino-3-phosphonopropionic acid (AP3), group-I–specific mGluR antagonist and phosphoserine phosphatase inhibitor,[57] also failed to block LTD production in GABA neurons (n = 4) (**Fig. 5B**). Data thus suggest that LTD induction in spinal GABA neurons is independent of groups I and II mGluRs.

4.7. Intracellular Ca^{2+} levels change similarly during the induction of long-term potentiation in spinothalamic tract neurons and long-term depression in GABAergic neurons

It is well known that a transient increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$ transients) after NMDAR activation is a determining factor for neuronal plasticity. It has also been proposed that the magnitude and pattern of intracellular Ca^{2+} increase determine the direction of neuronal plastic changes as to LTP or LTD.[17,29,34] To determine whether these findings also hold in the spinal cord (LTP in STTn and LTD in GABAn), we conducted 2 additional experiments. First, intracellular Ca^{2+} levels were measured before, during, and after the induction of LTP in STTn and LTD in GABAn by using a live-cell calcium imaging technique. Second, we tested the effect of a Ca^{2+} chelator, BAPTA (5 mM;

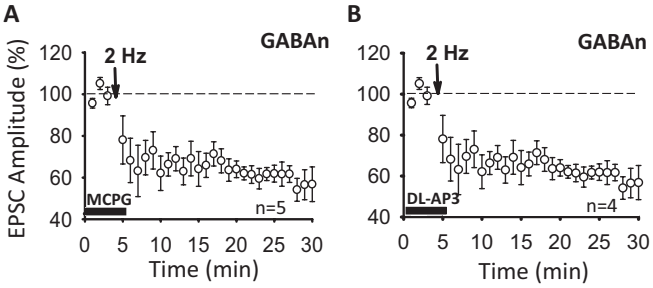

Figure 5. Neither MCPG (nonselective mGluR antagonist) nor AP3 (mGluR 1 antagonist and inhibitor of phosphoserine phostphatase) had an effect on long-term depression (LTD) induction in spinal GABAergic neurons (GABAn). (A and B) Spinal cord slices were superfused with either MCPG (500 μM in ACSF) or AP3 (30 μM in ASCF) for 5 minutes during excitatory postsynaptic current (EPSC) baseline recordings, and then afferent conditioning stimulation (ACS) was applied. Neither MCPG (A, n = 5) nor AP3 (B, n = 4) had any effect on the induction of LTD in GABAn.

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bis-[aminophenoxy] ethane-tetraacetic acid; applied into the cytoplasm of the cell by loading it in a patch pipette), on the induction of LTP in STTn and on the induction of LTD in GABAn. As shown in **Figures 6A and B**, both STTn and GABAn showed a rapid increase in cytosolic Ca^{2+} to peak levels during the application of ACS (2 Hz, 40 seconds, +30 mV holding potential), which was followed by a slow decline to baseline levels after ACS termination. The magnitude and pattern of the intracellular Ca^{2+} transients were almost the same in both STTn and GABAn, in response to the same conditioning stimulation (**Figs. 6A and B**). Furthermore, neither LTP in STTn nor LTD in GABAn could be elicited by ACS when the intracellular Ca^{2+} transients were buffered by loading a Ca^{2+} chelator, BAPTA (5 mM), into STTn or GABAn (**Figs. 6C and D**). The results indicate that the neuronal plasticity in STTn and GABAn share similar mechanisms of NMDAR activation and subsequent intracellular Ca^{2+} transients.

5. Discussion

There has been an unresolved issue about what determines the direction of synaptic plasticity because the 2 opposing forms of synaptic plasticity, LTP and LTD, were first observed from spinal DH neurons 2 decades ago.[44] We now show that these 2 opposing synaptic plastic changes depend on neuronal types: LTP in STTn and LTD in GABAn. Because STTn are pain transmission neurons and GABAn are inhibitory interneurons, the observed phenomenon is consistent with the neuronal changes in chronic pain conditions where pain transmission neurons show hyperexcitability while inhibitory function of the spinal cord is depressed.[20,21,45,55] After peripheral nerve injury, the sensitized spinal cord is known to induce decreased GABA immunoreactivity and GABAergic inhibitory transmission.[10,19,35] This study showed that LTD in GABAn further depresses inhibitory function, thus enhancing pain transmission in the sensitized spinal cord.

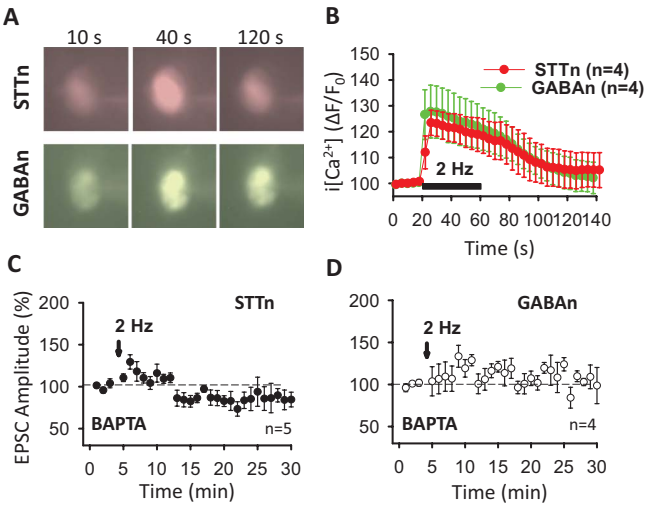

Figure 6. The induction of both long-term potentiation (LTP) in spinothalamic tract neurons (STTn) and long-term depression (LTD) in GABAergic neurons (GABAn) share the same mechanism of NMDAR-mediated rapid increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). (A and B)$[Ca^{2+}]_i$ levels were visualized with Oregon Green 488 BAPTA-1 (0.2 mM, administered intracellularly by preloading it into the patch pipette) before, during, and after 2-Hz ACS in an STTn and a GABAn (A). The averaged changes of $[Ca^{2+}]_i$ levels after 2-Hz ACS (B) show that intracellular free-Ca^{2+} levels in both STTn and GABAn increase at a similar rate and amplitude (n = 4 each). (C and D) Afferent conditioning stimulation with 2 Hz failed to induce either LTP in spinothalamic tract neurons (STTn) (C) or LTD in GABAn (D) when Ca^{2+} chelator, BAPTA (5 mM), was administered intracellularly by preloading it into the patch pipette.

Many different types of ACS have been used to mimic afferent inputs that cause synaptic plasticity. The most widely used ACS is the high-frequency stimulation consisting of bursts of 100 Hz (1 second long) repeated 3 times at a 10-second interval (total of 300 pulses for 30 seconds) with a holding potential of −50 mV.[16] This high-frequency ACS also consistently elicited plastic changes in the spinal cord in this study. However, such high-frequency bursts may not represent natural nociceptive afferent input to the spinal cord because the normal firing rate of nociceptors is much lower.[6,48,51] This study also used 3 other LTP-producing ACS protocols that were used in other brain areas: (1) continuous 10 Hz for 10 seconds (100 pulses) with a holding potential of +10 mV,[14] (2) continuous 2 Hz for 40 seconds (80 pulses) with a holding potential of +30 mV,[52] and (3) continuous 1 Hz for 100 seconds with a holding potential of −40 mV.[8] All of these 3 ACSs also produced LTP in STTn and LTD in GABAn. These 3 lower frequency stimulations (1, 2, and 10 Hz) are certainly within the natural firing rate of nociceptors.[6,48,51]

In most experiments, ACS was applied to the DREZ with a glass micropipette electrode. We had to search for a particular DREZ where stimulation consistently elicits LTP and LTD. Although this finding suggests that this particular zone is where the primary afferents are entering the spinal cord, stimulation of this zone will undoubtedly activate not only afferents but also ascending and descending tracts. To ensure that our observed synaptic plasticity is mainly due to afferent stimulation, we repeated some experiments with stimulation of the attached dorsal root with a suction electrode. Similar results were also reproduced by "chemical stimulation" by applying a mixture of NMDA and glycine in the perfusion fluid to activate NMDA receptors directly. Although it is not possible to use completely natural afferent stimulation in a reduced preparation (spinal cord slices), the same LTP and LTD were reproduced by 3 different means of stimulation. Therefore, we believe that the spinal LTP and LTD shown in this study is a consequence of primary afferent, perhaps nociceptive, stimulation.

Induction of LTD in inhibitory interneurons has not been consistent in different areas of the brain. In the hippocampus, GABAn show either mostly LTD[33] or both LTP and LTD, depending on the cell types.[36] Therefore, LTD is induced in a subpopulation of GABAn in the hippocampus. Although we were able to induce LTD consistently in GABAn in the spinal cord, we cannot conclude that LTD is induced in all spinal GABAn because only a subpopulation (~35%) of GABAn show GFP in our GAD67-GFP transgenic mice.[15] Because not all GABAn are the same in their characteristics and enzyme contents,[43] it is possible that the GFP negative spinal GABA neurons may respond differently from that of GFP^+ GABAn. Similarly, all our LTP recordings were done from STTn in the superficial lamina (I-IIa) of the DH although many more labeled STT neurons are detected in deep laminae (IV-V). Thus, it is possible that cell type–specific LTP shown in this study is limited only to STT neurons in the superficial lamina. Further studies are warranted to clarify this issue.

Historically, many aspects of LTP have been established from extensive studies in hippocampal neurons.[7] There are 2 well-known necessary conditions for synaptic plasticity: NMDA receptor activation and intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase.[30] Spinal cord LTP, in many ways, is similar to hippocampal LTP. Both show an enhanced responsiveness of the affected neurons with a prolonged time course and dependency on NMDA receptors for the initiation.[26,28,44,50] Our study demonstrates that both NMDA receptor activation and subsequent $[Ca^{2+}]_i$ increase are necessary conditions for the induction of both LTP in STTn

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and LTD in GABAn. Meanwhile, the maintenance of LTP in STTn or LTD in GABAn does not require NMDA receptor activation. NMDA dependency of the induction of spinal LTP and LTD is most likely related to the NMDA dependency of hyperalgesia and central sensitization after intense nociceptive inputs.[13,49,56]

The amplitude and duration of $[Ca^{2+}]_i$ transients are suggested as the key element for determining the direction of synaptic plasticity.[29] Abrupt and large $[Ca^{2+}]_i$ transients are linked to LTP induction, whereas moderate and prolonged $[Ca^{2+}]_i$ transients lead to LTD.[17,34] Furthermore, LTP can be depotentiated by lowering $[Ca^{2+}]_i$ levels in hippocampal neurons[11] and cortical pyramidal neurons.[17] The present data, however, show an almost identical abrupt and large $[Ca^{2+}]_i$ transient in both STTn and GABAn, but it induces opposite plasticity. Therefore, the role of $[Ca^{2+}]_i$ transients in synaptic plasticity in the spinal cord DH neurons seems to be different from other parts of the brain.

It is likely that STTn and GABAn activate different downstream signaling pathways with the same $[Ca^{2+}]_i$ transient. One can speculate that there may be differences in intracellular factors influencing the downstream signaling pathways. For example, excitatory projection neurons usually contain a high level of calbindin, whereas GABAn possess a high level of parvalbumin.[2,38] Long-term potentiation induction is facilitated by calbindin, while the excitability of interneurons is depressed by parvalbumin.[22] In addition, CaMKII and calcineurin are deficient in GABAn,[47] while PKCγ, which contributes to central sensitization[32] and LTP,[1] is found mainly in excitatory spinal pain transmission neurons.[42] This topic needs to be explored in future studies.

Another suggested factor that controls the direction of synaptic plasticity is the level of postsynaptic depolarization (holding potential) during the ACS: LTP and LTD would be induced when the depolarization is above and below the NMDAR activation threshold, respectively.[4] However, this study shows an opposite synaptic plasticity in STTn and GABAn with the same holding potential (+30 or +10 mV). The data in this study thus suggest that the direction of synaptic plasticity is not always determined by the extent of postsynaptic depolarization in the spinal DH neurons.

Metabotropic glutamate receptors are also involved in LTD in some cortical neurons.[9,23,27] However, the results of our pharmacological study suggest that LTD induction in spinal GABA neurons is independent of groups I and II mGluRs. Additional factors concerning synaptic plasticity that need to be addressed include: (1) whether the synaptic plasticity is through presynaptic or postsynaptic mechanisms, and (2) whether $[Ca^{2+}]_i$ transients are sufficiently established by Ca^{2+} entry through membrane channels (eg, NMDAR) or require Ca^{2+} release from intracellular storage.

In summary, the whole-cell patch recordings from identified spinothalamic tract (STTn) and GABAn in the mouse spinal cord showed that the same ACSs induced LTP in STTn but LTD in GABAn. The types of neurons rather than parameters of ACSs determined the direction of synaptic plasticity. Induction of both LTP in STTn and LTD in GABAn were dependent on NMDA receptor activation, and both were accompanied with a similar rapid rise in intracellular Ca^{2+} transients.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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