Activity-Dependent Axonal Plasticity: The Effects of Electrical Stimulation on Compound Action Potentials Recorded from the Mouse Nervous System *In Vitro*

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Abstract: The influence of electrical stimulation on the amplitude of the action potentials recorded from the mouse nervous system *in vitro* was investigated. Brief (1 s) high frequency (100 Hz) stimulation of the sciatic nerve induced a long-lasting increase in the amplitude of the compound action potential (CAP). Low frequency (1 Hz) stimulation delivered for 15 min attenuated the antidromically evoked potential recorded from hippocampal slices and CAP recorded from the sciatic nerve. The stimulation-induced decrease in the amplitude of CAP occurred in two phases. While during the first phase the decrease was reversible and calcium-dependent, the second, later phase was irreversible. The experiments with two stimulating electrodes activated separately revealed that the changes in the CAP amplitude were not related to unspecific electrode-tissue interactions. The attenuation in the CAP amplitude was accompanied by an increase and decrease of minimal and maximal thresholds, respectively. The stimulation of the sciatic nerve segments with twin pulses revealed that the velocity of CAP propagation and refractoriness were significantly diminished after LFS application. The stimulation-induced changes in CAP were correlated with decreased sodium channels antibody signal, indicating fall in the number of sodium channels. According to postulated hypothesis, the stimulation-induced influx of Na^+ during the first phase intensifies internalization of sodium channels. This amplified endocytosis is accompanied by activation of lysosomal pathways and subsequent hydrolysis of sodium channels leading to irreversible decline in the CAP amplitude. Described results indicate, that axons can contribute to neuronal plasticity.

Keywords: Axon, action potential, plasticity, sodium channels.

INTRODUCTION

One of the most basic properties of the nervous system is its plasticity. It is commonly interpreted as the ability of the nervous system to generate a modified response related to the prior activity [1-3]. While the plasticity could occur during axonal propagation of an action potential and/or transmitting of the information across the synapse, the overwhelming majority of the research on plasticity is devoted to investigate the synaptic processes. The best known examples of plasticity at the synapse are Long-Term Potentiation (LTP) [3] and Long-Term Depression (LTD) [4,5]. These two phenomena, expressed as change in the synaptic efficiency, are induced by repetitive stimulation of the CNS pathways. The mechanisms responsible for LTP and LTD may involve several changes in the synaptic processes [1, 2] including activation of specific genes [6]. However, in order to generate the synaptic response, the nerve terminal has to be invaded by an action potential. It is generally assumed that the action potential represents a steady signal resilient to persistent modifications, although short-lasting hyperpolarization of an axonal membrane following high-frequency stimulation [7, 8], and persistent hyperexcitability following axonal injury [9, 10] have been described. The mechanisms of these changes is not known although a massive influx of Na^+ and subsequent activation of Na^+-K^+ pump [8, 11-13], and/or modulation of protein biosynthesis [10] and new expression of sodium channels [14] have been implicated. The physiology of the axon might support the mechanisms of long-lasting modulation of an action potential. The activity of sodium and potassium channels, essential for generation and propagation of an action potential can be changed. The regulation of sodium channel activity is an intricate process complicated further by multiple forms of this channel distributed differentially in the CNS, and PNS [15, 16]. One of the major processes regulating properties of the voltage-gated Na^+ channel is phosphorylation/dephosphorylation [17-20]. The rising intracellular Na^+ concentration facilitates dephosphorylation of the channel and stimulates its incorporation into the membrane [13, 16, 21, 22], increasing sodium channel current [18, 19]. All these processes, activated by neuronal activity, can contribute to the persistent activity-induced change in the action potential and subsequent modulation of synaptic efficiency. The objective of described research was to verify the hypothesis suggesting that specific patterns of electrical stimulation can modify the amplitude and the shape of axonal action potential.

MATERIALS AND METHODS

The Preparation of Nerve Segments and Hippocampal Slice

CD-1 mice of both sexes, 1 to 3 months old were used to obtain either hippocampal slices or segments of the sciatic

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nerve. To prepare segments of the sciatic nerve the animals were decapitated, and the sciatic nerve was dissected out. Following dissection, the nerve was cut into 7-10 mm segments and placed for at least an hour in oxygenated Ringer's solution (33^{o}C). Subsequently, the individual segments were transferred to the recording chamber. To obtain hippocampal slices the animals were decapitated, the brain was removed and both hippocampi were dissected out. Each hippocampus was cut into slices (350 μm) with a manual tissue chopper, placed in an incubation chamber (33°C) containing Ringer's solution composed of (in mM): NaCl 124, KCl 3.1, KH_2PO_4 1.3, $MgSO_4$ 1.3 $CaCl_2$ 3.1, $NaHCO_3$ 25.5 and glucose 10. The chamber was constantly oxygenated with a mixture of 5:95% CO_2/O_2. Following a preincubation period, which varied from 1 to 3 hrs, slices were transferred to an interface-recording chamber, maintained at 33°C. The procedure used for preparation of biological preparations was approved by the Institutional Animal Care and Use Committee at CSI.

Electrophysiological Recordings

To follow antidromically evoked potentials recorded from hippocampal slices the stimulating and the recording electrodes were placed on Schaffer collateral fibers in CA1 hippocampal subfield, and pyramidal cell layer in CA3 hippocampal field, respectively. In order to eliminate any involvement of glutamergic neurotransmission, all experiments were performed on the slices incubated with 2 mM kynurenic acid.

To record the extracellular potential from the sciatic nerve, the recording electrode was placed inside the perineurium at the end of the segment, and the stimulating electrode was localized outside of perineurium. In this experimental arrangement several individual axons contribute to the recorded potential and therefore it is called "Compound Action Potential" (CAP). The distance between the stimulating and recording electrodes varied in different experiments and was adjusted to obtain good quality recording with CAP clearly separated from the stimulus artifact. Some experiments were conducted in Ca^{2+}-free Ringer's solution.

The stimulation and recording procedures were the same for both preparations unless the differences were specified in the text. At the beginning of the experiment the hippocampal slice, or the segments of the sciatic nerve was stimulated with the frequency of 0.03 Hz to stabilize the potential and to record the baseline represented by the potential stable for at least 10 min. This baseline served as a reference to calculate changes. The strength of the stimulation was adjusted to obtained maximal response. Following stabilization of the potential the preparation was stimulated either for 15 min with the frequency of 1 Hz (Low-frequency Stimulation, LFS), or with High Frequency Stimulation (HFS), which consisted of one or more episodes of stimulation with 100Hz lasting 1s each. The time interval between periods of stimulation is indicated in the description of individual experiments. The duration of single pulses was 0.1 ms, unless indicated otherwise. Following the stimulation with either LFS, or HFS, the stimulation with the frequency of 0.03 Hz resumed for the rest of the experiment.

To evaluate the velocity of the conductance of the action potential the delay measured from the stimulus artifact to the initiation of an action potential was divided by the distance between the stimulating and recording electrodes. In some experiments, two stimulating electrodes were placed on the segment of the sciatic nerve in different distances from the recording electrode. The identical stimulating current from a single source was applied alternatively to both electrodes. The control potentials were recorded following separate activation of each of these stimulating electrodes. Then, while the one of these electrodes remained inactive, the other one was used to stimulate the nerve with 1 Hz stimulation for 15 min. At the end of this stimulation period the potentials were recorded again after separate activation of each of the stimulating electrodes. The LTP program [23] was used for data recording and analysis.

Immunofluorescence Staining

In order to visualize sodium channels in the sciatic nerve we followed the published procedure [25] with some modifications. After fixation in 4% paraformaldehyde for 24 hours, the nerve segments were frozen and sliced into 15μm longitudinal sections with a cryostat at -20^{o}C. The immunofluorescence staining was performed on four sciatic nerve segments taken from four different animals. There were two segments, stimulated with LFS and taken immediately after the experiment was ended, and two control (not stimulated) segments from the same nerve of the same animal. Each segments yielded 56 slices and 8 slices of each segment, taken from the middle of the segment were analyzed. The sections were incubated for 1 h at room temperature in the solution of 2% non fat dry milk, 0.2% Triton X100 and 10% goat serum. Then the samples were incubated overnight at 4^{o}C with primary monoclonal antibody, anti-sodium channel (PAN) (sigma USA) dissolved in 2% non fat dry milk and 2% goat serum. Following rinse with PBS (3 times for 10 min each time), the samples were incubated for 1 h at room temperature in 2% non fat dry milk and 2% goat serum, and then for another hour in the same solution with the addition of goat anti-mouse Alexa fluor 488 antibody (Molecular Probes). Following rinse with PBS (3 times for 10 min each time) the sections were mounted on gelatin coated slides and covered with coverslip. Images were collected by Nikon eclipse 90i confocal laser scanning microscope and Nikon D-eclipse D1 camera. AutoDeblur v93 + AutoVisualize v93 was used in analyses. Since the slices were scanned at 1μm, each nerve slice of 15μm gave 15 of 1μm images. The intensity of immunofluorescence of sodium channels in a longitudinal slice of the mouse sciatic nerve was measured using MatLab software. To quantify the distribution and concentration of sodium channels, the slice photograph was imported into MatLab and converted into two dimensional matrix of equal number of pixels (1024 rows and 1024 columns, Fig. **11A**) and the averages of pixel intensity of all columns have been calculated. Then the part of the image presumably representing nodes of Ranvier were selected and enlarged until single pixel could be identified (Fig. **11B**). Ten pixels of the highest intensity from each side of the node (the total of 20 pixels from each node) have been selected to calculate the average intensity.

Statistical Analysis

Statistics and analysis were computed using Microsoft excel, SPSS, and MatLab software. Student's *t* test – paired and independent – was used to compare groups (two tailed).

One sample *t* test and One-Sample Kolmogorov-Smirnov test were used to determine statistical significant of percentage of change of control (zero is used as theoretical value). Conventional repeated-measure ANOVA followed by Tukey's *post hoc* was used to analyze data recorded at repeated points. Multiple comparisons were Bonferroni adjusted, equal variances assumed. In all tests p value of 0.05 was accepted as an indicator of statistically significant results.

RESULTS

The Effects of HFS on CAP

High frequency stimulation of the sciatic nerve exerted a biphasic effect. As illustrated in Fig. (**1**), the potential was significantly depressed immediately after HFS application. However, shortly after HFS termination, the amplitude of the CAP started to recover gradually and within 10-15 min the potential exceeded the value observed prior to HFS application. The potential remained elevated for at least one hr after HFS application (longer time not followed). The averaged results of these experiments, obtained from seven preparations demonstrated significant depression (-31.43±14.64%; p=0.001) during the first 10 min, followed by significant amplification (22.86±31.47%; p=0.02). The amplitudes of the depressed and amplified potentials were expressed as % of pre-stimulation baseline. In control experiments the amplitude of CAP remained stable for more than 3 hrs.

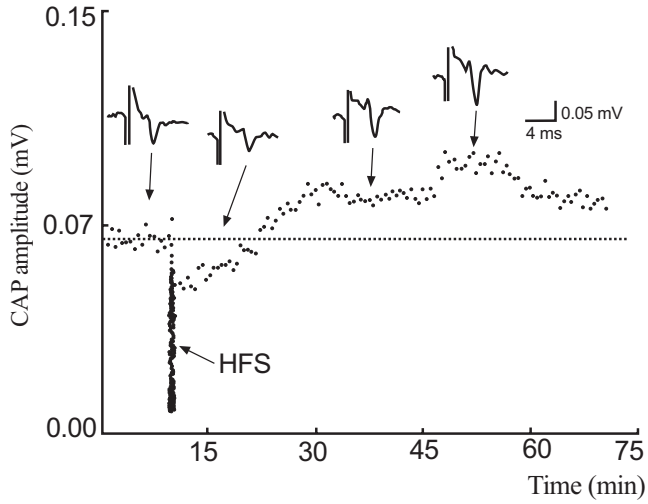

Fig. (1). The amplification of CAP, recorded from the sciatic nerve by high frequency stimulation (HFS). While the upper panel shows the shape of individual CAPs, the lower panel depicts the change of the potential during the entire experiment. Note that while the potential was briefly depressed during and immediately after application of HFS, it quickly recovered and became amplified shortly (15-20 min) after cessation of HFS.

The Effects of LFS on Antidromic Potentials Recorded from Hippocampal Slices

The antidromic potentials were recorded from the hippocampal slices in the presence of kynurenic acid, a blocker of glutamergic transmission. As shown in Fig. (**2A**), hippocampal population spike (PS) was significantly depressed after application of LFS (compare Fig. **2A1** and **2A2**). Fig. (**2B**) illustrates changes in CAP amplitude recorded during an individual experiment and Fig. (**2C**) shows the averaged changes recorded from 7 hippocampal slices.

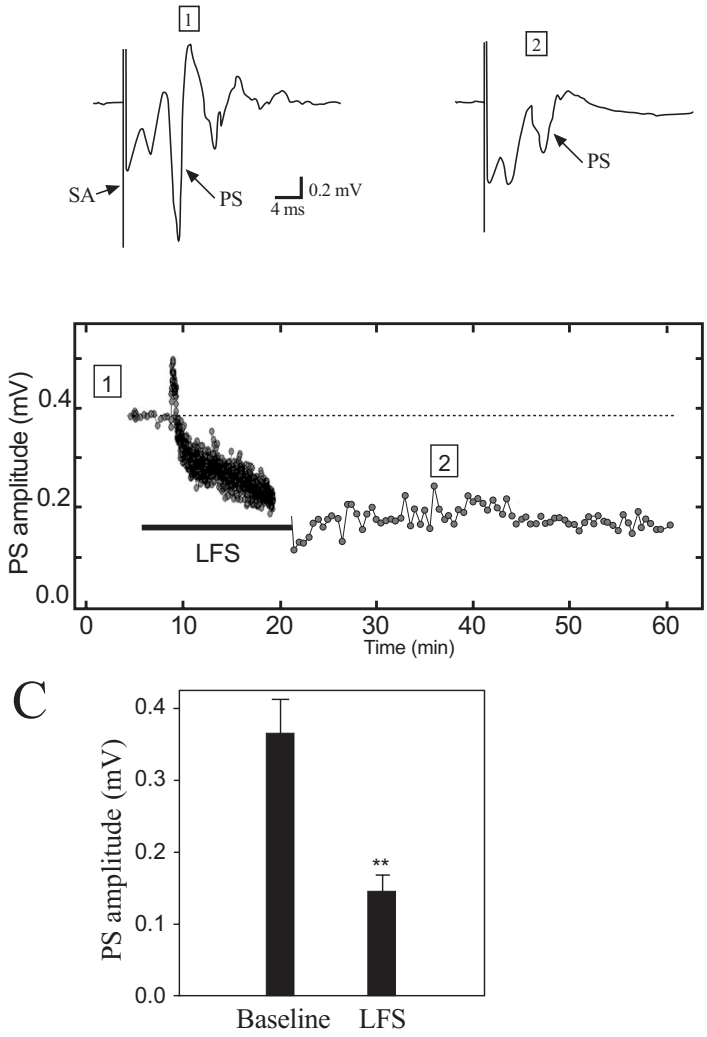

Fig. (2). The effect of LFS on an antidromically evoked potential recorded from hippocampal slices. **A**- an antidromically evoked population spike (PS) before (1) and after (2) application of LFS. SA - stimulation artifact. **B**- The changes in PS during an entire experiment. Numbers 1 and 2 indicate times when the records shown in A were taken. LFS low-frequency stimulation. **C**- averaged changes in PS amplitude induced by LFS recorded in 7 experiments (-61.1±16.6%, p< 0.014).

The Effects of LFS on the CAP Recorded from the Sciatic Nerve

As depicted in Fig. (**3**), LFS depressed the amplitude of the action potential recorded from the sciatic nerve. Fig. (**3A**) depicts individual potentials and representative experiment recorded before and after LFS application (upper panel and lower panels, respectively). Fig. (**3B**) shows cumulative data. Repeated ANOVA analysis (p=0.0007), followed by post hoc analysis showed statistically significant difference (p=0.0001) between potentials recorded before (minutes 0 - 12) and after LFS (minutes 30 - 40), respectively. Those changes were divided into two phases. As shown in Fig. (**3B**), the phase 1 included changes in the amplitude of CAP,

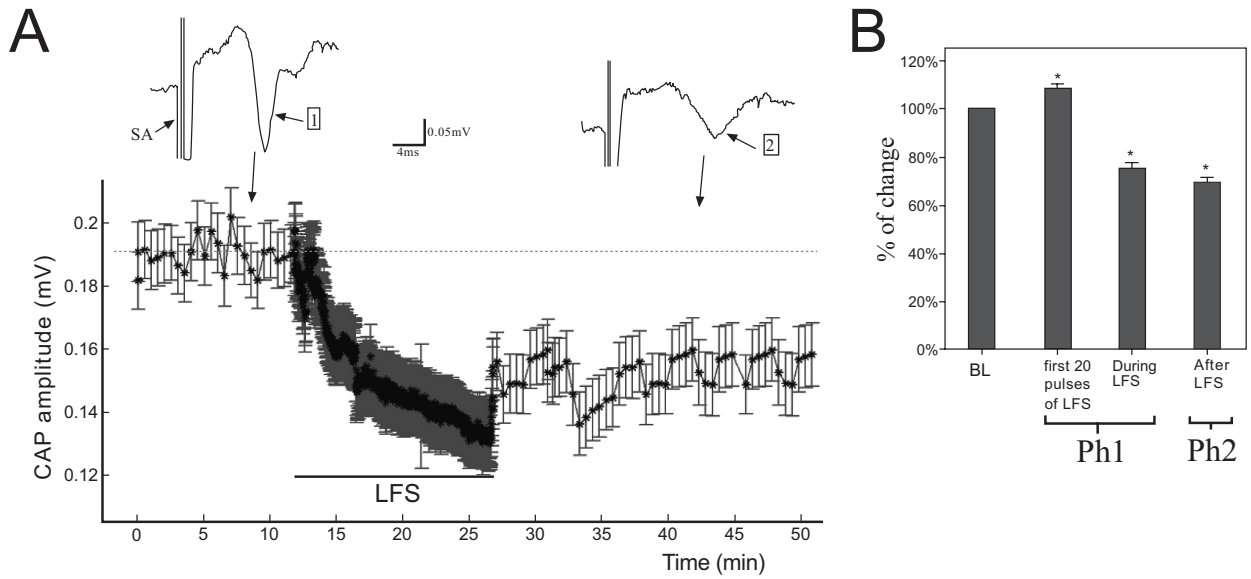

Fig. (3). The attenuation of CAP recorded from the sciatic nerve induced by low frequency stimulation (LFS). In opposite to HFS, Low-Frequency Stimulation persistently depressed CAP recorded from the sciatic nerve. **A**– the upper panel shows individual potentials recorded before (1) and 90 min after (2) application of LFS. The lower panel shows the changes in the CAP amplitude during entire experiment. **B**- Averaged changes in CAP amplitude observed in Phase 1 (first 20 pulses and during LFS) and in Phase 2 (after LFS). Note that there was a biphasic change in the CAP amplitude during Phase 1: an initial, brief increase in CAP during first 20 sec of stimulation was followed by a reduction in CAP. Data are represented as mean ± SEM.

which were observed during the initial part of phase 1 and included the response for 20 first pulses. The amplitude of CAP became enhanced for brief time period shortly after initiation of stimulation (Fig. **3B**). The average increase was 8.8±2.6%, (n=38, p=0.004). This transient increase was followed by a sharp, short-lasting drop of the potential during the second part of phase 1. This decreased potential partially recovered while LFS was still applied (Fig. **3B**). The average decrease in the CAP in this phase was 24.3±4.0% (n=38, p=0.0002, paired t-test). Upon termination of the LFS, the amplitude of the potential remained depressed (-30.5±3.6%, n=38, p=0.0005) for the remaining part of the experiment. In some instances the CAP was depressed by more than 50%. There was no correlation between the initial CAP amplitude and the magnitude of the depression. Thus, the observed changes were related to the treatment, and not to the physiological state of the preparation. One could not exclude the possibility, that depression of CAP was induced by unspecific reaction between the metal of the frequently activated stimulating electrode and the nervous tissue. To verify this assumption, we used two stimulating electrodes located along the axon 2-3 mm apart. The recording electrode was at the end of the axon, as in all other experiments. While only one of stimulating electrodes was used to stimulate the tissue with LFS, the CAPs induced by both of these electrodes activated separately before and after LFS application were recorded. As depicted in Fig. (**4A**), LFS induced the depression of the CAPs in response to the activation of either of these two electrodes. Thus, aLTD generalized across the whole length of the axon and was not localized to the stimulation site only. Repeated measure ANOVA followed by post hoc analysis showed statistically significant difference between potentials recorded with electrodes S1 and S2 before and after application of LFS (Fig. (**4B**), n=5, p<0.05).

There are at least two reasons which could be responsible for attenuation of CAP amplitude. Firstly, the application of LFS could simply accelerate the deterioration of the preparation, and the observed changes could be considered as unspecific. Secondly, the decrease could represent an important, physiologically relevant process initiated by LFS. Therefore, to distinguish between these two possibilities, we decided to determine, if LFS-induced attenuation is reversible. Following the depression of CAP, the segment of sciatic nerve was stimulated within five minutes after termination of LFS with either a few episodes of high frequency stimulation (HFS), or by application of a stronger stimulus, or with a stimulus of longer duration. As shown in Fig. (**5A**), LFS-induced depression was partially reversed by the first application of HFS (Fig. **5A**, point "a"). The second application of HFS 10 min later (twice with 10 sec interval) amplified the potential even further (Fig. **5A**, point "b"). Fig. (**5B**) shows the average depression of CAP by LFS and partial recovery induced by subsequent application of HFS. Similarly, the potential depressed by LFS has been temporarily reversed, when the duration (but not the intensity) of the stimulus was augmented from 0.1 ms to 0.3 ms (Fig. **6A**, n= 5, * BL/LFS, p=0.042; **LFS/0.3 ms, p=0.041; BL/0.3 ms, ns, p=0.48). All of these treatments reversed the depression of CAP if applied immediately, or at least within 10-15 min after cessation of LFS. Any of these treatments applied 40 min after cessation of LFS was ineffective and the depression could no longer be reversed (Fig. **6B**).

Thus, it is clear that while the attenuation of CAP by LFS did not damage the nerve, it occurred in stages. Therefore, one can speculate that in the sciatic nerve, LFS initiates a chain of reactions, whose duration extends beyond the period of stimulation. The first phase (Fig. **3B**, Phase 1) expressed as depression, lasting 10-15 min was reversible. However,

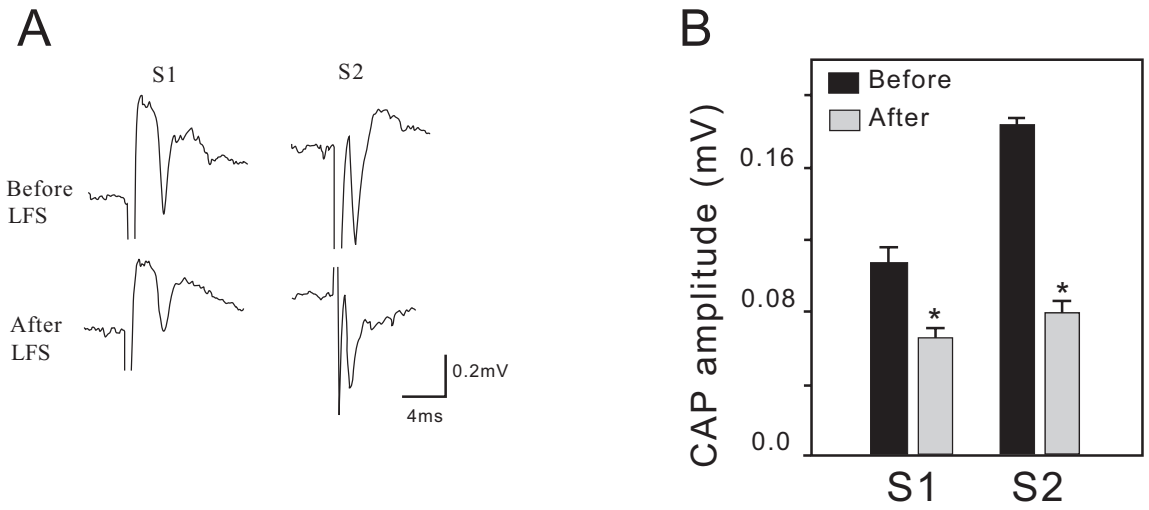

Fig. (4). The stimulation of the sciatic nerve with two electrodes. **A**- The CAPs amplitudes recorded when either S1, or S2 were activated before, and after LFS application. Note that regardless which stimulating electrode was activated, the CAP amplitude was depressed. In this experiment S1 was used to apply LFS. There was no change in observed results, regardless whether the recording electrode was located at the end of the nerve segment, or between both stimulating electrodes; **B**- The average results obtained with two recording electrodes. S1 and S2 represent the results recorded with electrode 1 and 2, respectively. Repeated measure ANOVA followed by post hoc analysis demonstrated statistically significant difference between potentials recorded with either S1 or S2 electrodes before and after application of HFS (n=5, $p<0.05$).

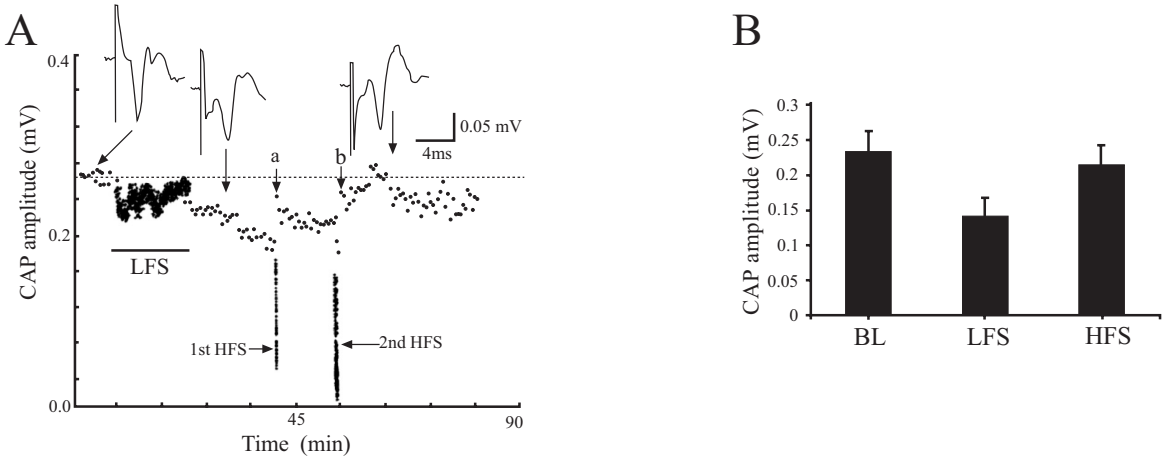

Fig. (5). The reversal of early phase (Phase1) of LFS-induced depression of CAP by subsequent stimulation with HFS. **A**- a representative experiment. The potential depressed by LFS partially recovered following 1st HFS stimulation (100 Hz/1s, marker a). The recovery of the potential was further enhanced by the 2nd HFS application (2x100 Hz applied for 1 s each with 10s intervals, marker b). **B**- Average of five experiments demonstrating the recovery of CAP following stimulation with HFS. The bar marked LFS shows the average of the potentials recorded in the time period between termination of LFS and initiation of HFS. The bar marked as HFS shows the average of ten potentials recorded 15 min after application of HFS. The difference between LFS and HFS is statistically significant (p=0.02).

the processes occurring during the second, later phase (longer than 15 min, Phase 2) made the depression long-lasting. It is important to note that the potential did not recover spontaneously and additional treatment was necessary to reverse the effect of LFS. This reversible, but time-dependent attenuation, could imply that all axons stimulated in this mixed nerve responded to LFS application in the same manner. To verify this assumption, we continued to characterize the LFS-induced attenuation of CAP applying stimuli of increasing intensities, presumably activating motor and sensory axons with different thresholds. Fig. (**7**) illustrates the relation between the strength of the stimulus and CAP amplitude before and after LFS application. LFS attenuated CAP in a very similar way regardless of the stimulus intensity, except at the supra maximal stimulus strength (2.22V and 3.22V), when the effect apparently became saturated. Thus, it is very likely, that different populations of axons stimulated in the sciatic nerve reacted to LFS in a similar way.

The Involvement of Calcium in LFS Effects

The calcium ions do not directly contribute to the generation and propagation of an action potential. In confirmation of this notion, we did not observe the difference between CAP recorded in normal and Ca^{2+}-free Ringer's. However, we found that application of LFS in calcium-free Ringer's is ineffective and has no influence on CAP amplitude (n=18, p=0.998, paired, t test). To verify this observation we per-

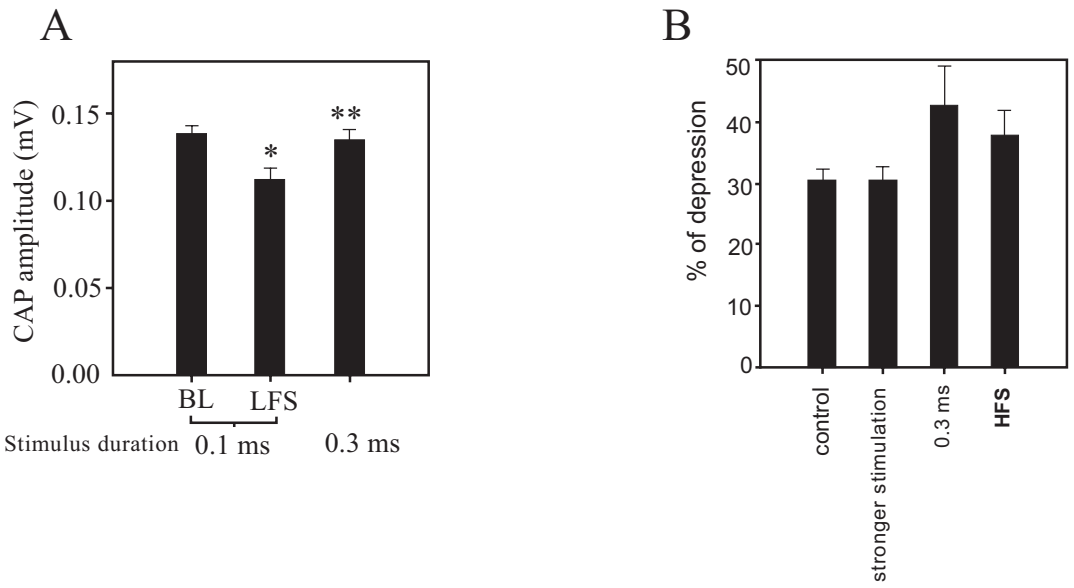

Fig. (6). The reversibility of LFS. **A**- The reversal of early phase (Phase1) of the depressive effect of LFS by an increase in the stimulus duration from 0.1 ms to 0.3 ms. The amplification was immediate, although the CAP returned to its depressed value when the stimulation with the pulse duration of 0.1 ms resumed; **B**- The irreversibility of the late phase (Phase 2) of aLTD. Higher stimulus strength (stronger stimulation), longer stimulus duration (0.3ms) and HFS (HFS) were applied subsequently to the same segments of nerves 40 min after induction of aLTD. While none of these treatments were able to reverse depression, stimulation with longer duration (0.3 ms) actually made the depression greater. The magnitude of the depression was calculated by subtracting post-LFS value from pre-LFS value and expressing this difference as % of pre-LFS value. None of these treatments reversed the depression of CAP in a significant way (n=5, p=0.157, repeated measure ANOVA).

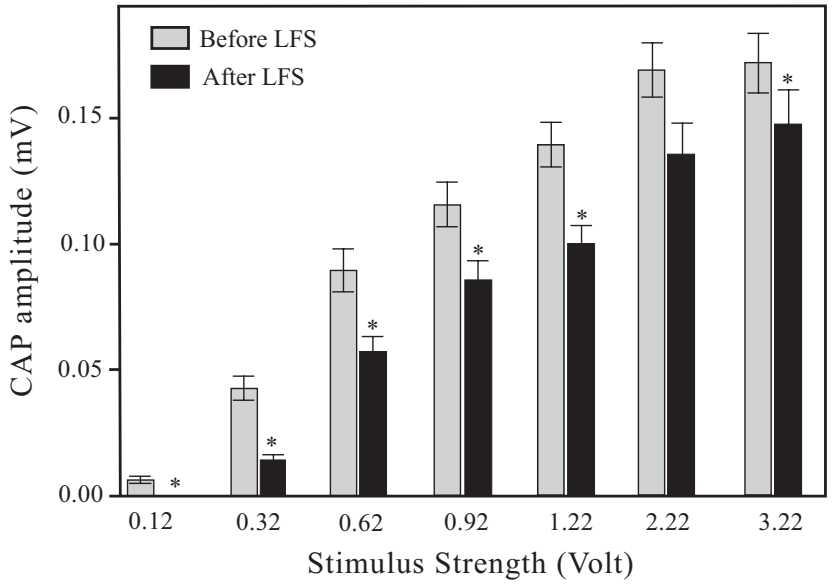

Fig. (7). The influence of the stimulus strength on the LFS-induced depression of CAP. The amplitude of CAP was measured at seven different stimuli strengths before and after application of LFS. The depressive effect was statistically significant at all intensities (n=9, $p<0.05$) except at supramaximal values (2.22V and 3.22V, $p>0.05$). Note that the difference in the amplitude of CAP evaluated before and after LFS application was very similar across different stimulus strength.

formed additional set of experiments. The LFS was applied for the first time in Ca^{2+}-free Ringer's and then several minutes later, second time after addition of Ca^{2+} (3.1 mM final concentration) to the Ringer's solution. While the first LFS application was ineffective, the CAP amplitude was reduced following second LFS application (n=6, p=0.009). Fig. (**8**) shows one of six experiments, which all gave very similar results. Additional control experiments revealed that applying of LFS twice (with 20 min delay) to the same preparation in Ca^{2+}-free Ringer's had no influence on the magnitude of the potential (n=5, $p<0.05$, paired t test).

The Influence of LFS Stimulation on Neuronal Refractoriness and Conduction Velocity

If two stimuli are applied to the segment of the sciatic nerve in a rapid succession, the second response will be smaller than the first response. This phenomenon is related to refractoriness of the nerve, and measures the ability of the

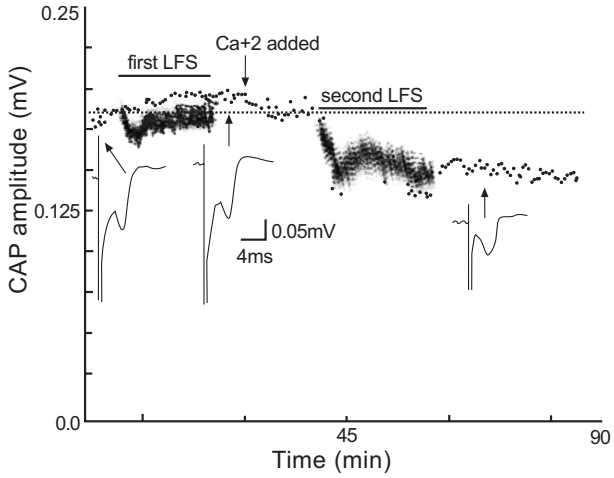

Fig. (8). The LFS-induced depression of CAP is calcium dependent. While the application of LFS had no effect on CAP recorded in Ca^{2+}- free Ringer's (the left part of the graph), addition of Ca^{2+} (3.1 mM) and subsequent application of LFS 15 min later (the right part of the graph) depressed the potential (n=6, p=0.009) (-22.41±13.16%).

nerve to recover completely from the previous activation. In order to evaluate the influence of LFS on the neuronal refractoriness, the segments of sciatic nerve were stimulated with paired stimuli at the interstimulus interval ranging from 9 ms to 75 ms, before and after LFS exposure. As shown in Fig. (**9A**) (upper panel) the second potential became equal to the first one at the interval of 75 ms. However, following exposure to LFS the second potential became equal to the first already at 15 ms. Thus, the LFS exposure reduced the refractoriness of the sciatic nerve by approximately 60 ms. The statistical analysis (repeated measure ANOVA followed by pair wise comparisons) showed statistically significant difference ($p<0.05$) except for the recovery points, which were 75 ms and 15 ms for controls and for LFS exposed preparations, respectively. Fig. (**9B**) shows the shapes of the potentials recorded at 15 ms interval. While the second potential is smaller than the first one before LFS application, it becomes equal to the first one after LFS application.

The nerve conduction velocity (NCV) of an action potential was estimated considering CAP delay. LFS exposure significantly reduced NCV from 42.0±2.8 m/sec (controls) to 28.8±4.3 m/sec in LFS (exposed, n=5, p=0.016). Thus, the reduction in the amplitude of an action potential, induced by LFS was accompanied by slower conduction of an action potential. This observation correlates well with the faster recovery from paired-pulse induced depression and reprimming of the channel. This slower conduction allows the channels to recover better before the arrival of delayed, second action potential.

The Effect of LFS on the Threshold Tested with Variable Duration of the Stimulus

The minimal threshold is defined as the least stimulus strength that can generate an action potential. It has been determined that changes in the peak and duration of CAP are strongly correlated and can be saturated by just-maximal amplitude. Since supra maximal stimulus intensity depressed CAP, the stimulus strength has been adjusted in all experiments until no longer changes in CAP time course and in its peak were observed. Fig. (**10A**) shows a change in a minimal threshold, evaluated before and after LFS exposure, tested with the stimuli of different duration. The minimal threshold not only increased following LFS, but the difference induced by LFS was much greater at longer stimulus durations, with the plateau at approximately 0.5 ms. These changes imply that either the excitability of axons was diminished, or new population of axons with low excitability was recruited after LFS exposure.

The maximal threshold is defined as the stimulus strength which is just enough to evoke the potential of a maximum value. The maximum threshold has decreased following exposure to LFS by nearly 20% and the decrease was not cor-

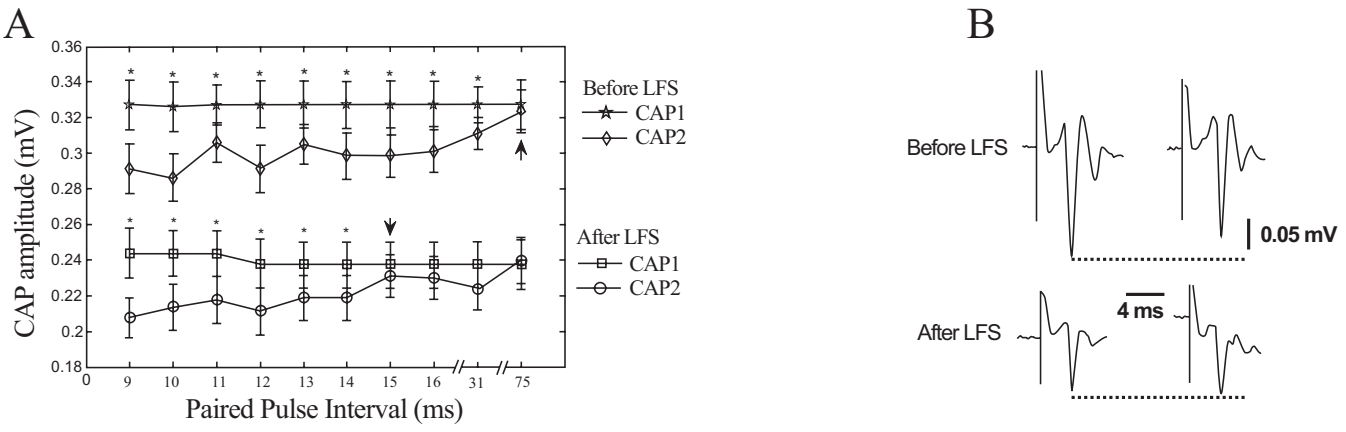

Fig. (9). The modulation of neuronal refractoriness by LFS. **A**- The fragments of the sciatic nerve were stimulated with two pulses applied with the time intervals ranging from 9 ms to 75 ms. While the upper panel illustrates the amplitude of the first (CAP1) and the second (CAP2) potential before application of LFS, the lower panel demonstrate these responses after application of LFS. Note that the difference between first and the second potential disappears at much shorter time interval (15 ms) after, than before (75 ms) the exposure to LFS; * - indicates the statistically significant difference ($p< 0.005$) in the refractory periods before and after LFS treatment, evaluated with t-paired test. **B**- typical potentials recorded from the control and exposed sciatic nerve stimulated with twin pulses at 15 ms intervals.

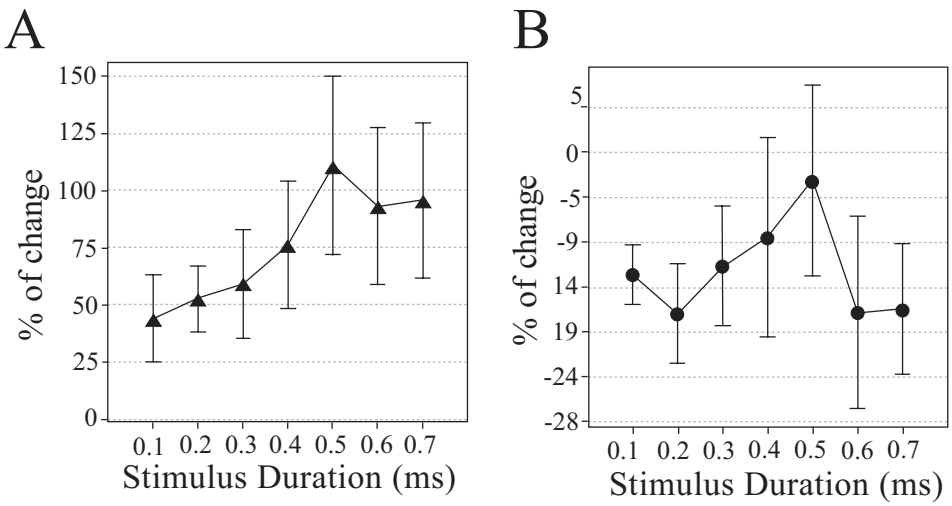

Fig. (10). The influence of LFS on the axonal minimal (**A**) and maximal (**B**) thresholds. **A**- The difference in minimal threshold evaluated at different stimulus duration before and after application of LFS. The difference in minimal threshold is positive indicating that LFS induces an increase in minimal threshold. This difference becomes greater, as the duration of the stimulus increases, to reach plateau at 0.5 ms; **B**- The difference in maximal threshold is negative indicating that LFS induces a decrease in maximal threshold. There is no correlation between the duration of the stimulus and the difference in maximal threshold.

related with stimulus duration (Fig. **10B**). The decrease in the maximal threshold would indicate that the maximal value of the action potential is reached faster after LFS exposure due to a faster saturation, and lower responsiveness of the sodium channels.

The Influence of LFS on the Number of Sodium Channels

The expression of the sodium channels was detected histochemically with sodium channel antibody. The intensity of the signal generated in the presence of this antibody was

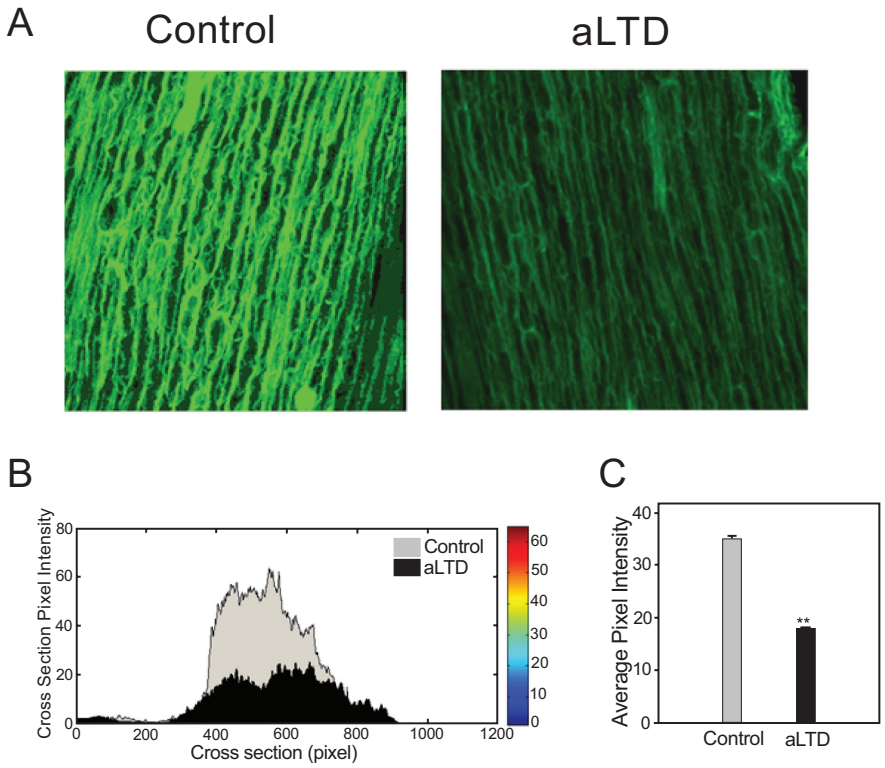

Fig. (11). The change in the number of sodium channels following exposure to LFS. **A**– an image of the representative slices from control (left) and LFS exposed (right) sciatic nerve. **B**- a representative Matlab analysis of a whole slice. A slice photograph was imported into Matlab, and then the whole photograph was converted into two dimensional matrix of equal number of pixels (1024 rows and 1024 columns) as described in the Methods. **C**- Average of pixel intensity of all columns of the matrix in control and LFS-exposed (aLTD) slices. The bars represent the averages of the pixel intensity from cross sectional pixel where the intensities in control and aLTD slices were the highest , ** $p<0.0001$, t-test.

interpreted as an indicator of the number of sodium channels. The analysis of the whole slice revealed a significant reduction in the signal representing the number of sodium channels from approximately 35 in controls (35.2± 0.33 SEM, n=14) to 18 (18.0 ±0.18 SEM, n=11) in the slices exposed to LFS (Fig. **11**). While Fig. (**11A**) illustrates a histochemical staining, Fig. (**11B**) and C show the distribution of the pixels at the cross section area of the slice, and the averaged pixels intensity, respectively. The subsequent analysis focused on the images of the nodes of Ranvier revealed that the color intensity at the nodes of Ranvier of aLTD slices is significantly reduced (54.09±3.2, n=14), as compared to control slices (165.43±9.04, n=11, p=0.0006, independent samples *t* test, Fig. **12**). Thus, induction of aLTD reduced the number of sodium channels much greater at the nodes of Ranvier (67%) than in the rest of the slice (50%).

The Influence of Blocking of K^+ Channels on aLTD

In 12 experiments, the segments of the sciatic nerve were exposed to LFS in the presence of 2 mM Tetraethylammonium (TEA), a blocker of K+ channels. There was no significant difference in the depression of CAP recorded from control preparations (-30.9±3.7%), and from the nerves incubated with TEA (-40.1±9.8%). However, TEA showed a trend to facilitate the induction of aLTD, since all nerves incubated with TEA expressed aLTD comparing approximately 70% of control nerves.

DISCUSSION

We have demonstrated that repetitive stimulation, applied to the segments of sciatic nerve according to the patterns which modify synaptic efficiency, induced long-lasting changes in the properties of an action potential. In an analogy to the phenomena observed in the CNS, we called these changes axonal long-term potentiation (aLTP) and axonal long-term depression (aLTD). The alterations in the action potential amplitude were related to the frequency of stimulation. Our results, obtained on the nerve segments, may not entirely represent events occurring *in vivo*. In our experimental design there is no feedback from somato-dendritic and synaptic compartments on the processes occurring in the stimulated axons. However, antidromic potentials recorded from hippocampal slices originated from the axons still connected to the cell bodies and the nerve terminals. Since the effects of LFS observed during testing of hippocampal slices paralleled the results obtained from the sciatic nerve, we concluded that the axonal plasticity is expressed in central and peripheral nervous systems. Moreover, the involvement of either the cell body and/or the nerve terminals is not critical for LFS-induced effects. Even if the connections between

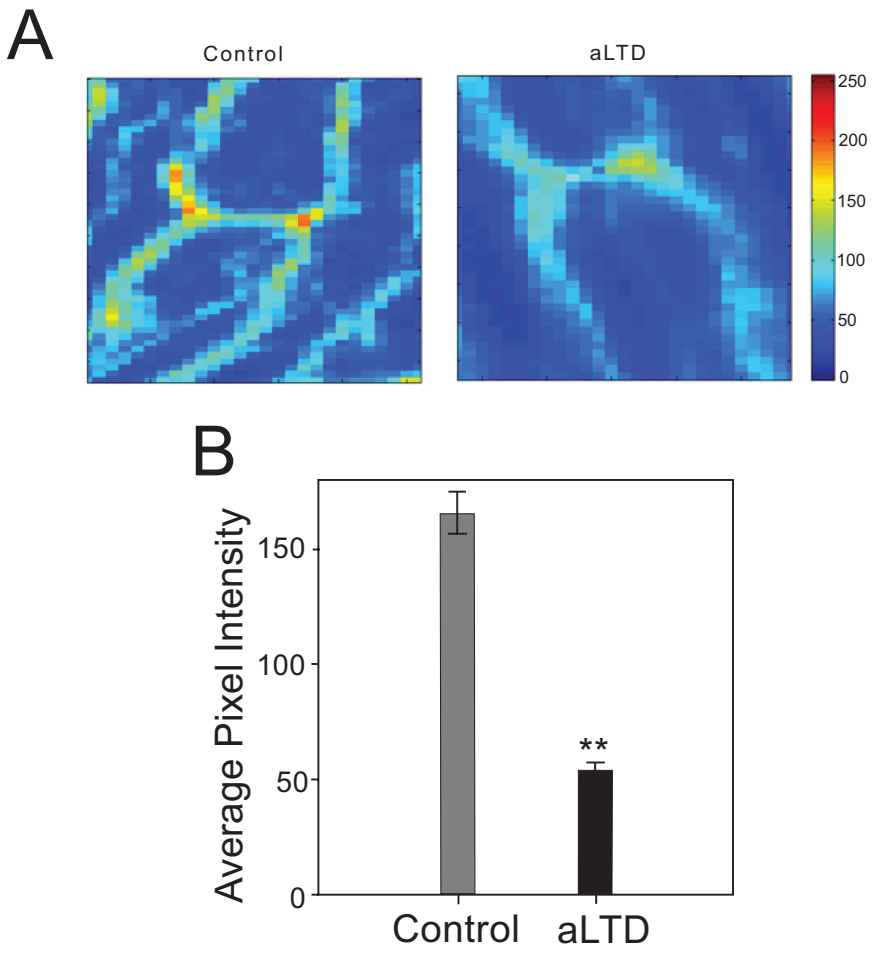

Fig. (12). The influence of LFS on the concentration of sodium channels at the nodes of Ranvier. **A**-The intensity of the pixels, representing the concentration of the sodium channels in the nodes of Ranvier in control (left) and LFS-exposed (right) preparations. The preparation was taken for staining 1 hr after exposure to LFS. The dark brown and dark blue colors on the scale represent the highest (250) and the lowest (0) density of the sodium channels, respectively. **B**- the averages of the pixel intensity evaluated at nodes of Ranvier, ** p<0.05, t-test.

the cell body and the axon were important for the effects of LFS, these interactions can be weaken or abolished in pathological conditions. Therefore, we consider our results physiologically relevant to the processes occurring *in vivo*, particularly in the view of recent report describing reduction in axonal activity during deep brain stimulation [24], persistent, axonal hyperpolarization [10], and modulation of the expression of sodium channels in response to neuronal activity [14, 25]. It should also be emphasized that our data can be interpreted only as an average change occurring in the whole nerve rather than changes taking place in individual axons.

Although the action potential has been generally considered as a steady signal, independent on the previous neuronal activity, there are reports describing the activity-dependent modulation of an action potential [10, 26-30]. The processes induced by LFS can be divided into two phases. The first phase started with a very brief period (15-20s) of amplification of an action potential. In an analogy to the process described in the hippocampus [28, 29], this short-term facilitation may be due to inactivation of A-type K^+ current. This facilitation was quickly overwhelmed by an attenuation of CAP which may have been due to either increase in external K+ concentration, and/or to an influx of Na^+ and subsequent activation of electrogenic Na^+-K^+ pump resulting in hyperpolarization of the membrane potential and partial conductance block [7, 8, 13, 30-32]. The channels responsible for this hyperpolarization could include large conductance sodium-dependent [33, 34], and the large conductance calcium-dependent potassium channels [35-37]. Although the depression continued even after the electrical stimulation was terminated, it could be reversed within next 15-20 min by the application of HFS or stronger stimulation. Apparently, the mechanism of CAP generation was still fully operational. The depression became even more pronounced later entering phase 2, when the decrease of CAP became irreversible. This phase can be correlated with the decrease in the histochemical staining aimed at detecting of the sodium channels. In correlation with electrophysiological data, we interpret these results as an indicator of decrease in the number of sodium channels. The staining showed sodium channels on the surface of the axon and inside of the cytoplasm. Therefore the reduction in the absolute number of sodium channels and not their redistribution was responsible for the decreases in their number. One can speculate that LFS application would initiate the influx of sodium ions (Fig. **13**). The elevated Na^+ concentration in the cytoplasm would reverse Na^+/Ca^{2+} exchanger triggering a rapid increase in intracellular Ca^{2+}.

That increase in intracellular Ca^{2+} concentration can reduce Na^+ conductivity and/or facilitate internalization of Na^+ channels [19, 38, 39] and their subsequent degradation in the lysosomes. This would correspond to phase 2 when aLTD becomes irreversible. The hydrolysis of sodium channel protein may occur with the participation of lysosme-degradative pathway [40], closely linked to endocytosis [41]. The lysosomes are present in the axons of PNS with the particularly high concentration at nodes of Ranvier [42].

Since blocking of the K^+ channels have no influence on the magnitude of aLTD, we favor Na^+ channels as being responsible for observed effects.

The axonal threshold is a membrane potential at which the opposite Na^+ and K^+ currents are equal and opposite of each other. At this point even a minute prevalence of the Na^+ conductance would lead to massive, self-accelerated influx of sodium and subsequent generation of an action potential. An increase in the minimal threshold, which accompanies

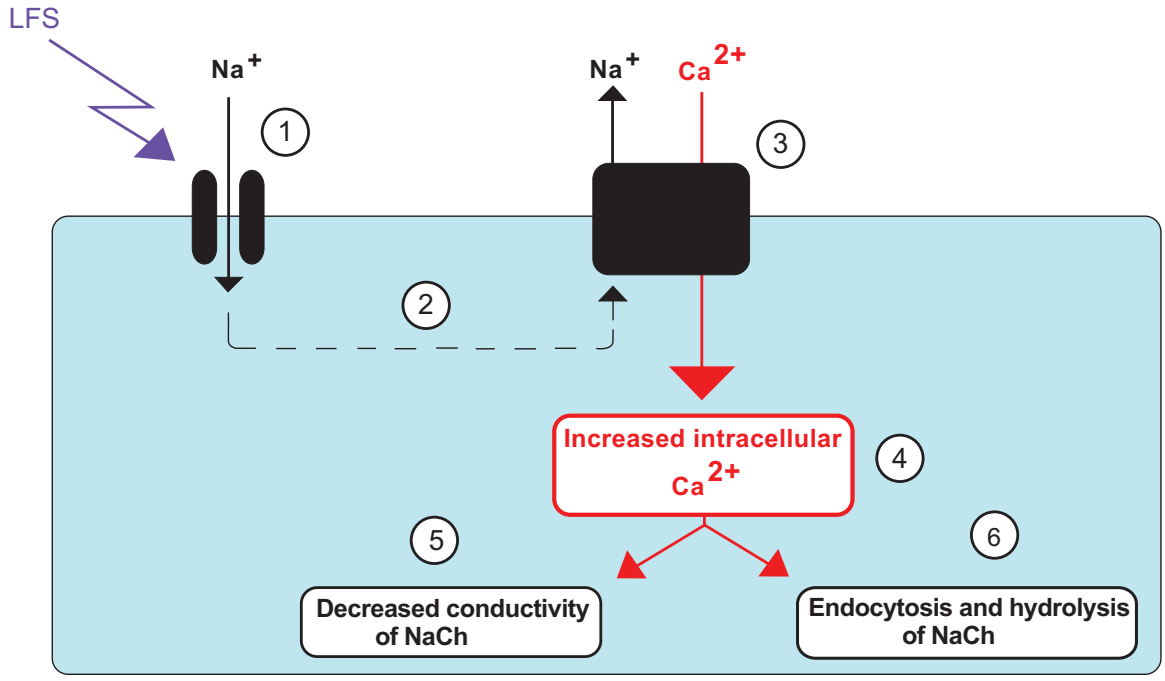

Fig. (13). The diagram illustrating LFS-initiated mechanism leading to accumulation of sodium channels (NaCh) in the cytoplasm and their subsequent degradation. LFS application (LFS) initiates the influx of sodium ions (**1**). The elevated Na^+ concentration (**2**) in the cytoplasm reverses Na^+/Ca^{2+} exchanger (**3**) triggering a rapid increase in intracellular Ca^{2+} (**4**). The elevation of intracellular Ca^{2+} concentration reduces the conductivity of sodium channels (**5**), and/or facilitates internalization and subsequent hydrolysis of Na^+ channels (**6**).

aLTD implies that LFS exposure delays the equilibrium between Na^+ and K^+ currents. LFS-induced increase in the axonal threshold can be interpreted, as one considers the nerve as either collection of axons reacting differently to repetitive stimulation, or set of neurons influenced by LFS in a similar way. The sciatic nerve is a mixed nerve consisting of the axons of different diameter. The largest diameter axons have the lowest threshold for extracellular current. Therefore one can speculate that LFS reduces and increases the overall contribution of large-diameter, and small-diameter axons to CAP, respectively. Thus, the contribution of different axons to CAP would shift towards reducing the involvement of large diameter axons on behalf of small-diameter axons. This notion is further supported by the observation that the largest percent difference between pre-and post-LFS values of CAP occurs with the lowest stimulus strength. Thus, the largest effect was most likely exerted on large-diameter, low-threshold fibers. Since the greater fraction of the stimulating current enters larger axons, they are stimulated more efficiently, and the largest effect was most likely exerted on these large-diameter, low-threshold fibers. As the contribution of these axons is diminished following LFS, the efficiency of stimulation is reduced and the threshold increases. Accordingly, since small-diameter axons have in general lower conductance rate, the conductance velocity of the nerve exposed to LFS was reduced. Since stimulus current will produce a greater depolarization in larger axons, we assume that just discussed process of the stimulation-induced endocytosis of sodium channels and their subsequent lysosomal hydrolysis would preferentially occurred in large-diameter axons. Therefore they are more prone to the effects of prolonged stimulation. Consequently, the change in the threshold induced by LFS was more pronounced at longer durations of the stimulating current.

One can not exclude the possibility that the same population of axons contributed to CAP before and after LFS application. In that case we would assume that all different axons undergo similar reduction in Na^+ channel concentration.

The induction and expression of aLTD were Ca^{+2} dependent in phase 1. There are large conductance calcium-dependent potassium channels present in the axons [43]. As discussed above, these K^+ channels could be responsible for the phase1 of aLTD. Apparently, in the absence of calcium phase 1 and subsequently phase 2 could not be initiated. Thus, the lowering concentration of Ca^{2+} could protect axon against detrimental effects of excessive stimulation. Although we did not evaluate the influence of Ca^{+2} specifically on phase 2, there are several Ca^{+2}-dependent processes like channels phosphorylation/ dephosphorylation [44], or trafficking which could contribute to the processes occurring at this phase.

Besides regulating the action potential, the reduction in the number of sodium channels can influence axonal function in a more general manner. As reviewed recently [45], several components of ion channels express biological activity not related to the conducting functions of the channel itself. For example, the β subunit of sodium channel which is a cell adhesion molecule linked to the cytoskeleton [46] could be involved in the regulation of trafficking of sodium channels, and might contribute to the results of our experiments.

The activity-dependent suppression of axonal reactivity may have clinical applications. The development of neuropathic pain after axonal damage in both peripheral [47-51] and central [52-55] nervous systems has been attributed to the injury-induced, axonal superexcitability accompanied by overexpression of voltage-gated Na^+ channels [56, 57]. Our results demonstrate that the axonal excitability and number of Na^+ channels can be attenuated by a specific pattern of electrical stimulation. Therefore, this specific pattern of electrical stimulation can be used to manipulate the density of sodium channels and to alleviate the source of the neuropathic pain.

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