THE ROLE OF GLUTAMATE IN AXONAL PHYSIOLOGY

by

AHMED ABOUELELA

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Abstract

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The information within the spinal cord and peripheral nervous system propagates along the nerves in the form of Compound Action Potentials (CAP). Although CAPs were always considered to be steady signals, with constant amplitude and velocity as determined by the conductive properties of the nerves our data show that exogenous glutamate increased the CAP. This increase in CAP was blocked after the addition of the general glutamate receptor antagonist kynurenic acid, the specific glutamate receptor antagonist MK801 and CNQX and prevented when these experiments were performed in a calcium-free medium.

The goal of this thesis was to examine the changes in axonal physiology in response to electrical stimulation and to pharmacological manipulation. We found that high frequency stimulation, or addition of exogenous glutamate (100 μM) increases the amplitude of compound action potentials (CAPs) in sciatic nerve preparations. These results were further extended and supported by immunohistochemical experiments showing that axolemma contains glutamate receptors (NMDA, AMPA/kainate and mGluR2), the excitatory amino acid transporter responsible for glutamate uptake (Excitatory Amino Acid Transporter-EAAT), and voltage-gated sodium and calcium channels. Thus, the axolemma of peripheral nerves expresses several proteins important for neuronal communication and modulation of the membrane excitability. Apparently, these proteins embedded into the axonal membrane, can under the influence of electrical stimulation or exogenous glutamate change membrane permeability and ionic

conductance leading to an increase in the amplitude of the compound action potentials observed in our experiments. Our results demonstrate of existence of axonal plasticity expressed as a change in the amplitude of the action potential following periods of changed activity accompanied by release of neurotransmitters. Therefore we suggest a mechanism of the process whereby electrical stimulation leads to increased axonal activity and subsequent release of glutamate that through activation of the glutamate receptors results in changes in the amplitude of CAPs. We term this phenomenon as axonal plasticity, which would represent one of the forms of neuronal plasticity. Neuronal plasticity is defined as a treatment-induced change in the neuronal response in spite of unchanged strength of the test stimulation. This observation was long described as a property of central synapses and thought to be the basis of learning (Malenka, 1994). Axonal plasticity , would constitute exclusive property of the axon and could contribute together with synaptic plasticity to modification of the efficiency of neuronal connections. This type of plasticity would be fundamentally different from the synaptic plasticity expressed in CNS in the form of Long-Term Potentiation-LTP (Bliss and Collingridge, 1993), Long-Term Depression-LTD (Dudek et al; 1992), and Spike Timing Dependent Plasticity (STDP) (Markram et al., 1997) which has been intensively investigated for last several decades. We assume that high frequency electrical stimulation induces the release of glutamate from stimulated axons. Subsequent increase in the extracellular glutamate concentration would be responsible for observed increase in CAP. Increase in the amplitude of CAP may be a result of: An increase in the number of activated axons (recruitment), 2) and/or increase in the amplitudes of individual potentials generated by single axons. The mechanisms responsible for each of these changes are very different. In the case of recruitment one can suggest paracrine action of glutamate which released from group of axons would enhance the CAP of their neighbors. The

increase in the action potential generated by individual axon could be due to a change in the threshold of this individual axon.

Our novel data together with published results clearly indicate that in spite of prevailing notion about “all-or-nothing” property of the action potential, axons and action potentials are capable of conveying the information in an analog manner (Clark and Hausser 2006). Presented results convincingly demonstrate that the amplitude of subsequently generated action potentials can change in a way correlated with the frequency of stimulation, or pharmacological treatment. In both cases the change occurred gradually with each evoked action potential slightly larger than its predecessor. This indicates that the effect was building step by step as the intraaxonal mechanisms have been recruited to contribute to the final effect. We have also observed reduced latency and increased area of CAP after glutamate application. The most obvious explanation for both phenomena would be a recruitment of additional, fast conducting axons which would shorten the latency and increase the area of CAP. Simultaneously this would increase the duration of the entire CAP, as slower conducting axons which contributed to CAP before the treatment would be activated as well.

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I would like to thank my mentor Dr. Wieraszko Andrzej for accepting me as a member of his lab, sharing his passion for science and for his directions, support and consideration.

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ABBREVIATIONS

Ach	acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ANOVA	analysis of variance between groups**.**
ATP	Adenosine Triphosphate
$[Ca^{2+}]_i$	Calcium Concentration
CAPs	Compound action potentials
CGF	Cuneate And Gracile Fasciculi
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione.
CNS	Central Nervous System
DL-TBOA	DL-*threo*-β-benzyloxyaspartate.
D-Asp	D-aspartic acid
ER	Endoplasmic Reticulum
EAATs	Excitatory amino acid transporters
Fluo-3	Is a fluorescence indicator of intracellular free calcium
GluTs	Glutamate Transporters
HFS	High Frequency Stimulation
IP3 receptors	Inositol Triphosphate Receptor
K^+.	Potassium
LFS	Low-Frequency Stimulation
LTP	Long-Term Potentiation
LTD	Long-Term Depression
MK801	Dizocilpine

Mg^{2+}	Magnesium
mGlur	Metabotropic Glutamatergic Receptors
Na^{+}	Sodium
Na^{+}/Ca^{2+} exchanger	Sodium Calcium Exchanger
NFDM	Nonfat dry milk
NGS	**N**ormal goat serum
NMDA	*N*-methyl-D-aspartate
nNOS	Neuronal Nitric Oxide Synthase
PBS	Phosphate-Buffered Saline
PLC	Phospholipase C
PMF	Pulsed Magnetic Field
P2X receptors	Purinoceptor7
RYR	Ryanodine Receptor
STDP	Spike Timing Dependent Plasticity
VSCCs	Voltage Sensitive Calcium Channels
VGLUT	Vesicular Glutamate Transporter

LIST OF FIGURES

A-Introduction:*

Synaptic transmission at a chemical synapse involves several steps. An action potential arriving at the terminal of presynaptic neuron causes voltage-gated Ca^{2+} channels to open. The influx of Ca^{2+} produces a high concentration of Ca^{2+} in the axon terminal; this in turn causes vesicles containing neurotransmitters to fuse with the presynaptic cell membrane and release their contents into the synaptic cleft (a process termed exocytosis). The released neurotransmitter molecules then diffuse across the synaptic cleft and bind to specific receptors on the post-synaptic membrane. These receptors cause ion channels to open, thereby changing the membrane conductance and membrane potential of the postsynaptic cell.

Glutamate is the most abundant neurotransmitter in the brain. Nearly all excitatory neurons are glutamatergic. Glutamate is a nonessential amino acid that does not cross the blood-brain barrier, and therefore must be synthesized in neurons from local precursors. The most prevalent precursor for glutamate synthesis is glutamine, which is released by glial cells.

Once released, glutamine is taken up into presynaptic terminals and metabolized into glutamate by the mitochondrial enzyme glutaminase. Glutamate can also be synthesized by trans-amination of 2-oxoglutarate; an intermediate of the tricarboxylic acid cycle. Hence, 4-9 nmol per mg of the glucose metabolized by neurons can also be used for glutamate synthesis (Pellerin and Magistretti 1994). The glutamate synthesized in the presynaptic terminals is packaged into vesicles by transporters, termed vesicular glutamate transporter (VGLUT). Once released, glutamate is removed from the synaptic cleft by the excitatory amino acid transporters (EAATs). There are five different types of high-affinity glutamate transporters, some of which are present in glial cells and others in presynaptic terminals. Glutamate taken up by glial cells is converted into glutamine by the enzyme glutamine synthetase. Glutamine is then transported out

of glial cells and into nerve terminals. In this way, glial cells cooperate with synaptic terminals to maintain an adequate supply of the neurotransmitter. This overall sequence of events is referred to as the glutamate-glutamine cycle.

The excitatory post synaptic potential in spinal motor neurons results from the opening of glutamate-gated channels permeable to both Na^+ and K^+. The ionic movement triggered by glutamate is similar to that produced by acetylcholine (Ach) at the neuromuscular junction. Glutamate receptors can be divided into two broad categories: Ionotropic receptors that directly gate channels, and metabotropic receptors that indirectly gate channels through second messengers. There are three major subtypes of ionotropic glutamate receptors: AMPA, Kainate, and NMDA. The action of glutamate on the ionotropic receptors is always excitatory, while activation of the metabotropic receptors can produce either excitation or inhibition.

B- Background

Information transfer in the nervous system occurs through generation of action potentials in neurons, and synaptic potentials at synaptic junctions. It is known that the magnitude of the synaptic potentials can be modified by their prior activity. This phenomenon, called synaptic plasticity, is expressed as a change in the amplitude of the synaptic potentials following specific pattern of neuronal activation. The best known examples are Long-Term Potentiation-LTP (Bliss and Collingridge, 1993) and long-term depression, LTD (Dudek et al., 1992). While presynaptic activation contributes to the generation of evoked potentials, it has to be preceded by the action potential generated at the axon hillock. Although the action potential has been always considered as a steady signal, resistant to any stimulation-dependent modifications, some recently published results (Carp et al., 2000; Meeks and Mennerick 2004; Debanne 2004), and the data from our laboratory (Ahmed and Wieraszko, 2009a) indicate that the properties of the action potential may be modified by axonal activity induced by either electrical or magnetic stimulation.. The mechanisms underlying these changes are not known, although excessive influx of Na^+, reversal of Na^+/Ca^{2+} exchanger (Morita et al., 1993; Kobayashi et al., 1997), modulation of protein biosynthesis (Weragoda et al., 2004) and/or changes in the expression of new sodium channels (Klein et al., 2003) are implicated. Thus, it is clear that neuronal excitability can be influenced by both synaptic and axonal plasticity.

The focus of this thesis is on ***axonal plasticity***, which is defined as a ***change in the amplitude of compound action potentials as it relates to prior axonal activities***.

We hypothesize that the change in the amplitude of compound action potentials may be mediated by glutamate that is released non-synaptically from stimulated axons and/or glial cells.

According to the classical definition, a neurotransmitter is a molecule which is released from presynaptic nerve terminals in response to stimulation and changes the neuronal membrane potential by binding to postsynaptic or presynaptic receptors. However, this classical view has been challenged by several reports (Meeks and Mennerick 2004; Debanne 2004), indicating that axons can release neurotransmitter-like substances without involvement of the synapses. For example, several reports described the Ca^{2+}-dependent release of messenger molecules from axons which have been stimulated chemically (Fisher et al., 2003; Sauer et al., 2001; Spitzer et al., 2008). The axonal release of ATP (Liu and Bennett, 2003) and peptides through vesicular exocytosis has also been reported (Spitzer et al., 2008). Electrophysiological experiments revealed that axons in the white matter of the central nervous system release glutamate, which excites neighboring cells (Kukley, et al., 2007; Ziskin, et al., 2007). Exposure of sciatic nerve segments to a specific pattern of magnetic (Ahmed and Wieraszko 2008; Ahmed and Wieraszko 2009a), or electrical (Ahmed and Wieraszko, 2009b) stimulation increases the amplitude of the evoked compound action potentials. Since these nerve preparations contained neither synaptic connections, nor cell bodies, the mechanism of the observed amplification had to be intrinsic to the axon. ***We hypothesize that stimulated axons release neurotransmitter(s), which interact with axonal auto-receptors, modifying the amplitude of compound action potentials evoked by subsequent stimulation***. Glutamate is a strong candidate for this function, since some components of the axonal glutamatergic system, like receptors (Stys and Lipton, 2007) and the high affinity uptake system (Carozzi, et al., 2008) have been identified. Our own research reveals that segments of the sciatic nerve and white matter of the spinal cord which have been stimulated either electrically, or magnetically, releases the non-hydrolizable radioactive glutamate analog D-aspartic acid (D-Asp) in a Ca^{2+}- dependent manner (Wieraszko, Ahmed,

2009a). Since D-Asp is considered an excellent marker of glutamatergic synapses (Muzzolini et al., 1997; Savage et al., 2001), one can assume that these segments of the sciatic nerve and spinal cord release glutamate. Furthermore, exogenous glutamate evoked the release of D-Asp as well, indicating the presence of receptors for glutamate that may modulate axonal physiology and eventually mediate axonal plasticity.

Preliminary results:

Results from our lab (Ahmed and Wieraszko, 2009a) indicate that high frequency stimulation or addition of glutamate to sciatic nerve preparation induced a dose-dependent release of [^{3}H]-D-Asp from these preparations. This suggested that high frequency stimulation may induce axonal plasticity in form of increases compound action potential (Figure 5) which can be mimicked by addition of exogenous glutamate (Figure 6). Additionally, these data provide a novel mechanism for axonal plasticity that is activity-dependent. Such a mechanism has been previously described for central synapses. Here we report a type of plasticity that is occurring in the axons. This plasticity can be induced by either repetitive electrical stimulation, or application of exogenous glutamate. Its expression is calcium-dependent. The relevance of this phenomenon at peripheral axons could be an axonal adaptation to modulate descending motor and ascending sensory information. Although at present we do not know which branch of the sciatic nerve is exhibiting plasticity (motor or sensory), this observation represent a novel mechanism by which axons can modulate the amplitude of CAP and therefore the levels of sensory input or motor output.

Experiments 1, 2, 3, and 4 described below and depicted in Figures 1, 2, 3, 4, 5, and 6 are included for illustrative purpose. Although I did not perform these experiments, I am familiar with methodology and I participated in the discussions related to the design and interpretation of these results.

1) The effect of electrical stimulation on Compound Action Potential recorded from the sciatic nerve in vitro

As depicted in Figure 1, high frequency stimulation (HFS) of the sciatic nerve resulted in a biphasic response. The potential was significantly depressed immediately after HFS application, but then the amplitude of the CAP started to recover gradually and within 10-15 minutes the potential exceeded the value observed prior to HFS application.

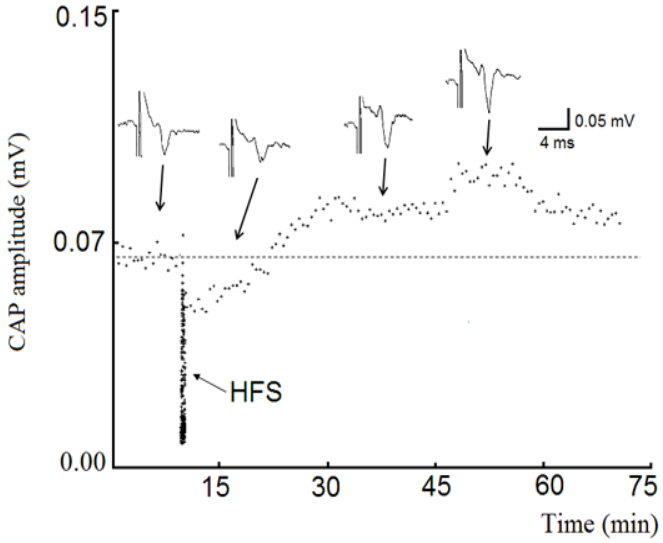


Figure 1 High-Frequency Stimulation (HFS) induced amplification of CAP amplitude recorded from sciatic nerve.

The potential remained elevated for at least one hour after HFS application (longer time not measured). The results of these experiments, obtained from seven preparations demonstrated a significant depression (-31.43±14.64%; $p<0.001$) during the first 10 minutes, followed by a significant increase (22.86±31.47; $p<0.02$) (Ahmed and Wieraszko, 2009a).

The stimulation of the sciatic nerve with Low-Frequency Stimulation (LFS, 1 Hz for 15 min) had an opposite effect and depressed the potential for the duration of the experiment (Figure 2, -30.5±3.6%, n=38, p=0.0005) (Ahmed and Wieraszko,2009a).

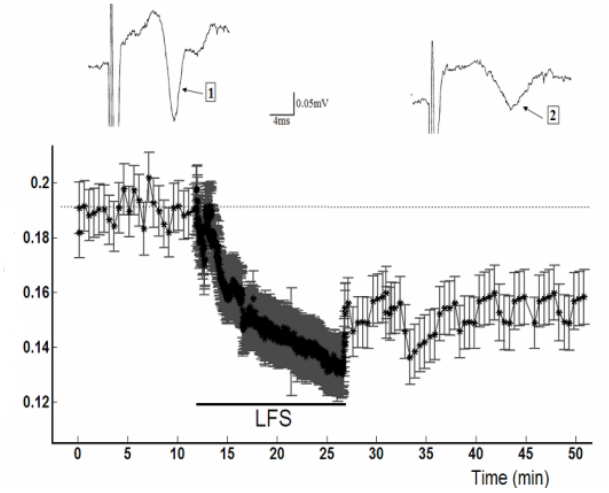


Figure 2 Low-Frequency Stimulation (LFS)-induced attenuation of the amplitude of CAP recorded from sciatic nerve.

Thus, depending on axonal activity, CAP can be either amplified or depressed. This is compatible to neuronal plasticity observed in almost every brain region examined and expressed as either Long-Term Potentiation, LTP (Bliss and Collingridge, 1993), or Long-Term Depression, LTD (Dudek et al., 1992). However, here the plasticity is mediated at the level of the axon which was thought to be a conductive part of the nervous system incapable of exhibiting plasticity but rather maintaining fidelity of neuronal transmission.

2) The effect of magnetic stimulation on CAP recorded from sciatic nerve and spinal cord dorsal columns in vitro

The segments of the spinal cord have been prepared, and the electrodes were placed as described by Matsumoto et al., (2005). Following decapitation, vertebral bone was removed. The spinal cord was longitudinally hemisected and dorsal column was dissected out. The segments of sciatic nerve or spinal cord have been exposed to pulsed magnetic fields in the recording chamber (Wieraszko et al., 2004). The preparation was electrically stimulated during the entire experiment. The pulsed magnetic field (PMF) was generated by DC-powered coils (Wieraszko, 2004), wrapped around an acrylic frame, which surrounds the interface-recording chamber. The field consisted of "on" phase (15 mT, 3sec) followed by "off" phase (0 mT, 3 sec). The preparation in the recording chamber was placed horizontally so that the PMF generated by the coils were perpendicular to the segment of the nerve, or spinal cord. The physical orientation of the preparation is important, because the flow of magnetic fields in relation to the preparation may affect the outcome (Walker et al., 2007; Greenebaum and Sisken, 2007). The magnetic field recorded with the magnetic probe located between recording and stimulating electrode was uniform within the recording chamber. Anti-ferromagnetic iridium/platinum (FHC, New Hampshire) and glass, stimulating and recording electrodes, respectively, located on both ends of the preparation were used. As suggested by Liboff (2004), the influence of the earth magnetic field on our results was ignored, since its intensity was 300 times lower that the signal used in our experiments. The activation and deactivation of the coils was achieved by a programmable timer. The averages of the CAP amplitude recorded in the first 20 minutes (baseline), were compared to the averages of the CAP amplitude recorded 30 minutes after the exposure to PMF.

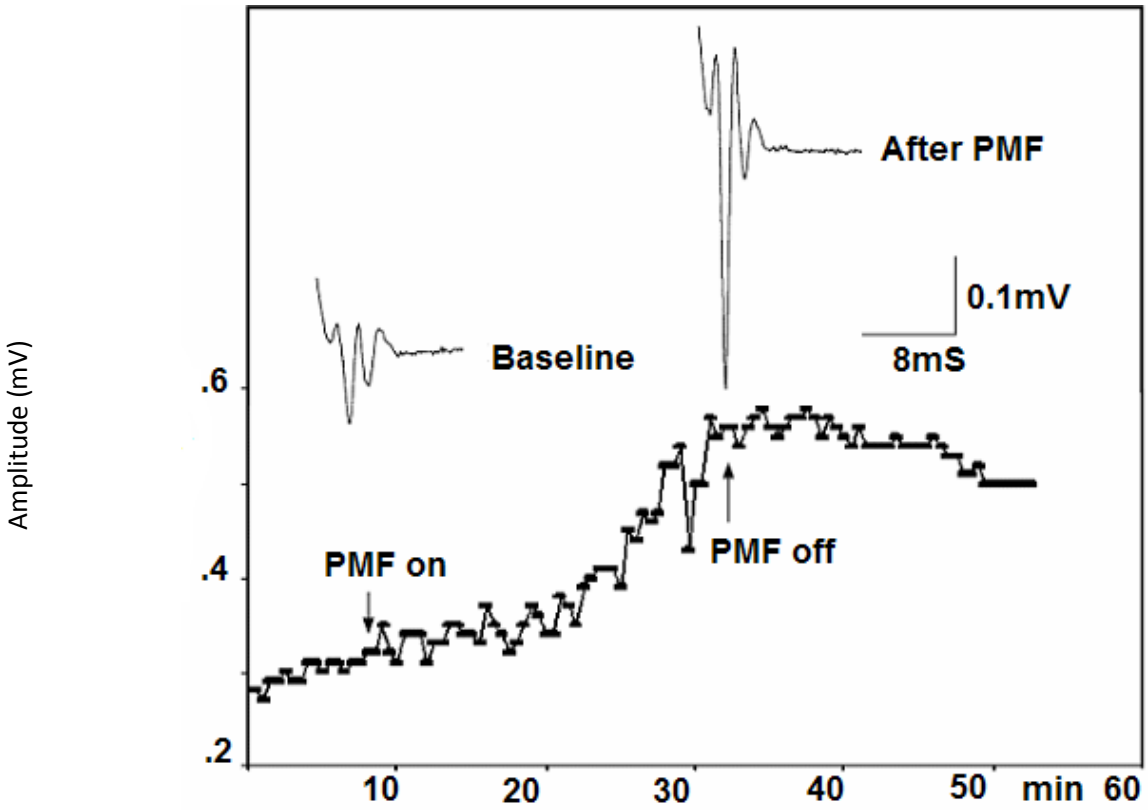


Figure 3 PMF-induced amplification of CAP recorded from sciatic nerve.

The results of representative experiments are shown in Figure 3. PMF (30 min exposure) significantly increased the mean amplitude of the CAP recorded from the sciatic nerve (n=10, 187±16%, $p<0.005$; Ahmed and Wieraszko, 2009b). The influence of PMF on CAP recorded from the spinal cord dorsal columns was tested as described above for sciatic nerves.

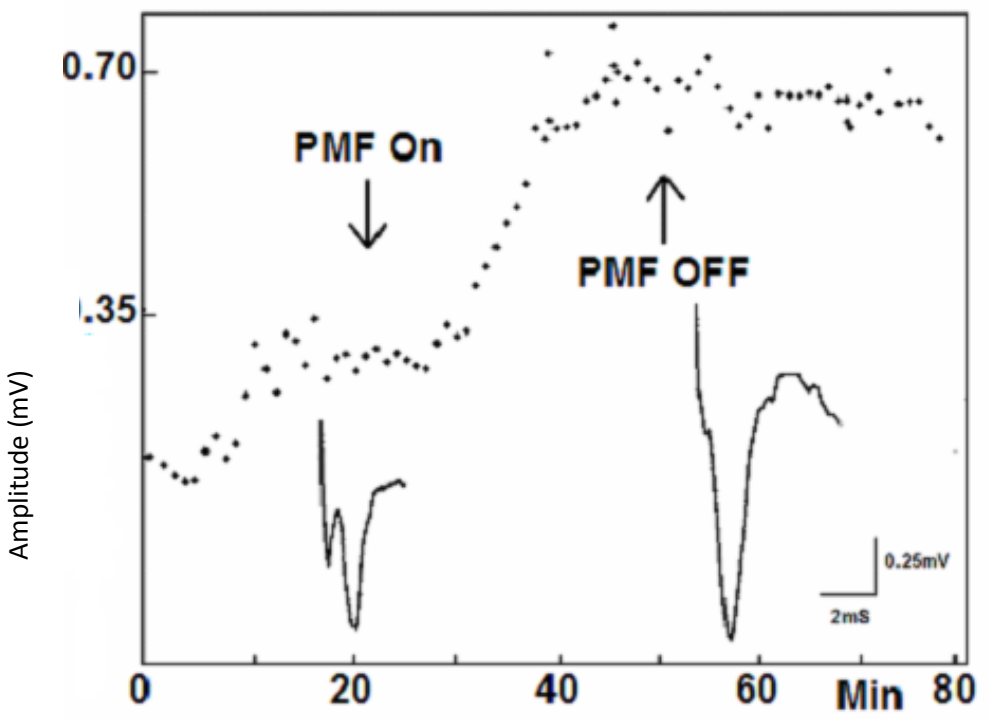


Figure 4 PMF-induced amplification of CAP recorded from spinal cord.

Figure 4 depicts typical experiments and in 5 additional experiments the amplitude of CAP recorded from the dorsal column of the spinal cord was elevated by 232 ± 19% (n = 5, $p<0.003$).

Since we used the dorsal column of the spinal cord that contains axon terminal of sensory neurons whose cell bodies are in the dorsal root ganglia in addition to ascending and descending projections to and from the brain, we suggest that this type of plasticity may be important for sensory modulation.

3) Release of glutamate analog from sciatic nerve and spinal cord white matter induced by electrical stimulation

The sciatic nerve and dorsal columns of the spinal cord were prepared as described above. We also used the same procedure to evaluate the release of glutamate analog from sciatic nerve and dorsal columns of the spinal cord as described in the method section. Following pre-incubation, the preparation was placed in a Brandel suprafusion system to evaluate release of [^{3}H]-D-Asp from the nerves exposed to electrical stimulation, as previously described (Wieraszko, 2004; Wieraszko and Ahmed, 2009a). The chambers used to evaluate the release during electrical stimulation were identical to those used for magnetic stimulation, except for the metal electrodes which were mounted inside at both ends of the chamber. These electrodes, connected to the stimulator (Brandel) were activated during electrical stimulation delivering pulses with the frequency of 10 Hz at 10 mA for 20sec. Prior to exposure to electrical stimulation, the segments of the nerve had been incubated in Brandel's chamber for 2hr in the Ringer's containing 0.26μM D-2, 3-3H Aspartic acid [^{3}H]-D-Asp without any perfusion to allow for uptake of the radioactive molecules in the tissue and to equilibrate the preparation. Following the pre-incubation period, the perfusion was initiated and continued without sample collection (pre-collection period) for the first 30 min to wash away any radioactivity not accumulated by the tissue. Then the collection of 2 min samples (1.8 ml) was initiated with the fraction collector and approximately 20 - 40 samples were collected in each experiment. In some experiments calcium-free Ringer's was used. The radioactivity in each collecting vial was quantified in the scintillation counter after addition of scintillation fluid and data were transferred into graphic form. [^{3}H]-D-Asp release increased almost immediately following stimulation and was significantly lower in Ca^{2+}-free Ringer's (Figure 5).

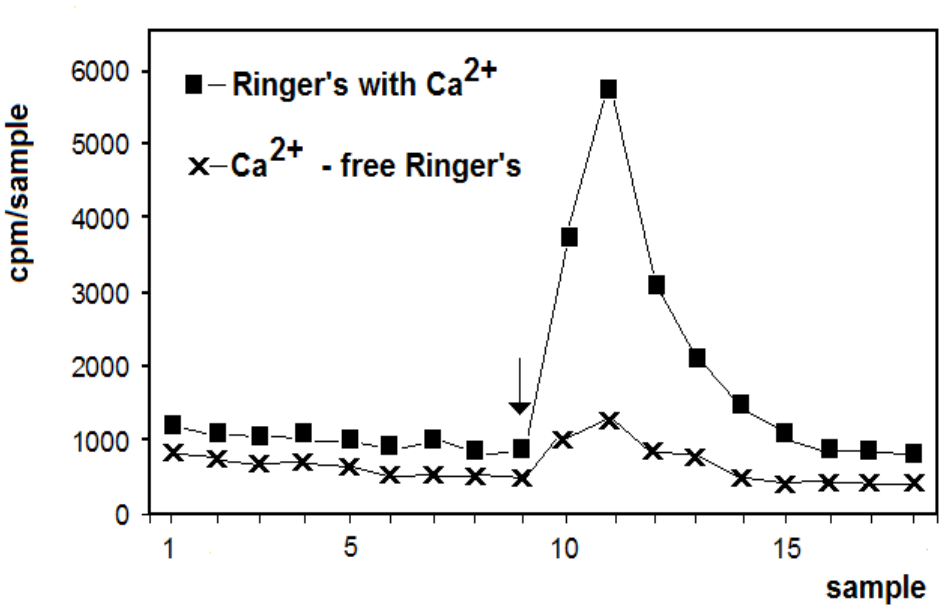


Figure 5 Electrical-induced release of [^{3}H]-D-Aspartate from sciatic nerve.

The average increase in [^{3}H]-D-Asp release was 184±24.5% (n=32). Ligation of the ends of the axons had no influence on the magnitude of electrically-induced release of [^{3}H-DAsp]. The background release in the experiments employing electrical stimulation was calculated by averaging the radioactivity in three samples before and after the stimulation and the magnitude of this release was expressed as a percent of this control background.

4) Glutamate-induced release of [^{3}H]-D-Asp from sciatic nerve and dorsal columns of the spinal cord

Since electrical stimulation of the sciatic nerve induced the release of the glutamate analogue we sought to stimulate these preparations with glutamate and measure the release of [^{3}H]-D-Asp to see if glutamate can mimic electrical stimulation. The segments of sciatic nerve or dorsal columns of the spinal cord were prepared as described above, placed in the Brandel Suprafusion system and preloaded with [^{3}H]-D-Asp. The procedure was as for electrical stimulation, but the preparations were exposed to different concentrations of glutamate instead of electrical stimulation. The exposure to glutamate evoked dose-dependent release of [^{3}H]-D-Asp from the sciatic nerve (Figure 6).

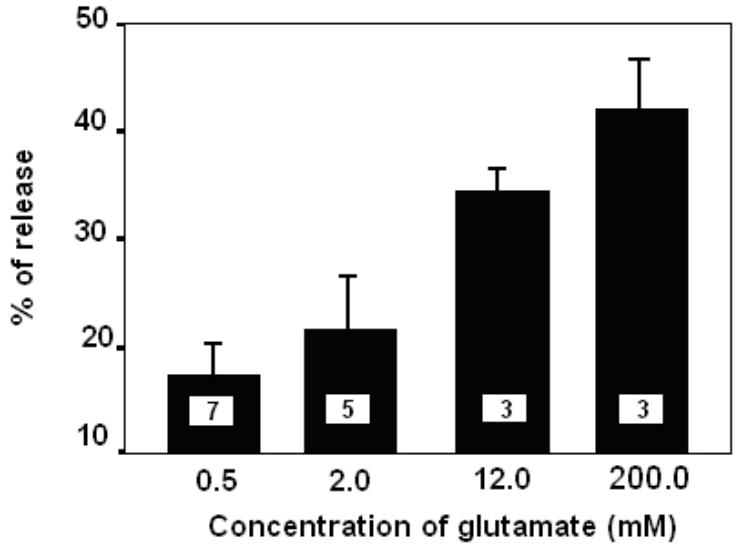


Figure 6 Glutamate-induced release of [^{3}H]-D-Asp from sciatic nerve. Number of experiments is shown inside the bars. Means ± SEM.

Similar findings were observed with the dorsal columns of the spinal cord. In two experiments with spinal cord 16 mM and 200 mM glutamate induced approximately 50% and 1500% increase in the release of [^{3}H]-D-Asp, respectively. These data indicate that exogenously added glutamate can mimic electrical stimulation in inducing [^{3}H]-D-Asp release from preloaded preparations of the sciatic nerve or dorsal column of the spinal cord.

Summary of the preliminary results

The information within the spinal cord and peripheral nervous system propagate along the nerves in the form of Compound Action Potentials (CAP). Although CAP were always considered to be steady signals, with constant amplitude and velocity as determined by the conductive properties of the nerves our preliminary data show that exposure of the sciatic nerve *in vitro* to electrical or magnetic stimulation increases the amplitude of CAP. The stimulation which increased the amplitude of CAP also induced the release of radioactive glutamate analog from preloaded axons. Moreover, our preliminary experiments showed that exogenous glutamate increased the CAP. This increase in CAP was blocked after the addition of the general glutamate receptor blocker kynurenic acid, the specific glutamate receptor antagonist MK801 and CNQX and abolished when these experiments were performed in a calcium-free medium.

Specific Aims

- Does glutamate play a role in axonal plasticity?
- Which type of glutamate receptor mediate axonal plasticity?
- What is the effect of calcium on axonal plasticity?
- What is the source of calcium involved in axonal plasticity?
- Which types of glutamate receptors are expressed?

RESULTS

5) Glutamate-induced increase of CAP recorded from the sciatic nerve in vitro

To further investigate the effects of glutamate on the sciatic nerve, we added glutamate to these preparations and evaluated the effects on the amplitude of the CAPs. Electrical stimulation induced an increase of the amplitude of the CAPs. The segments of sciatic nerve were prepared as described above in the method section following stabilization of the potentials induced by 0.03 Hz stimulation, 100 μM glutamate (dissolved in Ringer's solution) was added to the recording chamber (Figure 7A).

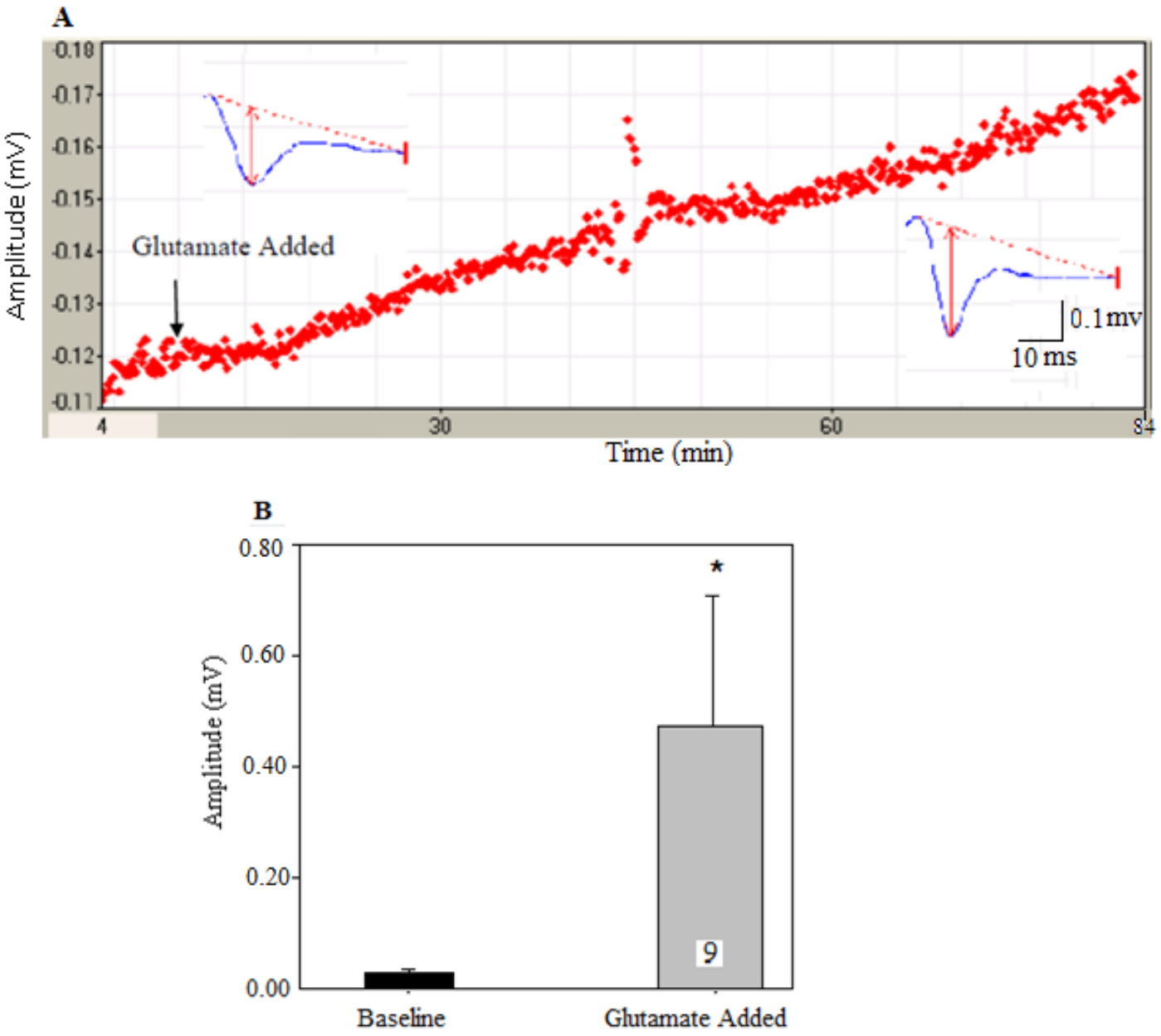


Figure 7 Glutamate-induced amplification of the amplitude of CAP. **A** Addition of glutamate (100 uM) significantly increased the amplitude of CAP. The glutamate was added to the recording chamber following 5 min period of baseline stabilization. **B** Averaged results of 9 experiments testing the influence of exogenous glutamate on CAP. Each bar represents mean ±SEM. * $p < 0.005$ from baseline. CAP was 0.04±0.01 mV, and 0.53±0.41 mV (average± SEM, $p<0.005$, n=9), before and after addition of glutamate, respectively.

The preparation was stimulated during entire experiment without any interruptions. As depicted in Figure 7B glutamate at 100 µM significantly increased CAP amplitude. Mean CAP amplitude before and after addition of glutamate was 0.04 ± 0.01 mV and 0.53 ± 0.41 mV, respectively, ($p<0.005$, $n = 9$).

Moreover 100 µM glutamate reduced the latency as depicted in Figure 8. The mean latency before and after addition of glutamate was 3.919 and 3.747 ms, respectively. ($p<.00003$, $n=10$).

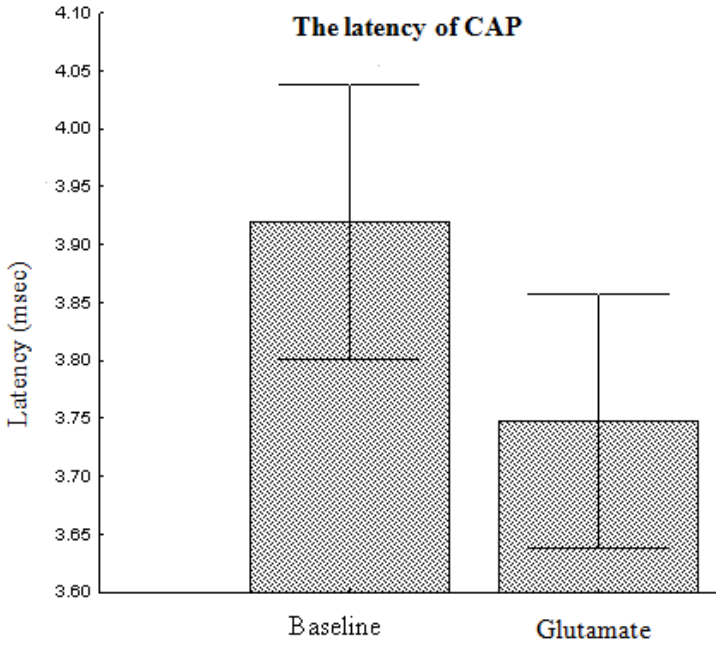


Figure 8 The influence of glutamate on the latency of CAP. Averaged results of 10 experiments illustrating the influence of glutamate on the latency of CAP. Each bar represents mean ±SEM. The mean latency before and after addition of glutamate was 3.919 and 3.747, respectively, $p<0.00007$.

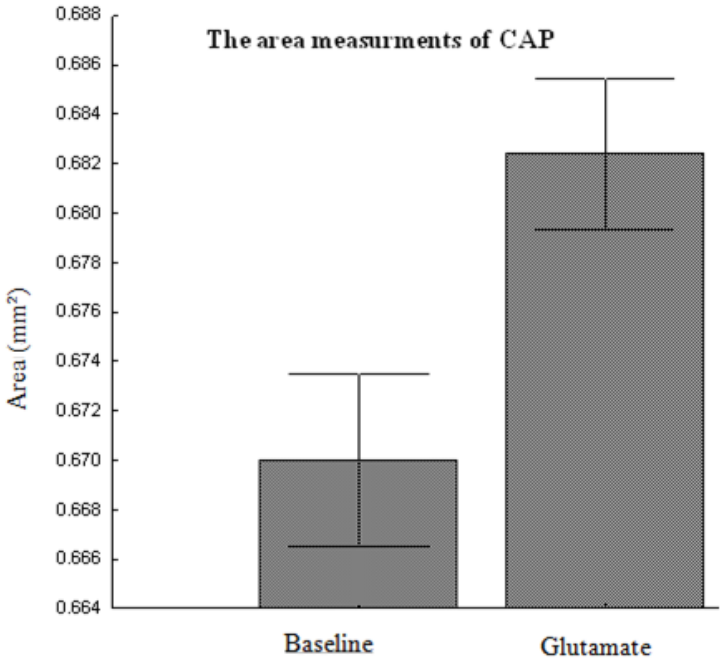


Figure 9 The influence of glutamate on the area of CAP. Averaged results of 12 experiments, displaying the effect of glutamate on the area of CAP. Each bar represents mean ±SEM. Mean area before and after addition of glutamate was 0.670 and 0.682, respectively, $p<0.002$.

The application of glutamate also increased the area measurement of the CAP: mean area before and after addition of glutamate was 0.670 and 0.682 cm^2, respectively ;($p<0.002$, $n = 12$, Figure 9). These data indicate that glutamate, exogenously added or released after electrical stimulation, has an enhancing effect on the CAPs.

6) Kynurenic acid blocked the effect of glutamate.

To determine the mechanisms of glutamate-mediated increase in the amplitude of CAPs we added glutamate in the presence of the general glutamate receptor antagonist kynurenic acid. We followed the same procedure as described in the previous section. Following stabilization of

the potentials, 2 mM kynurenic acid was added to the recording chamber. 5 minutes after the addition of kynurenic acid, 100 µM glutamate was added to the recording chamber (Figure 10A).

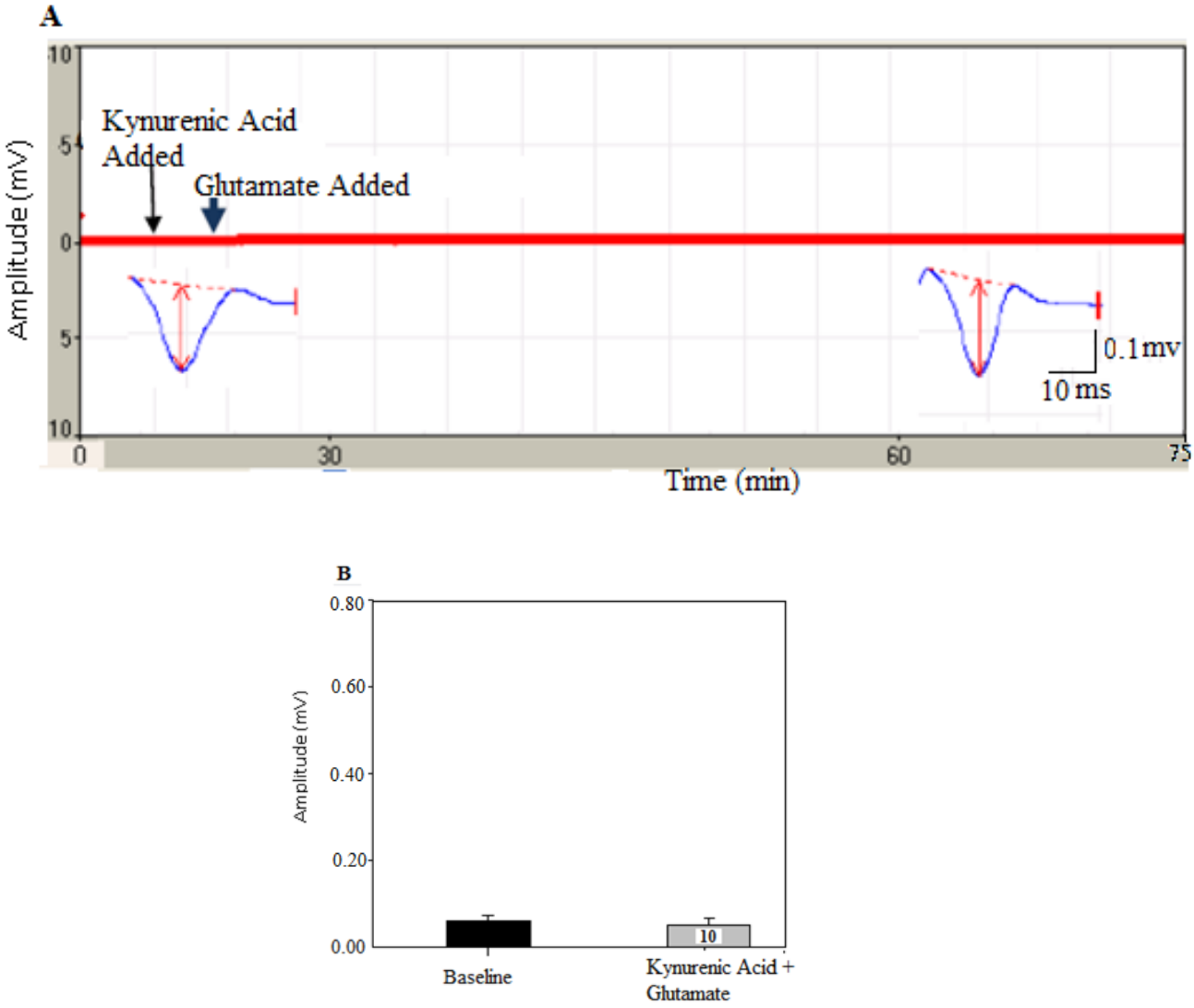


Figure 10 **Kynurenic acid blocked the effect of glutamate. A** Addition of 2 mM kynurenic acid eliminated glutamate-induced increase. Addition of kynurenic acid was followed 5 minutes later by addition of 100 µM glutamate. **B** The average results of 10 experiments evaluating the effect of glutamate on CAP in the presence of kynurenic acid each bar represents mean ± SEM. Mean before 0.07 ±0.02 mV, mean after 0.06 ±0.02 mV (average ± SEM, $p<0.04$, $n=10$).

As depicted in Figure 10B, addition of 2 mM kynurenic acid to the recording chamber blocked the increase of CAP induced by glutamate, [mean before 0.07 ±0.02 mV, mean after 0.06 ±0.02 mV SEM ($p<0.04$, $n=10$) (Figure 10B)]. Kynurenic acid did not affect the amplitude of CAP before the addition of glutamate.

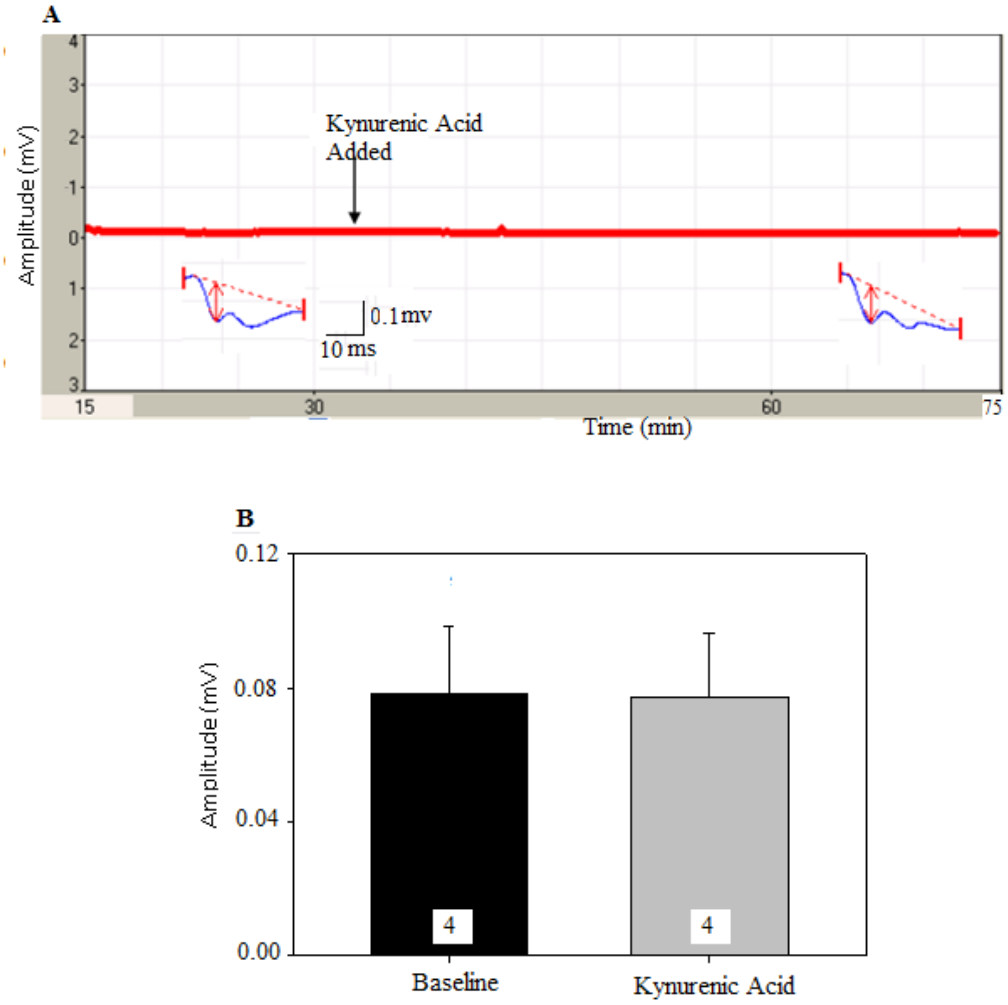


Figure 11 **The influence of Kynurenic acid on CAP**. **A** Addition of 2mM Kynurenic acid had no effect on the amplitude of CAP. The arrow shows the time of addition of kynurenic (20 minutes after starting of the recording). **B** The average of four experiments testing the influence of kynurenic acid on CAP. Means ± SEM. Mean before 0.08 ±0.02 mV, mean after 0.08± 0.02 mV SEM ($p= 0.7$, $n= 4$).

As shown in Figure 11, addition of 2mM kynurenic acid alone to the recording chamber did not change the amplitude of compound action potential. The CAP before 0.08 ±0.02 mV, and after 0.08± 0.02 mV addition of kynurenic acid, was respectively, $p = 0.7$, $n = 4$ .Thus, blocking glutamate receptors does not affect baseline axonal transmission, although activation of these receptors is required for observed increase of CAPs following electrical stimulation or addition of glutamate. Moreover blocking of glutamate uptake did not affect baseline axonal transmission (Figure 12).

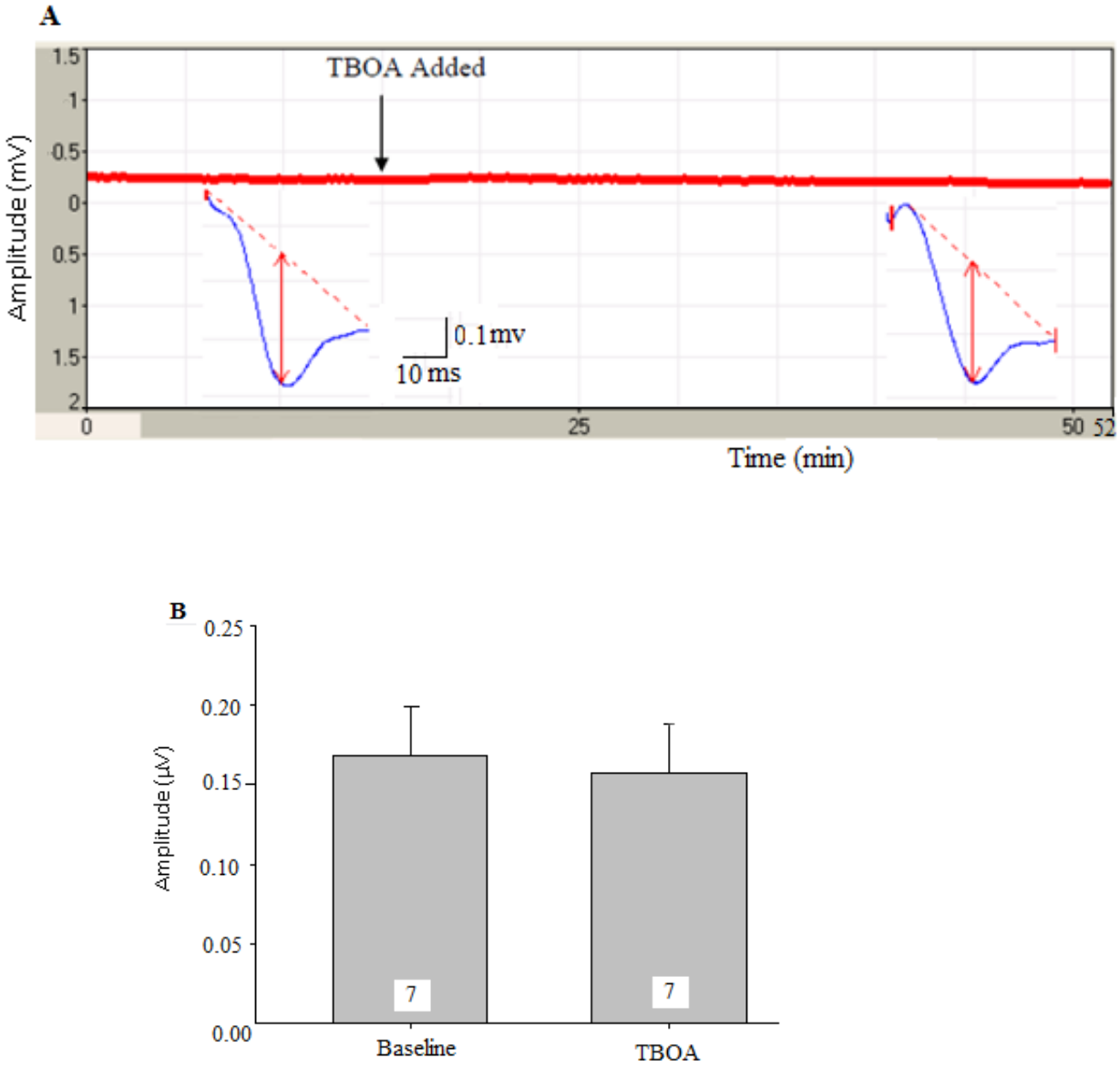


Figure 12 **The influence of TBOA on the effect of glutamate. A** TBOA did not increase the CAP. The arrow shows the time of the addition of TBOA15 minutes after starting of the recording. **B** Averaged results of seven experiments. Each bar represents mean ±SEM .The addition of 30 µM TBOA did not increase the compound action potential, ($p = 0.175$, $n=7$).

As depicted in Figure 12 A shows the time of addition of 30 µM DL-TBOA (a competitive blocker of excitatory amino acid transporters) to the recording chamber, Figure 12 B showed that the addition of 30 µM DL-TBOA did not increase the CAP , Mean CAP amplitude before and after addition of DL-TBOA was 0.168 ± 0.0299 mV and 0.157 ± 0.0305 mV, respectively, ($p = 0.175$, $n = 7$). This experiment confirmed our earlier observation that glutamate did not affect baseline axonal transmission and the baseline axonal conductivity is glutamate independent.

7) The NMDA specific blocker MK801 blocked the effect of glutamate.

Glutamate mediates its action through activation of ionotropic and metabotropic receptors. To further understand the mechanism of glutamate action on CAP, we used selective blockers for various ionotropic receptors. We followed the same procedure as in the previous experiment. Following stabilization of the potentials, 50 µM MK801 was added to the recording chamber followed 10 min later by the addition of MK801 (Figure 13A), 100µM glutamate was added to the recording chamber (Figure 13A).

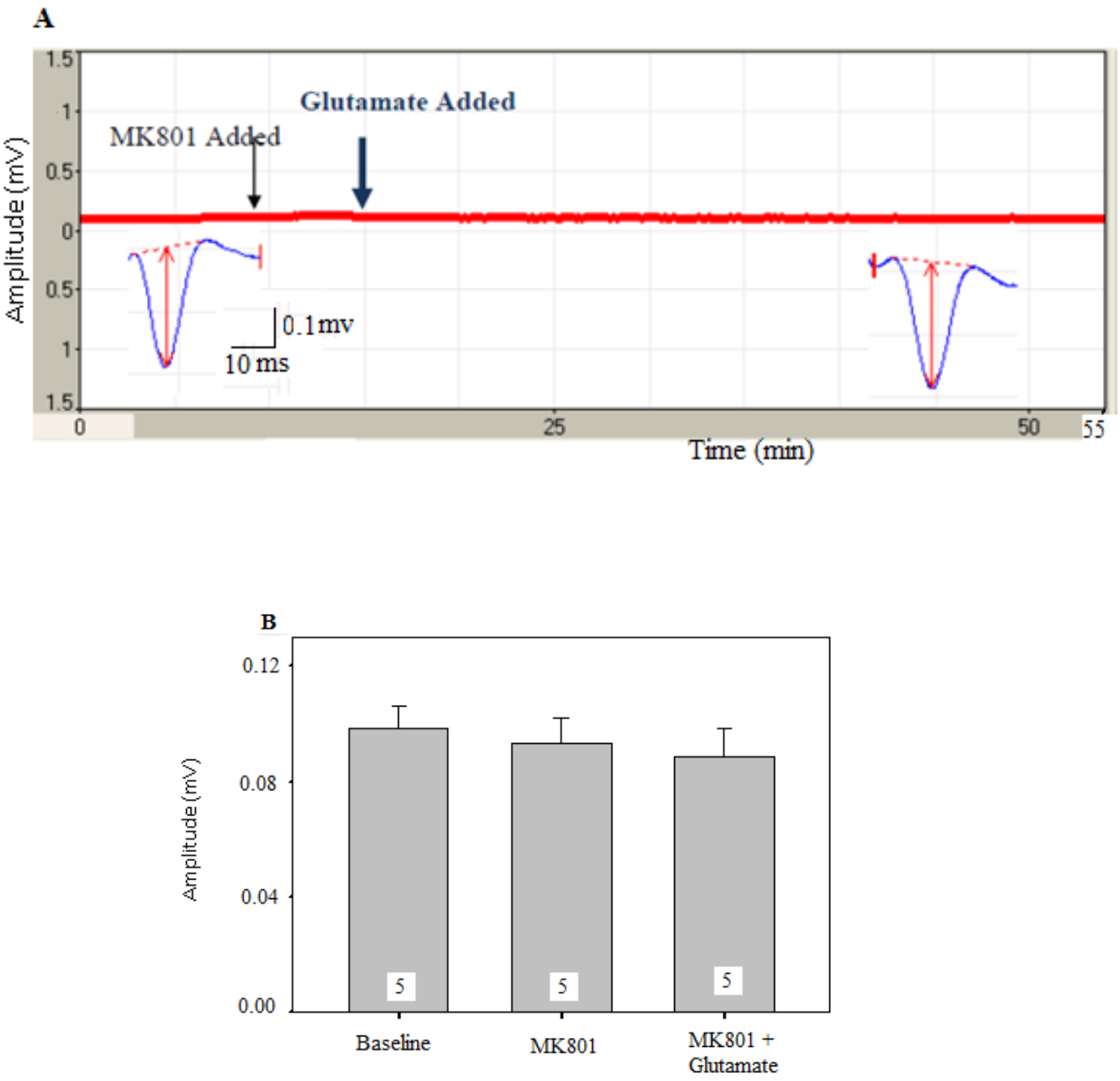


Figure 13 **The influence of MK801 on the glutamate effect on CAP . A** Addition of 50 µM MK801 was followed 5 minutes later by addition of 100 µM glutamate. **B** MK801significantly blocked the glutamate-induced increase of CAP. Averaged results of five experiments each bar represents mean ±SEM (p = 0.515, n = 5).

As depicted in Figure 13B, there is no statistical difference between the three groups {the first group is the average of the baseline, the second group is the average of CAPs after the addition of MK801 and the third group is the average after the addition of glutamate ($p = 0.515$, $n=5$)}. Addition of 50 μM MK801 to the recording chamber completely blocked the increase of CAP induced by glutamate. Therefore, exogenously added glutamate mediated the enhancement of CAP through activation of the NMDA receptors. The finding that MK801 did not affect the baseline amplitude of CAP indicates that glutamate receptor activation is not required for baseline axonal conduction but required for axonal plasticity involving glutamate.

8) The AMPA/kainate specific blocker CNQX abolished the effect of glutamate.

Sciatic nerve strips were prepared as described in the methods section. Following stabilization of the potentials, 250 μM CNQX was added to the recording chamber. Ten minutes after the addition of CNQX (Figure 14A), 100 μM glutamate was added to the recording chamber. As depicted in Figure 14B, there is no statistical difference between the three groups ($p = 0.745$, $n = 7$). Addition of 250 μM CNQX to the recording chamber blocked the increase of CAP induced by glutamate. (Figure 14B).

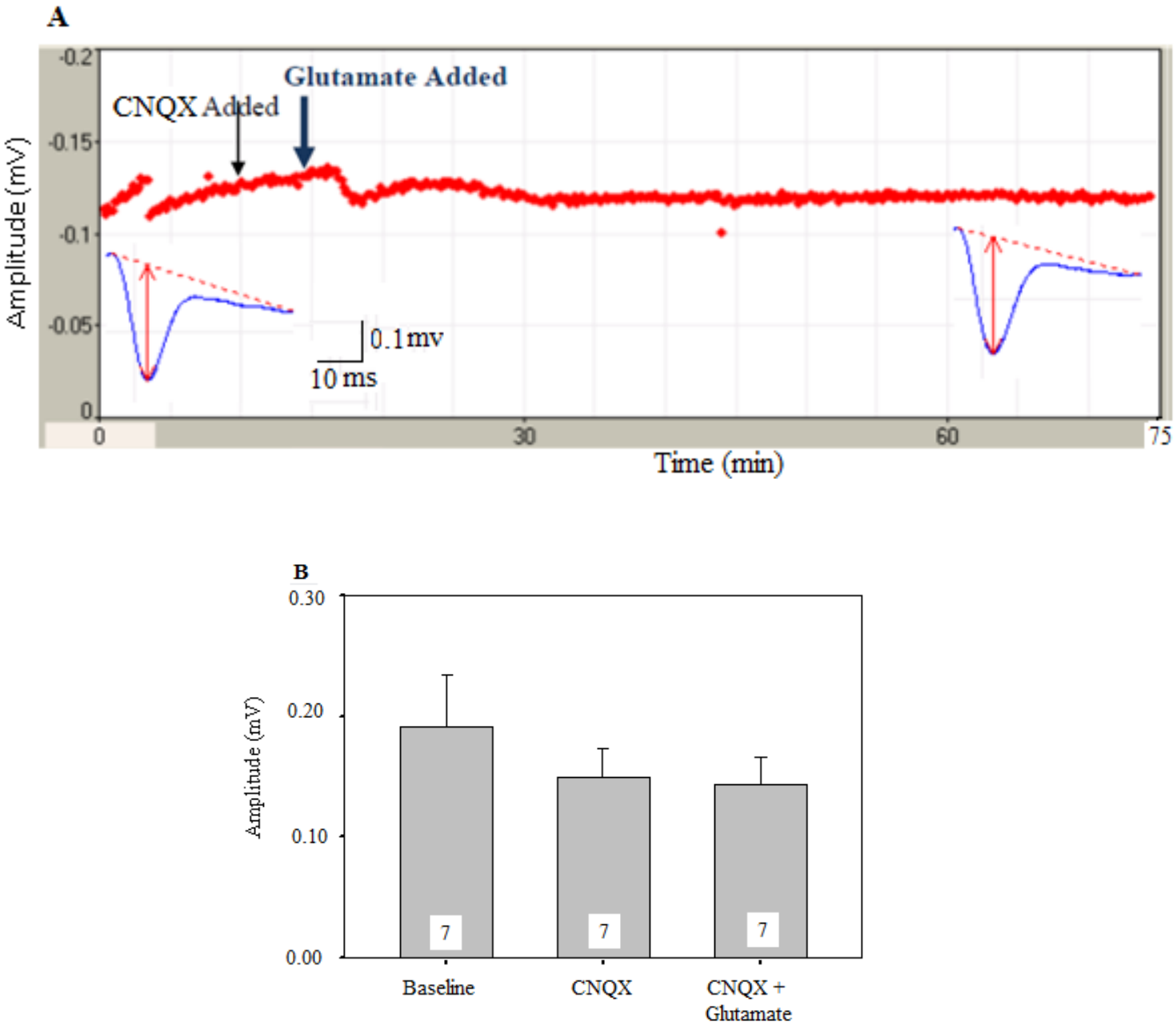
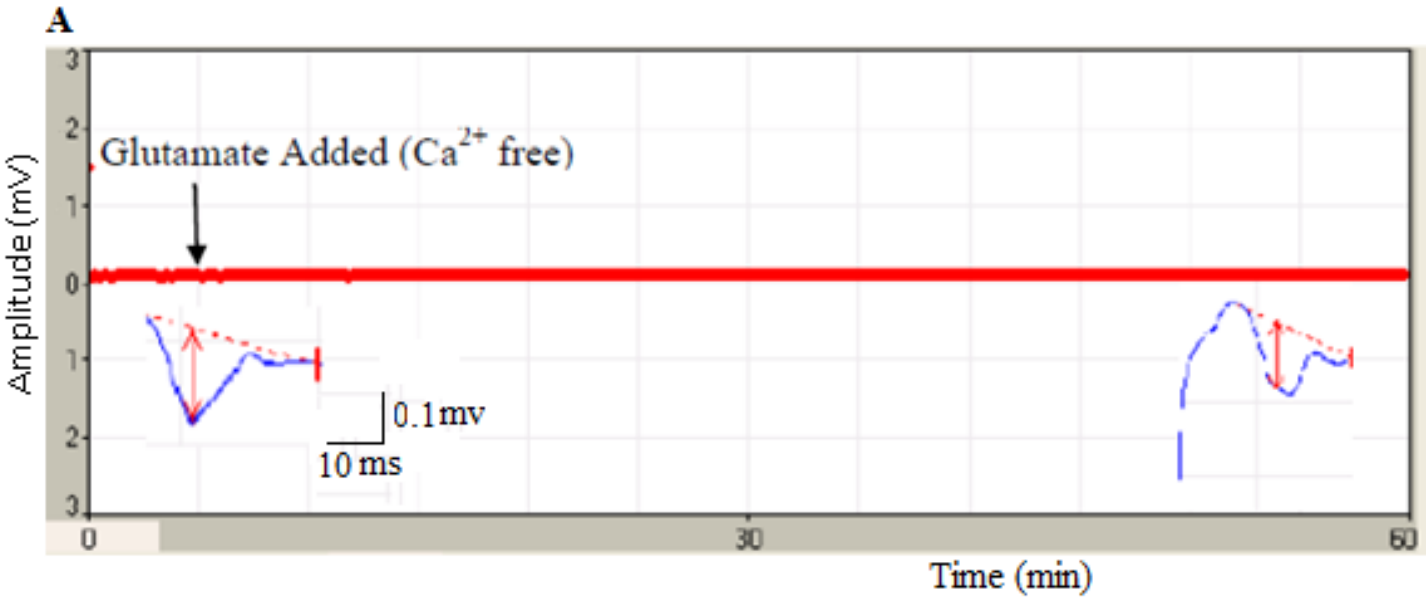


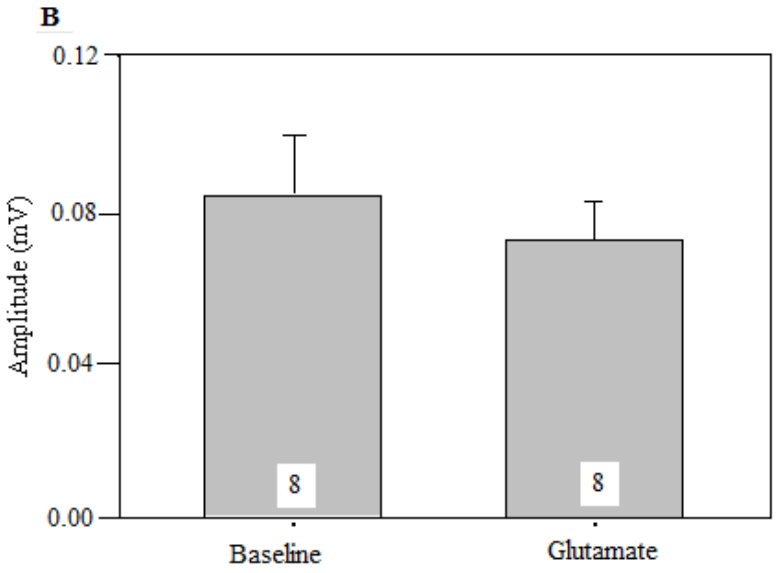
Figure 14 The effect of CNQX on glutamate-induced amplification of CAP . A Addition of 250 µM CNQX was followed 5 minutes later by addition of 100 µM glutamate. Addition of 250 µM CNQX to the recording chamber prevented the glutamate -induced enhancement of CAP. **B** Averaged results of seven experiments. Each bar represents mean ± SEM (p = 0.745, n = 7).

These data also show the necessity of AMPA/kainate receptors in the glutamate-induced increase in CAP. Activation of these channels results in membrane depolarization, removal of the magnesium block from the NMDA receptors and subsequent activation of these channels. Activation of AMPA/kainate class of ionotropic receptors precedes the activation of NMDA receptors and the expression pattern of these channels is complimentary, as they mediate fast and slow depolarization respectively. In glutamatergic synapses, both of these receptors are involved in mediating glutamate actions when they are co-expressed.

9) The presence of Calcium is essential for the increase in CAP.

The previous experiments showed that addition of glutamate to the sciatic nerve leads to an increase in CAP amplitude and this enhancement is mediated through activation of both AMPA/kainate and the NMDA receptors. While both receptors are excitatory AMPA/kainate are permeable to Na^{+} while NMDA receptors are permeable to $Ca^{2+.}$ Additionally, the NMDA receptors require binding of the agonist (NMDA or glutamate) and membrane depolarization that act to repel a Mg^{2+} from the pore of the channel making these channels coincidence detectors. To further understand the contribution of various glutamate receptor channels in mediating axonal plasticity, we used a calcium-free ringer's solution. This will abolish depolarization-induced calcium influx through calcium permeable channels, mainly the NMDA receptors and the voltage sensitive calcium channels. Sciatic nerve segments were prepared and after stabilization of the potentials induced by 0.03 Hz stimulation, 100 µM glutamate was added to the recording chamber (Figure 15A). Instead of using the regular Ringer's solution we used the Ca^{2+}-free Ringer's solution.





<u>Figure 15</u> The effect of glutamate on CAP amplitude in Ca^{2+} -free Ringer's. A the arrow shows the time of addition of glutamate. Glutamate was added 5 minutes after starting of the recording . **B** Averaged results of eight experiments. Each bar represents mean ±SEM. There was no statistical difference between these groups. Mean before 0.083 ± 0.016, mean after 0.072 ± .0122 SEM (p = 0.060, n = 8).

Figure 15B shows that when calcium was omitted from the recording medium, addition of glutamate had no effect on the amplitude of the CAP. The mean values of CAP recorded before and after addition of glutamate were 0.083 ± 0.016, and 0.072 ±.0122 mV, respectively ($p = 0.060$, $n = 8$). Interestingly, baseline CAP amplitude was not affected by the omission of calcium, indicating that baseline axonal conductivity is calcium independent. This is consistent with the very well known mechanism of propagation of the CAPs along axons that involve voltage sensitive sodium channels located at the nodes of Ranvier.

10) Dantrolene blocked the effect of glutamate.

On the basis of the previous experiments we concluded that the presence of extracellular calcium is essential for the increase of CAP after the addition of 100μM glutamate. The release of calcium from the internal stores has many sources including the mitochondria, and the endoplasmic reticulum (ER) which can release calcium through ryanodine receptors (Ouardouz et al; 2003). We thought to determine if the release of calcium from the internal stores plays a role in the increase of CAP after the addition of100 μM glutamate. Dantrolene inhibits release of calcium from intracellular stores via blocking ryanodine receptor (RYR).

We followed the same procedure as in the previous experiment. Following stabilization of the potentials induced by 0.03 Hz stimulation, 10 μM dantrolene was added to the recording chamber (Figure 16A). 10 minutes after the addition of dantrolene, 100 μM glutamate was added to the recording chamber.

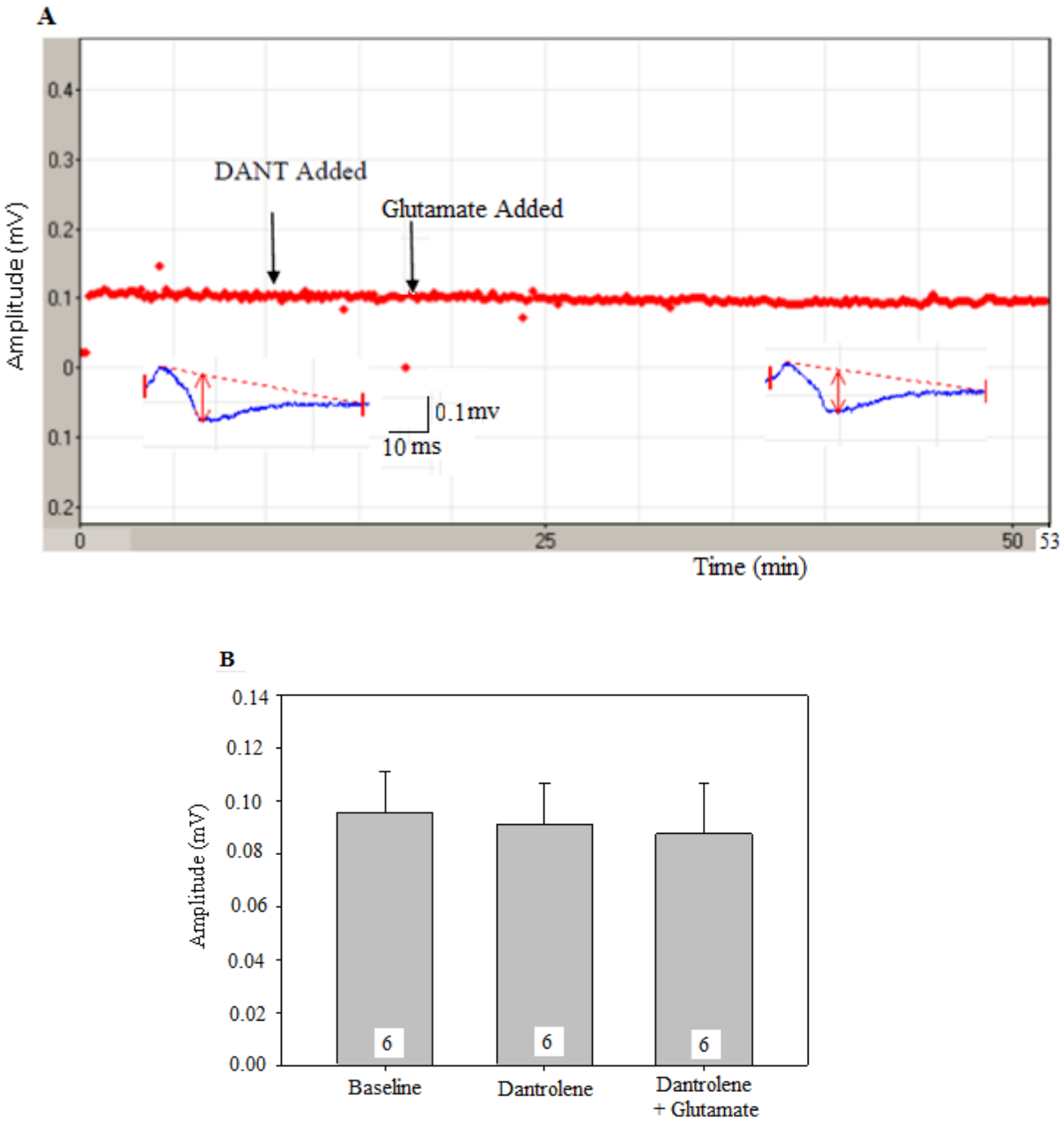


Figure 16 **The influence of dantrolene on the effect of glutamate . A** Addition of 10 µM Dantrolene {inhibits release of calcium from intracellular stores via inhibition of ryanodine receptor (RYR) was followed 10minutes later by addition of 100 µM glutamate. **B.** Averaged results of six experiments testing the influence of dantrolene on glutamate-induced CAP amplification. Addition of 10 µM Dantrolene to the recording chamber prevented the glutamate -induced enhancement of CAP. Each bar represents mean ±SEM ($p = 0.623$, $n=6$).

As depicted in Figure 16B, there is no statistical difference between the three groups ($p = 0.623$, $n=6$). Addition of 10 µM dantrolene to the recording chamber blocked the increase of CAP induced by glutamate. These data indicated that the intracellular calcium release through ryanodine channels is required to mediate the glutamate-induced increase in CAP.

11) Verapamil hydrochloride blocked the effect of glutamate.

Verapamil was used to block depolarization-induced calcium influx, through antagonizing voltage-sensitive calcium channels. Here, we used verapamil with glutamate to block calcium influx through voltage-sensitive calcium channels. Sciatic nerve preparations were carried out as previously described. Following stabilization of the potentials induced by 0.03 Hz stimulation, 10 µM verapamil was added to the recording chamber (Figure 17A). Ten minutes after the addition of verapamil, 100 µM glutamate was added to the recording chamber.

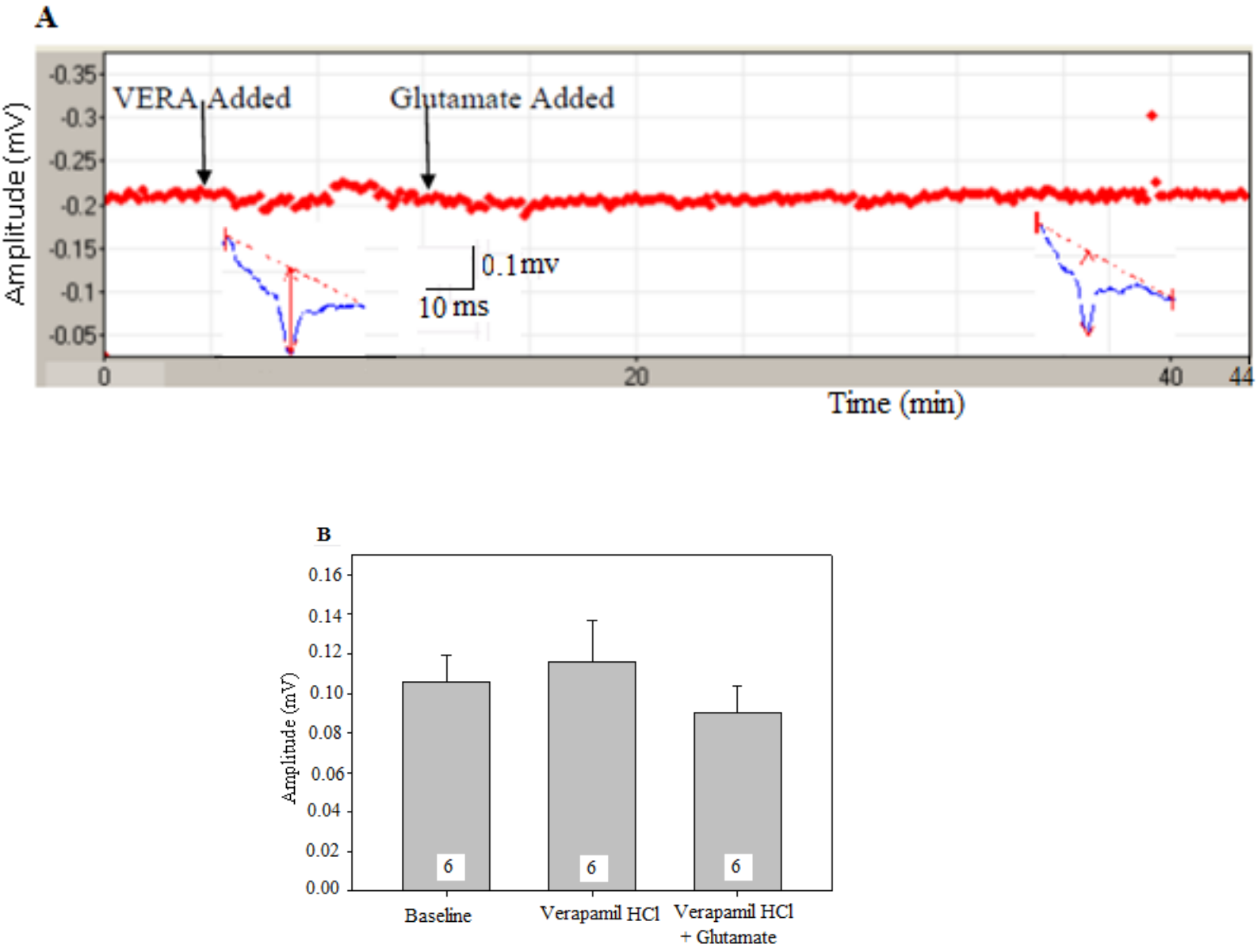


Figure 17 The influence of Verapamil on the glutamate effect . A Addition of 10 µM Verapamil (blocks depolarization-induced calcium influx, through antagonizing voltage-sensitive calcium channels) was followed 5 minutes later by addition of 100 µM glutamate . Averaged results of six experiments testing the influence of Verapamil on glutamate-induced CAP increase. Addition of 10 µM Verapamil to the recording chamber prevented the glutamate -induced enhancement of CAP. Each bar represents mean ±SEM (p = 0.529, n=6).

As depicted in Figure 17B, there was no statistically significant difference between the three groups ($p = 0.529$, $n=6$). Addition of 10 μM verapamil to the recording chamber blocked the increase of CAP induced by glutamate (Figure 17B). These data indicate that extracellular calcium influx through the voltage sensitive calcium channels was required to mediate the glutamate increase in CAP amplitude. This is consistent with the results of previous pharmacological manipulation where we reduced extracellular calcium or blocked the NMDA receptors with MK801. Thus, glutamate mediates the increase in CAP amplitude through activation of the glutamate receptors and depolarization of the membrane which subsequently activate the voltage-sensitive calcium channels leading to influx of extracellular calcium.

12) NMDA and kainate -induced increase of CAP recorded from the sciatic nerve in vitro.

To further understand the mechanisms of glutamate-mediated increases in CAP amplitude, we used the selective glutamate receptor agonists NMDA and kainite. Segments of sciatic nerve were prepared as described in the method section. Following stabilization of the potentials induced by 0.03 Hz stimulation the mixture of 10 μM NMDA and 10 μM kainate was added to the recording chamber.

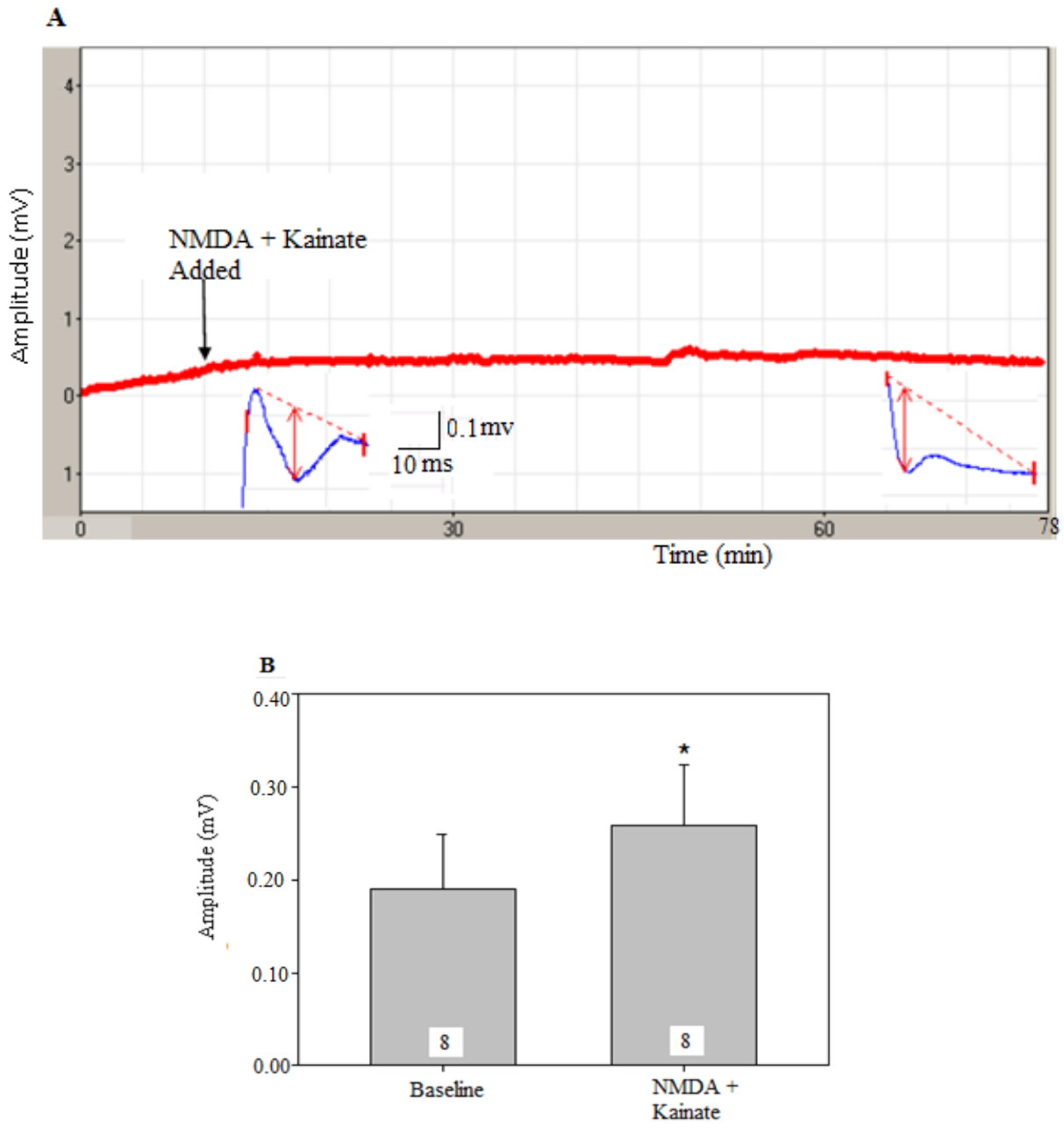


Figure 18 **The amplification of CAP amplitude by mixture of NMDA and Kainate .** **A**10 µM NMDA and 10 µM Kainate added simultaneously in reduced Mg (25%) significantly increased CAP. The arrow shows the time of the addition of 10µM NMDA and 10µM Kainate (10 minutes after initiation of the recording). **B** Averaged results of eight experiments. Each bar represents mean ±SEM. The mean values of CAP amplitude were 0.19±0.059 mV before and 0.258±0.0662mV after the addition of NMDA and Kainate (Average± SEM, $p < 0.005$, n=8).

As depicted in Figure 18B NMDA and kainate added simultaneously together significantly increased CAP amplitude. The mean values of CAP amplitude before and after the addition of NMDA and kainate were 0.19±0.059 mV and 0.258±0.0662 mV, respectively $p<0.005$, $n=8$ (Figure 18B). These data indicate that glutamate mediates its actions on the CAP through activation of the AMPA/kainate and NMDA receptors. Activation of the NMDA receptors requires membrane depolarization and the presence of the agonists at the receptor binding site. These results demonstrate the involvement of both channels in the mediating the glutamate effects.

13) Quantitative measurements of intracellular calcium using calcium imaging

Experiments performed in calcium-free environment showed that the presence of calcium was necessary for glutamate to induce the increase in the amplitude of the compound action potential at the sciatic nerve. We thought to verify this result by performing calcium imaging in the presence of glutamate and NMDA. Individual segments of the sciatic nerve were transferred to a 35 mm dish containing Ringer's solution and 5 μM Fluo3. We lowered the magnesium concentration in the Ringer's bath in some experiments to reduce the block of the NMDA receptors and allow calcium entry through these channels. Images were captured every 30 sec and addition of the drugs was done when baseline intensities were stable. To determine baseline $[Ca^{2+}]_i$, we took three frames before the addition of the drugs and the fluorescence intensity of these images was used as baseline fluorescent intensity. The changes in the fluorescent intensity in subsequent images after addition of the glutamate were expressed as per cent of control images recorded before glutamate addition.

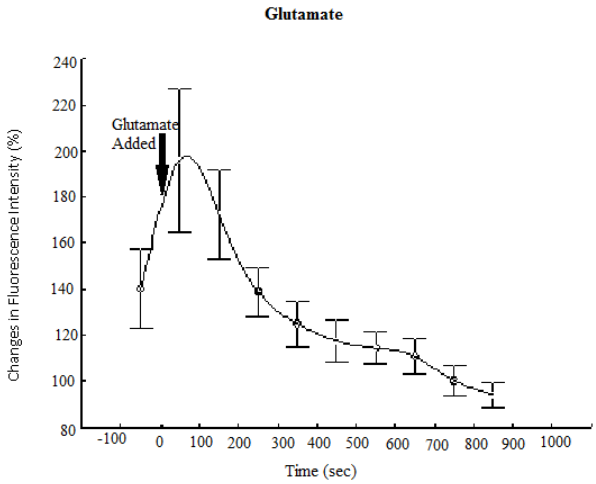


Figure 19 The influence of exogenous glutamate on fluorescence in the sciatic nerve. The increase in the fluorescence reflects the enhancement in free calcium concentration. The level of free calcium increased at the first 30 second after adding the glutamate but starts to decline after that. ($p < 0.05$, $n = 3$).

As depicted in Figure 19 there was significant increase in fluorescence intensity between the base line and the first two image (60 second) ($p<0.05$, $n = 3$) which revealed that the level of free calcium increased during the first 60 second after adding the glutamate. The rapid increase in calcium after addition of glutamate indicated that calcium entry was mediated through ionotropic receptors, probably the NMDA-regulated channels. Interestingly, the glutamate induced calcium increase in intracellular calcium concentration was not long lasting, indicating the presence of buffering mechanism that quickly reduces free axoplasmic calcium.

Next we added NMDA to selectively activate the NMDA receptors as these channels are permeable to calcium as well as Na^+.

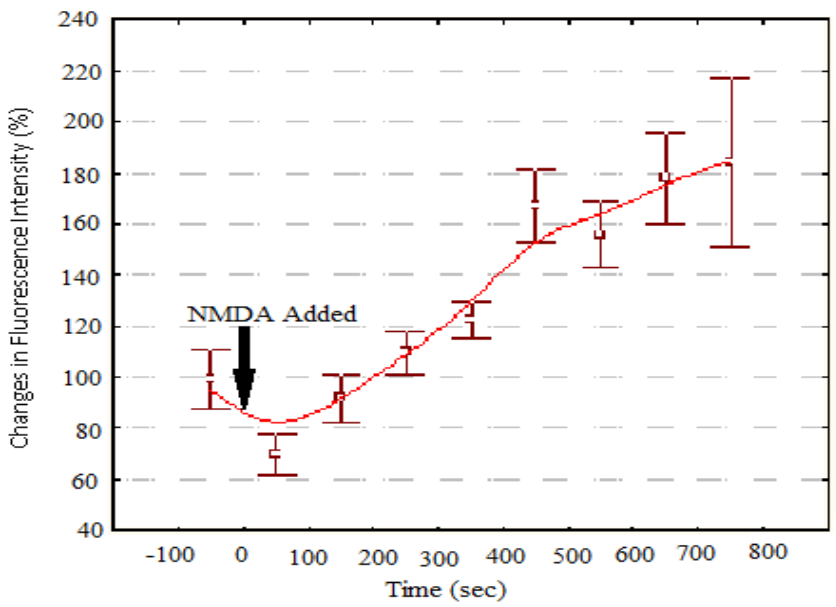


Figure 20 The influence of NMDA on intracellular calcium concentration measured in the sciatic nerve. There was significant increase in fluorescence intensity after the addition of NMDA ($p < 0.05$, $n = 3$). The rise continued till the end of the experiment.

As depicted in Figure 20 there was significant increase in fluorescence intensity after the addition of NMDA ($p<0.05$, $n = 3$). Unlike the glutamate-mediated increase in axoplasmic calcium, the NMDA induced a sustained increase in free calcium. This discrepancy between glutamate and NMDA could be due to the various channels activated by the addition of glutamate. While NMDA selectively activates only the NMDA receptors, glutamate activates both ionotropic and metabotropic receptors. The inhibitory nature of some of these metabotropic receptors may explain the transient nature in cellular response induced by glutamate.

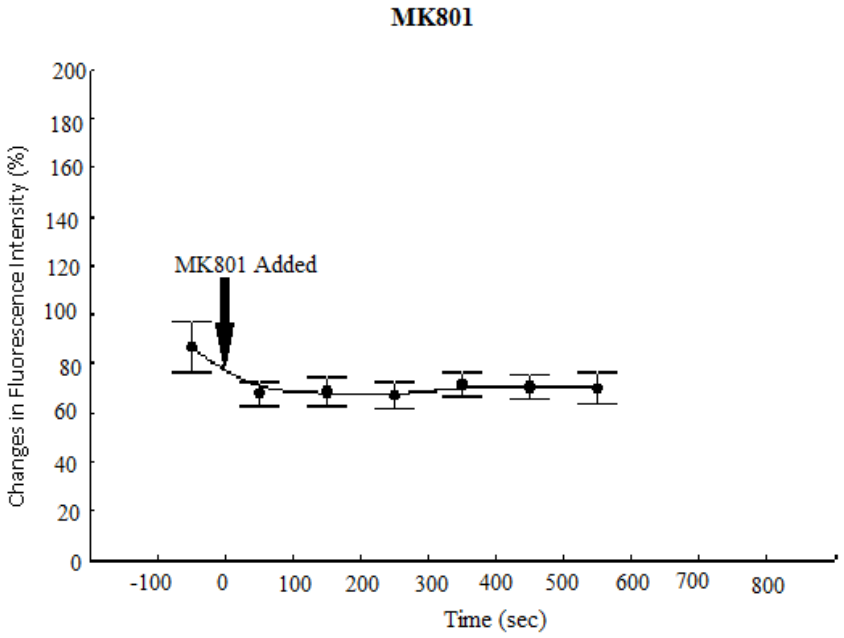


Figure 21 The influence of MK801 on intracellular calcium concentration . There was no change in the fluorescence intensity after the addition of MK801. (n = 3).

As expected, MK801 completely blocked the NMDA—induced increase in axoplasmic free calcium. Figure 22 shows a representative graph depicting changes in fluorescent intensities in the presence of NMDA and MK801. There was no increase in the fluorescence intensity after

the addition of MK801 (Figure 21) or after the addition of NMDA and MK801 to the recording chamber (Figure 22).

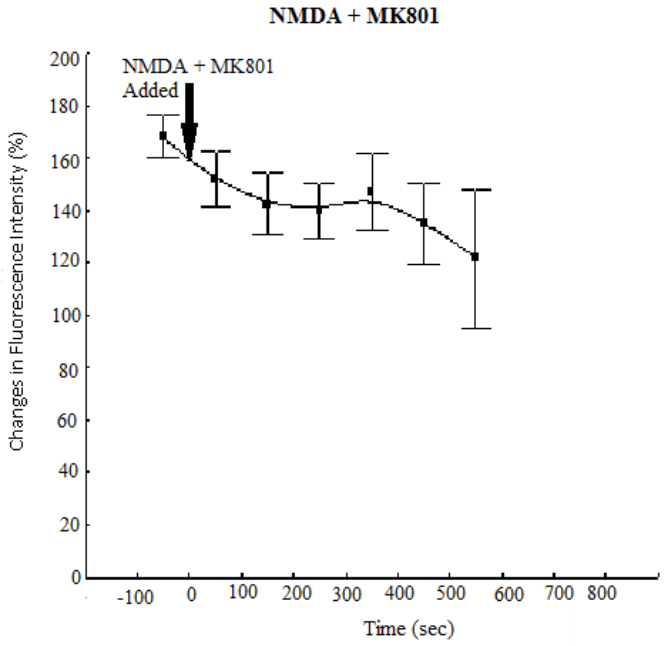


Figure 22 The influence of NMDA and MK801 mixture added simultaneously on the intracellular calcium concentration observed in the sciatic nerve . There was decrease in the fluorescence intensity after the addition of NMDA and MK801. (n = 3).

Thus, these data provide further evidence for the involvement of calcium in mediating the enhancement in the amplitude of CAP in the presence of glutamate.

14) Immunohistochemical characterization of channel expression on the axolemma.

While the data presented thus far use pharmacological agent to provide functional evidence for the presence of various channels on the membrane of axons within the sciatic nerve, we sought to use immunofluorescence to probe for the presence of channels and receptors, which will help us explain our pharmacological and electrophysiological observations. we found that axon within the sciatic nerve express voltage-sensitive calcium channels (Figure 23). This is

consistent with the effects of verapamil on CAP. Additionally, We found, as expected, that axons within the sciatic nerve express high level of voltage-sensitive sodium channels (Figure 24).

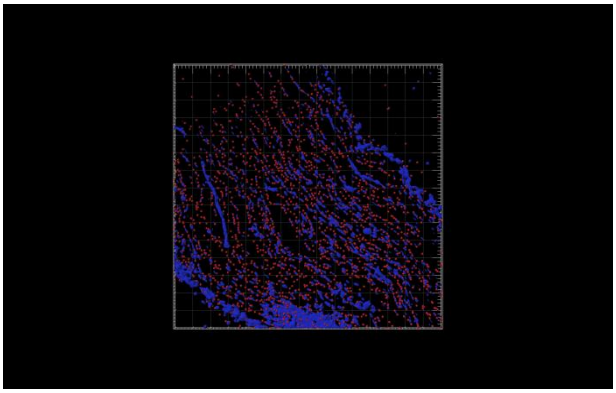


Figure 23 The sciatic nerve expresses voltage-sensitive calcium channels . Sections from sciatic nerve of 1-3 month old CD-1 mice immunostained with antibody against calcium channels (red).

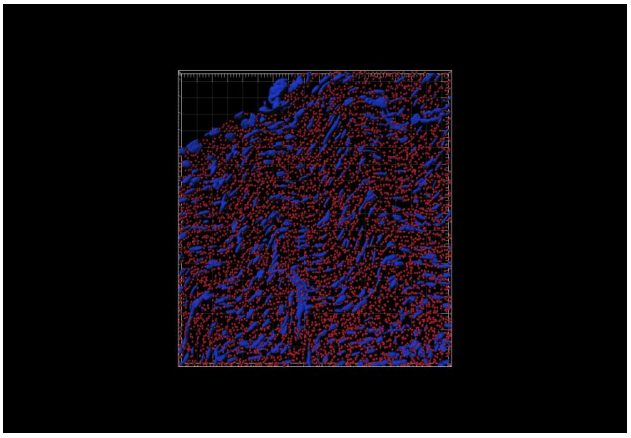
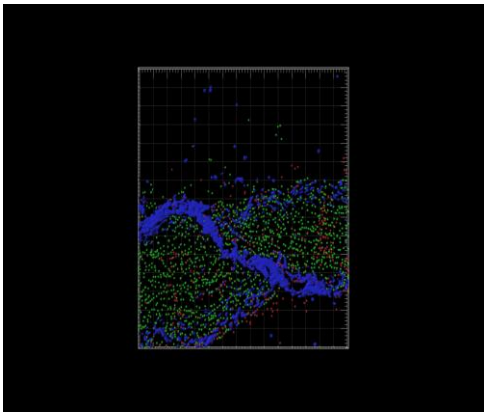
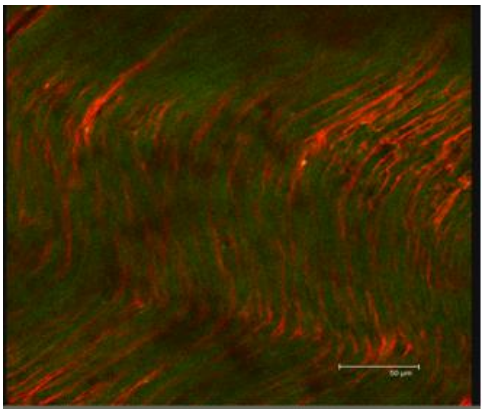


Figure 24 The sciatic nerve expresses high level of voltage-sensitive sodium channels . Sections from sciatic nerve of 1-3 month old CD-1 mice immunostained with antibody against sodium channell (red).



<u>Figure 25</u> The sciatic nerve expresses NMDA receptors M Glur.
Sections from sciatic nerve of 1-3 month old CD-1 mice immunostained with antibody against NMDA receptors (green). (red) mGlur.



<u>Figure 26</u> The sciatic nerve expresses AMPA/kainate and EAAT (excitatory amino acid transporter). Sections from sciatic nerve of 1-3 month old CD-1 mice immunostained with antibody against AMPA-Kainate receptors (green) and EAAT (red).

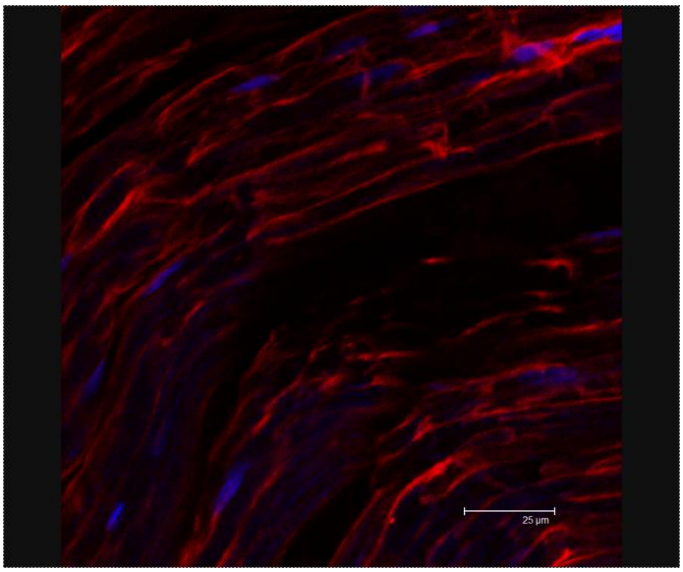


Figure 27 The sciatic nerve expresses NMDA receptors. Sections from sciatic nerve of 1-3 month old CD-1 mice immunostained with antibody against NMDA receptors (red).

We also found out that the axolemma expresses the major receptors for glutamate, both ionotropic and metabotropic, as evidenced by the presence of immunoreactive particles for AMPA/kainate (Figure 26); NMDA (Figure 25-27) and mGlur2 (Figure 25). Finally, we looked for the presence of glutamate transporter and found ample immunoreactivity for EAAT (Figure 26).

Therefore, we suggest that the presence of various channels and receptors on the membrane of axons within the sciatic nerve is responsible for mediating the effects of glutamate and electrical stimulation on the amplitude of CAP. These data also bring up a new model for axonal plasticity which was initially thought to occur only at the synaptic level. The presence of functional receptors and channels on the axolemma with the sciatic nerve strongly suggest a novel peripheral mechanism for information handling within these conductive structures in which CAP are either enhanced or depressed based on the frequency of these events.

Since sciatic nerves are mixed nerves, it is plausible to think that the sensory component of these fibers may exhibit such plasticity as well. This is consistent with observations obtained from the dorsal spinal cord which contains sensory neurons and is very rich in ascending sensory fibers. Such a novel mechanism of axonal plasticity within sensory fibers may serve to modulate ascending information as part axonal modulation of sensory information that can increase or decrease the threshold for perception based on modality.

Discussion

The goal of this proposal was to examine the changes in axonal physiology in response to electrical stimulation and to pharmacological manipulation. We found that high frequency stimulation, or addition of exogenous glutamate increases the amplitude of compound action potentials (CAPs) in sciatic nerve preparations. The glutamate-induced increase in the CAPs was calcium-dependent and could be abolished by glutamate receptors antagonists. These results were further extended and supported by immunohistochemical experiments showing that axolemma contains glutamate receptors (NMDA, AMPA/kainate and mGluR2), the excitatory amino acid transporter responsible for glutamate uptake (excitatory amino acid transporter-EAAT), and voltage-gated sodium and calcium channels. Thus, the axolemma of peripheral nerves expresses several proteins important for neuronal communication and modulation of the membrane excitability. Apparently, these proteins embedded into the axonal membrane, can under the influence of electrical stimulation or exogenous glutamate change membrane permeability and ionic conductance leading to an increase in the amplitude of the compound action potentials observed in our experiments. Our results demonstrate the existence of axonal plasticity expressed as a change in the amplitude of the action potential following periods of changed activity accompanied by release of neurotransmitters. Therefore we suggest a mechanism of the process whereby electrical stimulation leads to increased axonal activity and subsequent release of glutamate that through activation of the glutamate receptors results in changes in the amplitude of CAPs. We term this phenomenon as axonal plasticity, which would represent one of the forms of neuronal plasticity. Neuronal plasticity is defined as a treatment-induced change in the neuronal response in spite of unchanged strength of the test stimulation. This observation was long described as a property of central synapses and thought to be the basis

of learning (Malenka, 1994). Axonal plasticity would not be expressed at the synapses, would constitute exclusive property of the axon and could contribute together with synaptic plasticity to modification of the efficiency of neuronal connections. This type of plasticity would be fundamentally different from the synaptic plasticity expressed in CNS in the form of long-term potentiation-LTP (Bliss and Collingridge, 1993), long-term depression-LTD (Dudek et al; 1992), and spike timing dependent plasticity (STDP) (Markram et al., 1997) which has been intensively investigated for last several decades.

The model which we have selected for our research was represented by the segments of sciatic nerve maintained in vitro in oxygenated Ringer's solution. There are several properties of this model which make it especially suitable for our research. The open ends of the sciatic nerve seal within 2 hours following dissection. Therefore the integrity of cytoplasm and the axonal membrane is being preserved (Eddleman et al; 2000). Moreover, as demonstrated by our experiments and published data (Ahmed et al; 2009) the segments of sciatic nerve in vitro generate evoked action potentials, generated by both, myelinated and non-myelinated fibers. This indicates that basic properties expressed by axon in vivo like membrane potential, functional ion channels and enzymatic activity are preserved in the segments of the axon in vitro. Interestingly, those properties were preserved even in segments of sciatic nerve kept overnight in refrigerator (unpublished observation). It further supports the view that the sciatic nerve in vitro represents a reliable model, which can provide valuable information about properties of the axon in its natural in vivo environment.

Since segments of sciatic nerve do not contain synapse, any electrical activity and its modifications in response to an external stimulation had to have non-synaptic origin. The sciatic nerve is a mixed nerve containing both sensory and motor fibers and glia. Therefore, electrical

and chemical stimulations of the nerve preparation recruit both sets of fibers, and generated action potentials would propagate along the axon through orthodromic and antidromic propagation. Although we have not tested separately the effects of electrical stimulation and exogenous glutamate application on sensory and motor fibers in a mixed sciatic nerve preparation, we showed that in our experimental paradigm both types of these axons were activated in the same way and responded in a very similar fashion (Weragoda et al; 2004).We are convinced that CAPs recorded in our experiments represent the combined effects of sensory and motor neurons activation. However, since these two types of neurons respond to different types of modalities, the physiological consequences of activation of each of them will be different.

We assume that high frequency electrical stimulation induces the release of glutamate from stimulated axons. Subsequent increase in the extracellular glutamate concentration would be responsible for observed increase in CAP. The focus of our research was on the verification of this assumption.

Increase in the amplitude of CAP may be a result of:

1) An increase in the number of activated axons (recruitment), and/ or 2) increase in the amplitudes of individual potentials generated by single axons, 3) changes in extracellular resistance. The mechanisms responsible for each of these changes are very different. In the case of recruitment one can suggest paracrine action of glutamate which released from group of axons would enhance the CAP of their neighbors. The increase in the action potential generated by individual axon could be due to a change in the threshold of this individual axon. The alterations in the axonal environment due to axons' swelling and subsequent changes in the extracellular volume can also significantly contribute to modifications of CAP amplitude. It was suggested by Hamilton and collaborator (2010), that astrocytes swelling can induce the releases of glutamate

through volume regulated anion channel. They showed that ATP activates P2X receptors (purinoceptor7) which are permeable to ATP and glutamate which can be released in a non-exocytotic route. One cannot exclude activity-induced axonal alterations which could lead to non-exocytotic release of glutamate through P2X channels. This would constitute additional source of glutamate during exercise which we will explain in more details later.

Although action potentials in peripheral nerves have been always considered as steady signals, resistant to any stimulation-dependent modifications, some recently published results (Carp et al., 2000; Meeks and Mennerick 2004; Debanne 2004), including the data from our laboratory (Ahmed and Wieraszko, 2009a) indicate that the properties of the action potential may be modified by specific axonal activity. Moreover, it has been shown that sciatic nerve releases glutamate in response to repeated stimulation in vitro (Wieraszko and Ahmed 2009a).

We hypothesize that the change in the amplitude of CAPs may be mediated by glutamate that is released non-synaptically from stimulated axons and/or glial cells due to increase in the intracellular free Ca^{2+} concentration. Our data indicate that both the release of Ca^{2+} from the internal stores and the influx of extracellular Ca^{2+} can contribute to glutamate-induced elevation in intracellular calcium concentration. Our main hypothesis is based on the fundamental observation showing that application of exogenous glutamate on axonal preparations in vitro induces persistent (1 hour.) increase in the amplitude of CAP. This result is in agreement with the report by Matsumoto and collaborators (2005) demonstrating a significant 10% increase in CAP recorded from sensory axons of cuneate and gracile fasciculi (CGF) of the thoracic spinal cord of young rats following addition of 100 µM glutamate. The addition of 100 µM NMDA evoked even higher, 20% increase. The effects of glutamate and NMDA were almost eliminated by D (-)-2-amino-5-phosphonopentanoic acid (NMDA receptor antagonist) in very young

animals (post natal day 0-6, and 0-14), although the effect of glutamate in spinal cords of older animals (post natal day 7-14) was not attenuated by blocking of NMDA receptors. The results of Matsumoto and collaborators (Matsumoto et al., 2005) are in accord with our data showing the importance and ability of glutamate to change the axonal excitability. Although our and their data were obtained on different models, similarities of the results indicate that basic axonal properties are alike across different species.

Since the effect of glutamate was blocked in our experiments by glutamatergic receptors general antagonist, kynurenic acid we concluded that glutamate-induced increase in the amplitude of CAP is very likely to be mediated through glutamatergic receptors. The application of selective antagonists of these receptors revealed that both NMDA and AMPA/kainate receptors are involved in observed effects. Thus, the glutamate-induced increase in CAPs was a result of combined interaction of various glutamate receptors upon their activation.

As it is generally accepted (Malenka, 1994) NMDA receptors are coincidence detectors and require the concomitant agonist binding to the receptor and postsynaptic membrane depolarization. The latter is critical for magnesium removal from the pore of the channel. In this paradigm, activation of the AMPA/kainate receptors may serve the function of membrane depolarization and unblocking of the NMDA receptors. Indeed, in the presence of CNQX, an AMPA/kainate receptor blocker glutamate failed to induce an increase in CAPs amplitude suggesting that the activation of the AMPA/kainate receptors and membrane depolarization precedes NMDA receptors activation. This is consistent and in analogy with the role that NMDA receptors play in the synaptic plasticity in the CNS (Malenka, 1994).

Due to neuronal plasticity the efficacy of synapses is constantly affected by synaptic activity. Furthermore these changes can be either incremental or decremental rendering the

synapses more or less responsive, respectively. Many presynaptic and postsynaptic mechanisms have been shown to underlie these changes in synaptic efficacy and have been termed long term potentiation LTP (Bliss and Collingridge, 1993) or long-term depression LTD (Dudek et al., 1992) to signify increases or decreases in synaptic potency, respectively. Although we named process observed in our axonal preparation as axonal plasticity, this phenomenon is fundamentally different from just mentioned expressions of synaptic plasticity in CNS. There are no synapses in the axonal segments tested in our experiments and molecular mechanisms underlying observed changes have to be different than that involved in synaptic plasticity.

Similar to the observation in central circuits where LTP have been reported (Bliss and Collingridge, 1993) (Dan and Poo, 2006) in our peripheral sciatic nerve preparations we found that the amplitude of the response to electrical stimulation was significantly increased in the presence of glutamate.

Although it may be counter intuitive at first and neuronal plasticity in general is considered as a property of central neuronal circuits where it is thought to be the neuronal substrate for learning, here we report axonal plasticity at the level of conductive fibers in the PNS. This increase in the amplitude of CAPs in response to high frequency stimulation or to the addition of exogenous glutamate could be of physiological significance. One may envision a physiological condition where frequent or repetitive movement such as in the case of walking might create condition that mimics the high frequency stimulation in our experiment. Subsequent increases in the amplitude of CAPs could make the execution of the motor task more efficient. The frequent generation of action potentials traveling along the sciatic nerve would lead to the release of glutamate that through activation of axonal glutamate receptors and ensuing increase in the intracellular calcium would lead to increases in the amplitude of CAPs followed by an

increase in acetylcholine release at the neuromuscular junction. This in turn would lead to an increase in the amplitude of the end plate potentials which might change the muscle performance. Moreover, if the activity of the axon would be changed for a long time, it could even alter the type of the muscle fiber itself.

Skeletal muscle is not only the most abundant tissue in the human body, but also one of the most adaptable. Persistent training with weights can double or triple a muscle's size, whereas disuse, as paralysis or in space travel, can shrink it by 20 percent in two weeks. Muscle is actually a bundle of cells, also known as fibers, kept together by collagen tissue. A single fiber of skeletal muscle consists of a membrane, many scattered nuclei that contain the genes and lie just under the membrane along the length of the fiber, and thousands of inner strands called myofibrils located within the sarcoplasm. Filling the inside of a muscle fiber, the myofibrils are the same length as the fiber and are the part that causes the cell to contract forcefully in response to nerve impulses. Motor nerve cell, or neuron, extends from the spinal cord to a group of fibers, making up a motor unit. In leg muscles, a motor neuron controls, or "innervates," several hundred to over a 1,000 muscle fibers. However, when extreme precision is needed, for example, to control a finger, an eyeball or the larynx, one motor neuron controls only one or at most a few muscle fibers.

The actual contraction of a myofibril is accomplished by its tiny component units, which are called sarcomeres. Within each sarcomere are two filamentary proteins, known as myosin and actin, whose interaction causes the contraction. During contraction a sarcomere shortens as the actin filaments at each end of a central myosin filament slide toward the myosin's center. One component of the myosin molecule, the so-called heavy chain, determines the functional characteristics of the muscle fiber. The heavy chain exists in three different types designated

together with the fibers which contain them as I, 2a and 2 x. Type I fibers are also known as slow fibers while type 2a and 2x are referred to as fast fibers. The difference in the contraction speeds of the fibers is a result of differences in the way the fibers break down adenosine triphosphate (ATP) in the myosin heavy chain region to derive the energy needed for contraction. Slow fibers rely more on relatively efficient aerobic metabolism, whereas the fast fibers depend more on anaerobic metabolism. Thus, slow fibers are important for endurance activities and sports such as long-distance running, cycling and swimming, whereas fast fibers are important for the power pursuits such as weight lifting and sprinting.

Buller and collaborator (1960) showed that certain properties of a muscle were dependent on the source of its innervation. This interrelationship was demonstrated by denervating a fast and a slow muscles and then surgically reuniting the proximal nerve segments to the foreign distal nerve stump. They found out that the contraction time of the slow soleus muscle had become more like that of the fast muscle and vice versa. Moreover, as suggested by Roy and colleagues (1985) the neural influence on muscle properties is shared with other control systems. This finding is of primary importance for our results. As mentioned above, the persistent change in the amplitude of CAP could change the properties of muscle unit by changing the type of individual muscle fibers. It could also explain the mechanism of changes in the type of muscles following specific physical exercise performed by marathon runners versus sprinters.

The sciatic nerve is a mixed nerve containing both sensory and motor fibers. Electrical stimulation of the nerve preparation recruits both sets of fibers through orthodromic and antidromic conduction. Although we have not tested separately the effects of electrical stimulation and exogenous glutamate application on sensory and motor fibers in a mixed sciatic nerve preparation, we hypothesize that in our experimental paradigm both types of axons were

activated in the same way and responded in a very similar fashion. The results of the experiments reported by Matsumoto and collaborators (2005) strongly support our hypothesis. They performed hemi dissection of dorsal spinal cord tracts that contain axons of ascending, sensory nerves, and showed significant 10% increase in CAP recorded from sensory axons of cuneate and gracile fasciculi (CGF) of the thoracic spinal cord of young rats following addition of 100 µM glutamate. The addition of 100 µM NMDA evoked an even higher (20%) increase in CAP. Thus, sensory fibers alone can exhibit plasticity as measured by an increase in the amplitude of CAPs with constant strength of the stimulus. The sciatic nerve potentiation of the response of sensory fibers will lead to a heightening of sensory perception by either lowering of the axonal threshold or the recruitment of more axons. Sensory neurons from a given dermatome are grouped into fascicule. In this paradigm of axonal plasticity, over- activation of sensory fibers by a given modality could lead to non- synaptic release of glutamate by those fibers, making them more responsive to their specific modality. Additionally, glutamate through paracrine action would partially activate neighboring fibers. This in turn will lead to sensitization of all sensory fibers emanating from the dermatome where the sensory fibers were over activated. For example a slight burn of the skin, although activating directly nociceptors and their respective fibers only would, by paracrine action of axonally-released glutamate render the dermatome very sensitive to touch, vibration or pressure sensations. Another possible explanation for the observed increase in the amplitude of CAPs in response to glutamate additions would be increased recruitment of fibers. When glutamate is added to the recording bath, subsequent stimulation resulted in a significant increase in the amplitude of CAPs. Thus to reiterate our earlier assumption, the frequent activation of the motor nerve can change the muscle fiber from slow to fast or recruit more slow than fast fibers based on the exercise program. In parallel, potentiation of CAP in the

sensory neurons would reduce the threshold of perception making the pathway more responsive to stimulation. Thus, we are convinced that CAPs recorded in our experiments represents the combined effects of sensory and motor neurons activation. Since the overwhelming majority of the nerves in human body are mixed, intense stimulation of the peripheral nerve via either motor or sensory input would most likely affect not only the nerves carrying information of stimulated modality, but the adjacent nerves of other modalities as. This assumption, if true would have far-reaching consequences implying mutual interactions and reciprocal influence between different types of axons within peripheral nerves.

All cellular components of white matter, including glial cells and axons are endowed with Ca^{2+} -permeable receptors, pumps and channels in the plasma membrane, as well as those in organelles. Intracellular Ca^{2+}overload resulting from deregulated activity of the channels, such as those opened by glutamate and ATP, is deleterious (Matute et al., 2010) . White matter axons and glial cells control intracellular Ca^{2+} concentrations through a complex interplay between Ca^{2+}flux across the plasma membrane and Ca^{2+}release from and sequestration into internal stores. Two of the major players in Ca^{2+}signaling that are relevant to white matter pathophysiology are glutamate and ATP, which are released from axons and glial cells (Matute et al., 2010). This notion about importance of Ca^{2+} in neuronal physiology is in agreement with our results showing that glutamate had no effects on the amplitude of the CAP in the absence of calcium (Figure 15).

As anticipated, baseline CAP amplitude was not affected by the omission of Ca^{2+} , indicating that baseline axonal conductivity is calcium independent. This is consistent with the very well known mechanism of propagation of CAPs along axons (Stys, 2005), which involves voltage sensitive sodium channels located at the nodes of Ranvier. Our quantitative

measurements of intracellular Ca^{2+} using Ca^{2+} imaging confirmed that an increase in the intracellular calcium concentration is essential for the increase of CAP after the addition of 100 µM glutamate. As depicted in Figure 19 there was significant increase in fluorescence intensity between the base line and the first image (30 second) which revealed that the level of free calcium increased during the first 60 second after adding the glutamate. The rapid increase in calcium after addition of glutamate indicates that calcium entry is mediated through ionotropic receptors most likely linked to NMDA channels. Interestingly, the glutamate induced increase in intracellular calcium was not long lasting, indicating the presence of buffering mechanism that quickly reduces free axoplasmic calcium. As depicted in Figure 15, extracellular calcium is essential to increase the CAP. The glutamate-induced increase in CAP was not observed in the calcium free Ringer's solution, and was prevented by blocking the L type voltage gated Ca^{2+}. These results confirmed that the entrance of extracellular Ca^{2+}through the L type Ca^{2+} channels and the presence of extra cellular calcium is essential for increase the CAP (Figure 17).

As depicted in Figure 20 selective activation of NMDA receptor-linked channels by addition of NMDA itself induced significant increase in the fluorescence (in reduced magnesium Ringer's solution) indicating an elevation of intraaxonal free calcium concentration. Unlike the glutamate- mediated transient increase in axoplasmic calcium lasting 60 second only (Figure 19), the NMDA induced a sustained increase in free calcium for 900 second (Figure 20). This discrepancy between glutamate and NMDA could be due to the various channels activated by the addition of glutamate. While NMDA selectively activates only the NMDA receptors, glutamate activates both ionotropic and metabotropic receptors (Ouardouz et al., 2009). Since some of the later are inhibitory, simultaneous activation of different types of glutamate receptors can yield

attenuation of free calcium release after the first 60 second instead of enhancement for the entire 900 second observed after NMDA addition.

The segments of the axons were kept in our experiments in oxygenated Ringer's. However, one could argue that although the addition of 100 μM glutamate increased the CAP of the sciatic nerve, there were some negative effects caused by inadequate environmental conditions in the incubation chamber which could affect our data. It has been reported (Goldberg et al., 2007), that transient removal of oxygen and glucose from the unmyelinated primary murine cortical culture compartment which contains the axon resulted in irreversible loss of growing of the axonal length and labeled neurofilament. While this injury was not prevented by addition of ionotropic glutamate receptor antagonist it was blocked by antagonists of voltage-gated sodium channels, and removal of extracellular calcium. Likewise, neither blocking of NMDA receptors, nor activation of these receptors with kainate or NMDA had an effect on the extent of the injury induced in these cultures by oxygen and glucose deprivation. Thus, these results indicate that in spite of known excitotoxic effect of glutamate (Domercq et al., 2005), intense activation of ionotropic axonal glutamate receptors is not sufficient to cause injury in isolated, unmyelinated cortical axon. The ability of the segments of axons in vitro to generate evoked action potentials indicates that the most important and sophisticated property of these axons is preserved. Indeed, loss of this ability is considered as a good indicator of axonal damage (Stys et al., 1990). This reinforces our previous conclusions and indicates that exogenous glutamate used in our experiments was not detrimental but beneficial for improving axonal ability to conduct action potentials (see Figure 7) , and can indeed mimic the in vivo condition.

One can speculate that this increase in CAP represents plasticity in the peripheral nervous system which occurs in response to specific stimulus. This increase in CAP is required when we

learn new skills like walking, fast repetitive movements or therapeutic exercises which require activity of both motor and sensory nerves. The neuromuscular junction is a synapse between a motor neuron and a muscle. As an action potential reaches the end of a motor neuron, voltage gated calcium channels open allowing calcium to enter the neuron. Calcium facilitates vesicle binding and neurotransmitter release from the motor neuron into the synaptic cleft. In vertebrates, motor neurons releases acetylcholine (ACh), a small molecule neurotransmitter, which diffuses through the synapse and binds its receptor on the muscle end plate, opening ligand gated sodium channels, depolarizing the muscle fiber and causing a muscle contraction. So the observed increase in CAP in our experiment can be beneficial increasing muscle strength by the enhancement of neuronal conduction of the action potential. Besides increasing neuromuscular activity by physical exercise, one could envision an increase in CAP following a local application of exogenous glutamate in the vicinity of the nerve in vivo.

As reported recently by Ouardouz and collaborators (Ouardouz et al., 2009) the release of calcium from the intracellular stores is essential to increase the compound action potential. In agreement with these results, blocking of Ryanodine channels which release calcium from the axoplasmic reticulum prevented glutamate-induced enhancement of CAP (Figure 16). Indeed, our calcium imaging experiments showed an increase in free calcium concentration following addition of glutamate. These results confirmed that the elevation of intracellular calcium concentration is essential to the increase of CAP. Apparently, both influx of calcium from extracellular environment and from intracellular stores cooperate with each other and are essential for observed effects. Although it could seem to be a far reaching analogy, the form of synaptic plasticity, expressed as LTD in the cerebellum also requires elevation of intracellular

calcium concentration which must be simultaneously supplied from intracellular and extracellular environment.

NMDA and kainate (10 μM each) increased CAP only when added simultaneously to the segments of axon maintained in magnesium reduced environment (Figure 18). Similarly, glutamate and NMDA increased free cytoplasmic calcium level only when added together in reduced magnesium environment. However, we did not observe the same increase of free cytoplasmic calcium when we added either NMDA and MK801 or MK 801 by itself, or glutamate or NMDA were added in a regular Ringer's solution containing physiological magnesium concentration.

As mentioned before, one of the basic properties of our model is lack of synapses. Therefore we attributed all observed effects of glutamate to purely axonal mechanisms. However, axon does contain the structures, called nanocomplexes, which are reminiscent of synapses. Their function can contribute to the effects observed in our experiments. Nanocomplexes are clusters of signaling molecules (glutamate receptors, Ca^{2+} channels, nNOS ({neuronal nitric oxide synthase}, RyRs,) which are organized along the internodal axolemma under the myelin sheath in discrete "axonal nanocomplexes". Their cellular arrangement looks similar to the structures observed at the post-synaptic membrane of conventional interneuronal synapses. Although over-activation of nanocomplexes during disease can lead to a lethal release of Ca^{2+} from intra axonal stores (Ouardouz et al., 2003), the discreet interaction of exogenous glutamate with these nanocomplexes, and subsequent limited elevation of intracellular calcium concentration could explain the increase in CAP in our experiments. One can conclude that the source of calcium and duration of its glutamate-induced increase is critical for observed effects. By analogy, both forms of synaptic plasticity, LTP and LTD are induced by the enhancement of

intracellular calcium concentration evoked by electrical stimulation. However, depending on the pattern of this stimulation and subsequent elevation of calcium concentration, two different groups of enzymes, either kinase or phosphatases would be activated resulting in an increase, or decrease in synaptic efficiency, respectively.

Myelin, produced and maintained by Schwann cells in the peripheral nervous system insulates 99% of the surface of axons, providing a low capacitance, high resistance protective covering which supports efficient saltatory impulse propagation (Stirling et al., 2010). Myelinated axons are crucial for the reliable transmission of signals and conduct more rapidly than nonmyelinated axons of the same size within the nervous system. The nodes of Ranvier are highly enriched in sodium channels which conduct inward depolarizing currents; whereas internodal/juxtaparanodal potassium channels maintained electrical stability and polarization. The regulated activity of these channels is essential to support normal axonal physiology. The synaptic model's features important for memory storage include localized depolarization, the restriction of the alterations to intensely depolarize synaptic regions and finally the dependence of alterations on protein synthesis (Martin et al., 2000; Kandel, 2001). Those features were observed by Weragoda and colleagues (2004) in injured axons. According to these authors axonal injury can trigger a focal depolarization followed by a local decrease in action potential threshold and subsequent long term hyperexcitability (LTH) which expresses memory-like properties. The axons of Aplysia californica's tail sensory neurons and tail motor neurons displayed similar localized LTH after peripheral depolarization produced by 2 minutes exposure to elevated extracellular [k^+] (Weragoda et al., 2004).The induction and the expression of LTH were prevented by local application of the protein synthesis inhibitors anisomycin or rapamycin. They also reported restriction of alterations to intensely depolarized regions, and dependence of

the alterations on local rapamycin sensitive protein synthesis. These results suggest that axons may offer an opportunity to define fundamental mechanisms of neuronal plasticity that were important in the evolution of memory (Weragoda et al., 2004).

One can suggest that exogenous glutamate will facilitate entrance of calcium through the NMDA receptors and will initiate calcium-induced calcium release from the endoplasmic reticulum. Calmodulin, a calcium binding protein that interacts with sodium channel increases the channel current amplitude in a calcium dependent manner. This will change the inactivation phase of voltage gated sodium channels increasing CAP (Waxman 2006).

It is well documented, that influx of sodium ions into axoplasm and subsequent membrane depolarization is an essential step in initiation of the action potential. However, besides its role in generation of the action potential, sodium ions exert significant influence on intracellular calcium concentration. As demonstrated by Nikolaeva and collaborators (2005), the release of calcium from intra axonal stores is highly dependent on Na^+ influx and reversal of the mitochondrial Na^+/ Ca^{2+} exchanger (Nikolaeva et al., 2005).

The intracellular calcium stores include the mitochondria and the endoplasmic reticulum (ER). While endoplasmic reticula can release calcium through activation of ryanodine (Ouardouz et al., 2003) and IP3 receptors (Tian et al., 2008), mitochondria can release the Ca^{2+} via their Na^+/ Ca^{2+} exchanger (Nikolaeva et al., 2005). When a combinational treatment was applied to block IP3 receptors and mitochondrial Na^+/ Ca^{2+} exchanger, attenuation of intra-axonal calcium release was much more effective than any of these treatments applied alone. Although this observation suggests that both of these pathways contribute to the intra -axonal calcium concentration and work independently (Nikolaeva et al., 2005), mitochondrial calcium is

not likely to be involved in CAP increase observed in our experiments. As depicted in Figure 13, 10 μM dantrolene, which blocks IP3 receptor-linked intracellular calcium channels, completely eliminated the increase of compound action potential. Although the contribution of other internal stores of calcium were not tested in our experiments our results confirmed the Ryanodine channels as the only valued source of intra axonal calcium required for glutamate-induced CAP increase. Therefore we would like to suggest that influx of calcium via NMDA receptors-linked channels initiates calcium release from internal, ryanodine receptor-regulated channels by the mechanism described for cardiac muscle (Stirling et al., 2010). This suggestion is in line with the data reported recently (Ouardouz et al., 2006) showing that interfering with L-type Ca^{2+} channel sensor can inhibit coupling to axonal ryanodine receptor and prevent the release of calcium from endoplasmic reticulum. Our results depicted in Figure 17 supports this observation showing voltage gated Ca^{2+} channels as essential to increase the CAP, as their blockade abolishes the increase in CAP. Moreover, the blocking of L- type Ca^{2+} channels strongly inhibited the intra axonal Ca^{2+} increase mediated by the GluR6 Kainate receptors (Ouardouz et al., 2009).

Interestingly, it has been demonstrated (Ouardouz et al., 2006) that activation of mGluR1 receptors activated PLC, leading to IP3 production and subsequent Ca^{2+} release from internal stores. These data combined with the results showing inhibition of CAP increase by kynurenic acid (Figure 10B) which among others blocked also the mGlur receptors gives a strong support for interpretation of our results.

The research by Ouardouz and colleagues (2009), suggest that GluR5 kainate receptor mediated intra axonal Ca^{2+} responses show some dependence on extracellular Ca^{2+}. The

majority of GluR5 dependent axonal calcium increase occurs via G proteins and the activation of PLC, as well as the release of calcium from intraaxonal IP3 dependent Ca^{2+} stores.

By contrast, Glur6 receptor mediated intra axonal calcium responses involve depolarization dependent Ca^{2+} release from ryanodine sensitive calcium stores, involving L- type voltage gated calcium channels that function as voltage sensors rather than calcium permeable pores (Ouardouz et al.,2009). These results support our results and introduce possible mechanism of the increased CAP. Namely, exogenous glutamate could activate the mGluR5 receptors that would activate PLC leading to IP3 production, release of calcium from internal axonal stores .It also could activate Glur6 receptor that can further increase the calcium release through Ryanodine sensitive calcium receptors and subsequent change in activation of sodium channels (Waxman 2006).

Mechanism of increase in the amplitude of CAP.

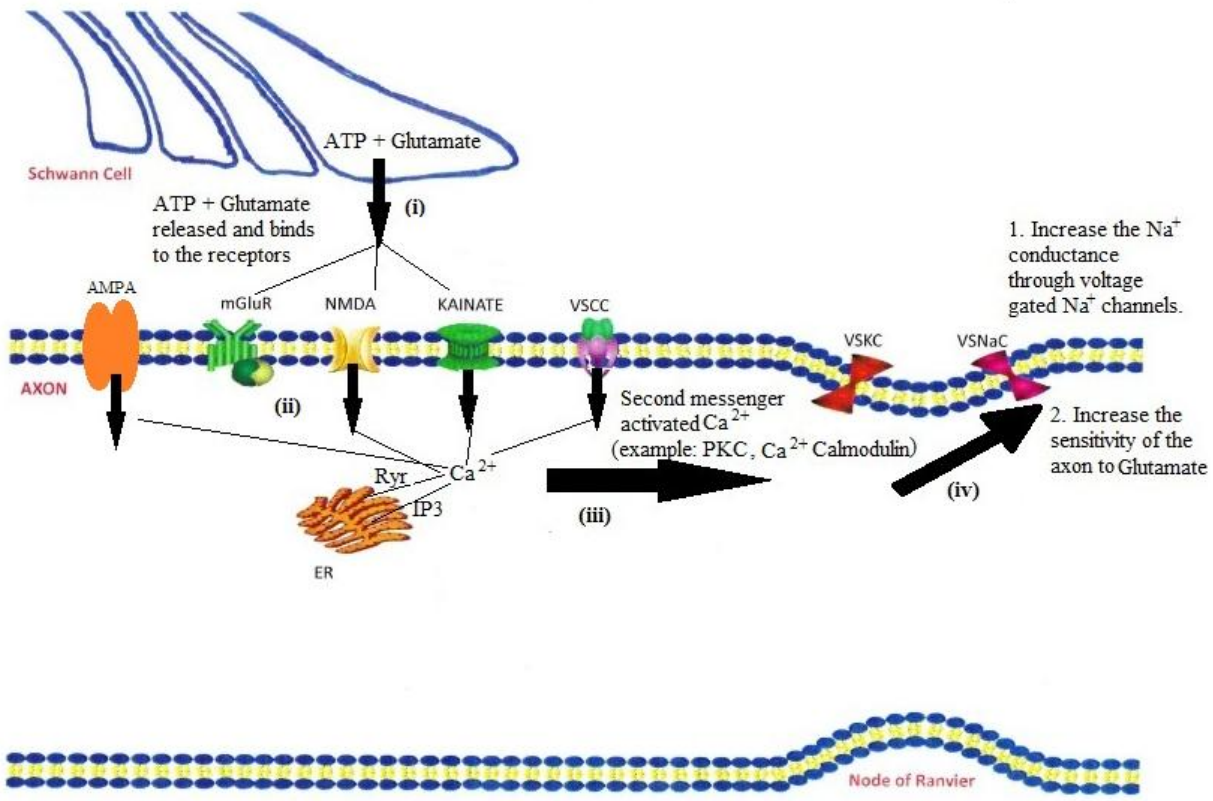
As a summary of our discussion one can depict the sequence of events which would lead to the axonal plasticity expressed as changes in the action potential characteristics.

1. The electrical stimulation employed in our experiments is a standard procedure widely used to stimulate nervous system in vitro. Considering synaptic and non synaptic responses of different neuronal preparations recorded in vitro it is generally accepted that this type of stimulation induces the activity of neuronal preparations which very closely resembles activity of these structures in vivo. Just to reiterate, electrical stimulation of the segments of the sciatic nerve in vitro with different frequencies used in our experiments is supposed to mimic normal and enhanced nerve activity in vivo. While in intact animal action potential generated at the axon hillock would propagate along the entire length of the axon, we have stimulated electrically short axonal segments in vitro. However, since stimulated

axons generated action potentials which looked like those generated in vivo, we consider the method of stimulation and our model as a good representation of in vivo conditions.

2. Repetitive generation of the action potentials in the peripheral nerve would result in non synaptic, glial and/or axonal release of glutamate. This release could occur at activated nanocomplexes, which would mimic the synaptic action, releasing glutamate in exocytotic or via non-exocytotic manner. Repetitive axonal activity could trigger release of ATP from Schwann cells. This extracellular ATP could in turn activate the P2X channels which open large conductance pore permeable to ATP and glutamate (Hamilton and Atwell 2010).
3. In order to analyze the mechanism of stimulation-induced amplification of the action potential we have applied exogenous glutamate. This procedure would eliminate the first step in vitro (electrical stimulation and generation of the action potentials) and in vivo (generation of the action potentials) necessary to observe axonal plasticity and would allow us to analyze the molecular background of this plasticity. It is not known exactly what would be extracellular concentration of glutamate during repetitive axonal stimulation, although it is known that it can reach higher micromolar levels during synaptic activity in the CNS. We have selected 100 μM concentration of exogenous glutamate considering the fact that this exogenous glutamate would have to penetrate through epineurium, perineurium and endoneurium to reach the axonal membrane. One also has to keep in mind that exogenous, extracellular glutamate would be quickly cleared by the axonal uptake system. Moreover, the notion about excitotoxicity of glutamate in CNS (Matute et al., 2007a) may not apply to PNS. Goldberg and colleagues (2007) tested the role of glutamate receptors in the vulnerability of isolated axons by blocking NMDA and non-NMDA receptors during oxygen and glucose deprivation. Although transient removal of oxygen

and glucose had significant and detrimental effects on axonal physiology, the injury was not prevented by addition of ionotropic glutamate receptor blockers and could not be reproduced by glutamate receptor agonist. Similarly the GluR agonist kainate and NMDA applied for two hours did not cause injury within twenty four hours after their application (Goldberg et al., 2007). This suggests that intense activation of ionotropic glutamate receptors is not sufficient to cause injury in isolated cortical axons. These results support our hypothesis that the glutamate applied in our experiments at a concentration of 100 μM may be beneficial.



<u>Figure 28</u> Proposed mechanism

The repetitive activities (firing of the action potentials) will release Glutamate with ATP from Schwann cell (and possibly from the axon itself). Glutamate will bind to ionotropic and metabotropic Glutamatergic receptors. The opening of glutamate-gated channels (AMPA, NMDA, and kainate) will allow influx of Ca^{2+}. The depolarization of the membrane will activate the voltage gated calcium channels and NMDA linked channels as well.

1-The influx of calcium will induced calcium release via IP3 and ryanodine channels into axoplasm and subsequent calcium-induced calcium release from AR (axoplasmic reticulum) via cardiac type calcium induced calcium release. Other sources of intracellular calcium do not play a role and are not shown.

2- The increase of intra-axonal Ca^{2+} will activate second messenger (e.g. Protein Kinase C (PKC) or Ca^{2+} Calmodulin) this in turn will increase sensitivity of axon to glutamate through trafficking of glutamatergic receptors and also will increase the conductance of Na^{+} through voltage gated Na^{+} channels. Consequently, this will increase CAP.

4. The regulation of extracellular glutamate concentration and the consequences of the activation of glutamatergic receptors are complex. Glutamate activates ionotropic and metabotropic receptors which are expressed on axons and in glial cells in grey and white matter (Matute et. al., 2007a). In addition to glial cells, axons are also endowed with GluRs and GluTs. Thus axons in the dorsal column of the spinal cord are depolarized by the activation of AMPA receptors (Ouardouz et al., 2006). Nevertheless the axonal AMPA are formed by the GluR4 subunit, while the kainate receptors are composed of at least GluR5 and GluR6 subunits which are located at the internodes (Ouardouz et al., 2009 a,b).The oligodendrocyte lineage expresses functional AMPA and Kainate receptors throughout a wide range of developmental stages and species including humans (Matute et. al., 2007a). Recent findings suggest that synaptic-type glutamate signaling operates between axons and their supporting glial cells. Glutamate reuptake will be a necessary component of such a system (Arranz et al., 2008). EAAC1 is the major glutamate transporter detected in oligodendroglia cell membranes in both developing and mature optic nerve, while GLT-1 was the most heavily expressed transporter in the membranes of astrocytes (Matute C., 2011). In mature axons EAAC1 was abundant at the node of Ranvier. Moreover in addition to oligodendroglia and astrocytes, axons represent a potential source for extracellular glutamate and have the capacity for Na^+ -dependent glutamate uptake (Arranz, et al., 2008). Glutamate uptake from extracellular space by specific glutamate transporter, is essential for the prevention of excitotoxicity. Thus it appears that all glial cells differentially express the three major GluTs present in CNS. These transporters keep extracellular glutamate at the

low basal level of 1-2 μM. The major GluT expressed by axons is GLT1 (EAAT2) and some significant levels of GLAST (EAAT1). During depolarization the GluTs contribute to glutamate release in white matter via the reversal of Na^+ dependent glutamate transporter.

5. The increase in the extracellular glutamate concentration, occurring due to either repetitive axonal activity, or to application of exogenous glutamate would result in elevation of intraaxonal free calcium concentration. There are several mechanisms which could/would participate in this process. Exogenous glutamate would interact with NMDA receptor linked channels which would allow influx of extracellular calcium into axoplasm. Simultaneously interaction of glutamate with AMPA receptors would depolarize the membrane inducing opening of voltage-gated calcium channels which would then contribute to the intracellular calcium elevation. These channels are apparently linked to and could open Ryanodine channels allowing calcium from internal stores to diffuse to the axoplasm. In parallel, the enhancement in intracellular calcium due to opening of NMDA and voltage-gated calcium channels would stimulate generation of the second messenger, IP3 and subsequent, additional influx of calcium from the internal stores through IP3 regulated channels. Our results convincingly demonstrate that all these sources of calcium contribute together to increase intracellular calcium concentration. The action of the channels involved has to be synchronized and they have to be activated simultaneously in order to induce amplification of CAP. Therefore one can assume that the intra axonal increase in calcium concentration has to be compartmentalized. It would correspond to location of individual calcium channels and it would involve specific domains of axoplasm likely to be localized in the proximity of nanocomplexes. The role of NMDA receptors,

which seem to be crucial for axonal plasticity, supports our assumption. The addition of glutamate or NMDA in regular Ringer's did not induce intracellular calcium level unless the concentration of magnesium has been reduced facilitating opening of NMDA receptor-linked channel. Moreover, while an increase in intracellular calcium concentration lasted 60 seconds only after addition of glutamate, calcium stayed elevated for about 900 seconds after NMDA addition. Thus it is likely that selective activation of NMDA receptors by NMDA addition activates ionotropic mechanisms and only then an increase in CAP is induced. On the other hand, the addition of glutamate might activate buffering system that allows the increase in intracellular calcium to last 60 second only.

6. The leading role of NMDA receptors in axonal plasticity noticed by us was also reported by others. Matsumoto and colleagues (2005) found that 100 µM glutamate and 100 µM NMDA increased the CAP recorded from dorsal column axons of neonatal rats. Apparently NMDA receptors are involved in axonal plasticity not only in adult animals (our data) but in young animals as well. This would underline the significance of axonal plasticity which is expressed already early on in life. Axonal AMPA receptors are weakly permeable to Ca^{2+} the entry of which in turn stimulates the release of calcium from the axoplasmic reticulum by opening intracellular Ryanodine calcium channels (Ouardouz et al; 2009a). Moreover axonal kainate receptors with the GluR 5 subunit are coupled to phospholipase C activation. The activation of GluR5 kainate receptors occur via a G-protein coupled PLC-dependent synthesis of IP3 which in turn activates IP3 receptors on the AR. This could be representing another mechanism which would increase the release of calcium from the AR. In addition activation of kainate receptors with GluR6 sub-unite induces a small amount of calcium entry that stimulates nitric oxide synthase, as well as a

local depolarization which activates L type calcium channels and Ryanodine receptors in the axoplasmic reticulum (Ouardouz et. al., 2009b). The release of calcium from the internal stores at the nanocomplexes can activate second messenger which phosphorylate the sodium channels.

7. Homeostasis of intracellular calcium is extremely important in neuronal tissue and calcium itself is considered a second messenger. The increase in calcium concentration significantly changes neuronal physiology in acute and permanent manner. There are many possible mechanisms which could be initiated by elevated calcium concentration. Some of them are particularly relevant for these considerations. The entrance of calcium through the NMDA receptors and through voltage gated calcium channels will change the relative expression of different Na^+ channel isoforms (Waxman et al., 1993). or will activate slow-inactivating sodium channels (Waxman et al., 1993).Calmodulin, the calcium binding protein that interacts with voltage gated sodium channels increases the channel current amplitude in a calcium dependent manner (Waxman 2006). Apparently, the expression of axonal plasticity does not always require calcium. As reported by Weragoda and his colleagues (Weragoda et al., 2004) the axons in Aplysia undergo long-lasting changes in the excitability following specific treatment. Although these changes expressed as long-term hyperpolarization were calcium independent, their presence supports our claims about axonal plasticity. It should be emphasized, however that changes in the axonal excitability demonstrated in our experiments were very strongly calcium-dependent. Thus, one can assume that axons in the animal's kingdom demonstrate at least two forms of plasticity with different cellular mechanisms. Nonetheless the expression of axonal plasticity in Aplysia supports the validity of our notion of axonal plasticity as well.

7. Our novel data together with published results clearly indicate that in spite of prevailing notion about “all-or-nothing” property of the action potential, axons and action potentials are capable of conveying the information in an analog manner (Clark and Hausser 2006) Presented results convincingly demonstrate that the amplitude of subsequently generated action potentials can change in a way correlated with the frequency of stimulation, or pharmacological treatment. In both cases the change occurred gradually with each evoked action potential slightly larger than its predecessor. This indicates that the effect was building gradually as the intraaxonal mechanisms have been recruited to contribute to the final effect. We have also observed reduced latency and increased area of CAP after glutamate application. The most obvious explanation for both phenomena would be a recruitment of additional, fast conducting axons which would shorten the latency and increase the area of CAP. Simultaneously this would increase the duration of the entire CAP, as slower conducting axons which contributed to CAP before the treatment would be activated as well. There is also another explanation in a view of the results reported by Sasaki and collaborators (Sasaki et al., 2011). They have determined that individual action potentials recorded from hippocampal single axons increased their duration following application of exogenous glutamate. The effect was initiated at the point of glutamate application indicating that the properties of the action potential were modified during its propagation along the axon, not at the point of generation. As in experiments reported by Sasaki’s group (Sasaki et al., 2011), the glutamate effect in our experiments was blocked by antagonists of AMPA receptors, but was not affected by glutamate uptake inhibitors. Therefore one cannot exclude the possibility that exogenous glutamate would also modify the rate of propagation of and the size of the action potentials in individual axons of sciatic

nerve tested in our experiments. Change in these single axons would then modify the properties of CAP. The glutamate-induced axonal plasticity may, as a consequence of axonal repeated activation represent beneficiary effect exerted in neuromuscular system by extensive physical activity. Indeed, the presence of several ions channels, carriers and receptors visualized in axonal membranes by us apparently creates environment permissible for modification of the action potential during its propagation down the axonal length. The recordings from individual axons using our experimental model would be necessary to verify this assumption.

Materials and methods

Animals

CD-1 mice of both sexes, 1 to 3 months old, kept in normal day light/dark cycle were used in all experiments. All mice were housed in groups of three in a pathogen-free room maintained on a 12 hour light/dark cycle and given food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY, and were in conformity with National Institutes of Health Guidelines. The number of mice sufficient to provide statistically reliable results was used in these studies.

Preparation of sciatic nerve

CD-1 mice of both sexes, 1 to 3 months old were used to obtain segments of the sciatic nerve. The animals were sacrificed by cervical dislocation, both hind limbs were skinned, the origin of the sciatic nerve was exposed and sciatic nerves were dissected out. The nerves were cut into 7-10 mm segments and placed for at least one hour in oxygenated Ringer's solution (33^{o}C) 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% CO2/O2. Following a preincubation period, which varied from 1 to 3 hours. individual segments were transferred to the recording chamber containing continually oxygenated Ringer's solution(33°C). In some experiments Ringer's solution with reduced magnesium concentration (325 µM $MgSO_4$), or calcium-free ($CaCl_2$ was omitted) was used.

The procedure used for preparation of biological preparations was approved by the Institutional Animal Care and Use Committee at CSI.

Electrophysiological recordings

The compound action potential, which reflects the number of activated sensory, motor, myelinated and unmyelinated axons, was recorded as follows:

The recording electrode (wire) was placed inside the epineurium at an end of the segment, and the stimulating electrode was localized outside of epineurium. In this experimental arrangement several individual axons contributed 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.

Since CAP is a macroscopic reflection of the single axon measurement of the fiber volley (Meeks and Mennerick 2004) one can make inference about processes occurring in a single fiber by measuring compound action potential. In order to characterize the properties of CAP, the nerves were stimulated in all experiments with the frequency of 0.03 Hz which was applied during entire duration of each experiment without any interruptions.

Following stabilization of the potentials induced by 0.03 Hz stimulation, the different chemicals, depending on the type of the experiment, were added to the recording chamber. The duration of single pulse was 0.1 ms.

The latency and the area measurements

The latency was measured as distance between the start of the stimulus and the beginning of the first negative of the compound action potential (Figure 29). Approximately 4-5 samples of the compound action potential were selected before and after the addition of glutamate respectively. They were averaged and statistical significance was calculated using the paired t test (Figure 8).

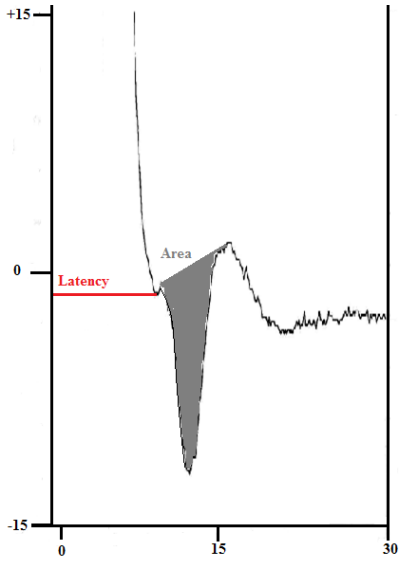


Figure 29 Example of how latency and area measurements were taken. A As displayed by the red line, the latency measurement was taken from the first low point to the measurement to the y-axis. **B** As displayed by the grey area, the area measurement was taken as the area of that. The scale master was the instrument used to measure the area.

The area of the compound action potential was measured by the scale master. The scale master is an instrument which enables the user to measure any irregular shape or area. Approximately 4-5 samples of the compound action potential were selected before and after the

addition of glutamate respectively (Figure 29). All measurements were averaged and statistically calculated using the paired t test (Figure 9).

Immunnohistochemistry

The nerves were fixed in 4% paraformaldehyde for 24hours and subsequently placed in 30% sucrose solution for 24 hours. 30µm cryosections were prepared and transferred onto slides for the immunnohistochemistry processing as follows.

The cryosectioned sciatic nerve slides were washed with phosphate-buffered saline (PBS; 0.01M, ph7.4) 3 times (10min each), permeabilized and blocked with 2% nonfat dry milk (NFDM) in PBS, containing 0.02% TritonX-100 and 10% Normal goat serum (NGS) for 1hr, and incubated overnight (at 4^{o}C) with the primary antibody after 3 washes with PBS. The in vitro expression of various channels and receptors was analyzed with the following antibodies (diluted in NFDM-PBS with 2%NGS): rabbit anti- GluR 2/3 (1:500; Millipore Corp. MA, USA) , mouse anti- EAAC1 (1:500; Chemicon International Inc., CA, USA) , mouse anti- NMDA R2B (1:500), rabbit anti-Ca (1:500), mouse anti-Na (1:500) and rabbit anti- AMPA (1:500). The following day, the samples were blocked for 1hr with 10% NGS in NFDM-PBS, and then washed and incubated for 1 hr at room temperature with secondary antibodies diluted in NFDM-PBS with 2%NGS.

The dilution of the secondary antibodies were 1:250 Cy5 (Cy5 is a far-red and near-infrared emitting dye which is ideal for fluorescence measurements ; Jackson ImmunoResearch laboratories, Inc., PA, USA) (goat anti-mouse) and Alexa fluor 488 (1:250; Santa Cruz Biotechnology, Inc., CA, USA) (goat anti-rabbit). The nuclei were counterstained and finally cover-slipped with Prolong Gold antifade reagent with DAPI (Invitrogen, Eugene, OR, USA) .

Images were captured using confocal microscopy (Leica SP2 AOBS).Using a 488 nm Ar/Kr laser for the Alexa fluor 488,543 nm HeNe laser for Cy5 and 405 nm diode laser for DAPI.To determine relative changes in protein expression, the gain and offset was identical for all comparisons. Images were reconstructed from Z stack using Leica software.

The evaluation of intracellular calcium with imaging quantitative measurements

Our electrophysiological experiment performed in calcium-free environment showed that the presence of calcium is necessary to observe glutamate-induced increase of the compound action potential recorded from the sciatic nerve. We thought to verify this result by performing the calcium imaging in the presence of glutamate, NMDA, and MK801.

The nerves were cut into 1 cm segments and placed for at least 30 minutes in oxygenated Ringer's solution (33^{o}C). Next, individual segments were transferred to another dish containing Ringer's solution. Fluo3 (5 μM) was added and the dish was kept in incubation chamber for 30 minutes. Since there were no changes in calcium images in normal Ringer's solution, the Ringer's with reduced magnesium concentration was used to evaluate the effect of glutamate, NMDA and MK801 on intracellular calcium accumulation. The piece of sciatic nerve was mounted in the recording chamber and was kept in position by placing it under a piece of wire in the shape of horseshoe with a nylon net stretched across it.

Confocal images of cellular fluorescence were obtained using a Leica inverted microscope (Leica SP2 AOBS) equipped with a dry 10 x HC PL fluoter 10x 0.3 dry objectives and a water immersion lens. The excitation wavelength used was 496 nm-Ar\Kr laser and, the emission wavelength of the ionophore measured between was 509-596 nm.

In all experiments, fluorescence was measured in the sciatic nerve. All recordings were performed at room temperature (22–25°C). At the end of each experiment, fluorescence intensities were calculated.

The averaged intensities of the first three images of each experiment were used as a base line in all the experiments. Therefore we took three base scans and then recording chamber was loaded with one of the following: glutamate (100 µM), NMDA (10 µM), MK801 (50 µM), and the images were then taken every 30 seconds. The changes in free calcium concentration inside the axon were evaluated by comparison of the baseline fluorescent intensity with the intensity after addition of given molecule. Data were expressed as percent changes in fluorescent intensities.

Chemicals used

All tested chemicals, prepared as stock solutions in Ringer's were added directly to the recording chamber in the volume ranging from 10 µl to 100 µl to obtain desired final concentration.

NMDA - *N*-methyl-D-aspartate, an agonist of NMDA receptor; (Sigma).

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic; an agonist of AMPA glutamatergic receptor; (Sigma).

Kainic acid; C10H15NO4 It is a specific agonist for the kainate receptor used as an ionotropic glutamate receptor ; (Sigma).

Kynurenic acid – 4-Hydroxyquinoline-2-carboxylic acid, a broad spectrum antagonist of glutamatergic receptors; (Sigma).

MK801 or Dizocilpine **-** a non-competitive antagonist of the NMDA receptor; (Sigma).

CNQX - 6-cyano-7-nitroquinoxaline-2, 3-dione, a competitive AMPA/kainate receptor antagonist; (Sigma).

DL-TBOA - DL-*threo*-β-benzyloxyaspartate, a competitive blocker of excitatory amino acid transporters; (Tocris).

Verapamil, an L-type calcium channel blocker; (Tocris).

Dantrolene sodium, an intracellular calcium release blocker acting on the ryanodine endoplasmic reticulum receptor; (Tocris).

Fluo-3 is a fluorescence indicator of intracellular free calcium (Ca^{2+}). It is used to measure Ca^{2+} inside living cells in confocal laser scanning microscopy using visible light excitation (compatible with argon laser sources operating at 488 nm). Fluo-3 is essentially nonfluorescent compound, but upon binding of Ca^{2+} its fluorescence increases sharply with an emission maximum at 525 nm; (Invitrogen).

Ringer's solution: NaCl 124, KCl 3.1, KH_2PO_4 1.3, $MgSO_4$ 1.3, $CaCl_2$ 3.1, $NaHCO_3$ 25.5 (Sigma), and glucose 10 ; (Fisher).

Statistics:

All data were post analyzed offline and statistics were computed in Statistica V 6.1 (Statsoft, Inc. Tulsa, OK) and MatLab software . Data were analyzed with ANOVA and paired t-test unless specified otherwise. All data are expressed as mean ± SEM.

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