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CALCIUM HOMEOSTASIS AND

MODULATION OF NOCICEPTIVE SYNAPTIC

TRANSMISSION

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I hereby confirm that this thesis was independently written by me. No material was used other than that referred to. Sources directly quoted and ideas used, including figures, tables, sketches, drawings and photos, have been correctly denoted. Those not otherwise indicated belong to the author. No part of this thesis has been submitted to obtain an academic degree.

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SUMMARY

Calcium Homeostasis and Modulation of Nociceptive Synaptic Transmission

This study was designed to improve our knowledge regarding mechanisms of nociceptive signaling at spinal cord level. One of the forms of spinal cord synaptic transmission modulation is central sensitization, a manifestation of synaptic plasticity at spinal cord level, which was found to be present at many chronic pain syndromes. Understanding of synaptic transmission mechanisms and modulation is crucial for resolution of persistent difficulties in finding an effective chronic pain treatment.

Various experimental approaches to tackle the mechanisms of central sensitization have been employed in the past because of the complex nature of the phenomenon under investigation. This study deals mainly with development of a calcium imaging technique with a final goal to study mechanisms of central sensitization *in vitro*, on population of dorsal horn neurons.

In our experiments we have analyzed synaptically evoked intracellular calcium changes as a result of dorsal root stimulation in a superficial dorsal horn area in spinal cord slices. We have found two types of responses in the dorsal horn neurons. First type showed intracellular calcium increase during the dorsal root stimulation. The second type of response was usually significantly delayed after the stimulation and was due to calcium release from internal stores. The calcium release from internal stores due to afferent stimulation was not previously shown to be present in these neurons. The intracellular calcium

increase during the delayed response was not dependent on activation of ionotropic glutamatergic receptors, suggesting that it was mediated through metabotropic receptor pathway. This could have a significant role in the induction of some types of chronic pain syndromes, since intracellular calcium increase is thought to be a key trigger point in spinal cord neurons sensitization.

Intracellular calcium concentration and its dynamic changes are precisely regulated by number of sophisticated mechanisms. Intracellular calcium buffers were shown to play an important role in the calcium homeostasis. In our work we have concentrated on the possible role of calcium binding proteins parvalbumin (PV), calbindin (CB), and calreticulin (CR) in pathological pain states. We have used a model of acute unilateral knee joint arthritis to study changes in PV expression in the spinal cord dorsal horn and changes in PV, CB and CR expression in spinothalamic tract (STT) neurons. In our experiments, arthritis induced significant downregulation of PV in the ipsilateral dorsal horn neuropil. Some of the results also suggested that these changes could occur in GABAergic neurons, present in this dorsal horn area. In retrogradely labeled STT neurons, PV and CB were significantly upregulated, while the number of CR positive neurons did not differ in our experimental model. These results suggest that change in CBP expression could have a significant effect on Ca homeostasis and possibly modulation of synaptic activity.

Using the spinal cord slices calcium imaging we have developed an *in vitro* model of dorsal horn neurons sensitization. Bulk loading of the slice with the fluorescent probe enabled us to study intracellular calcium changes in a population of dorsal horn neurons at the same time. This is of great advantage compared to the patch clamp technique that enables to study just one or two

neurons simultaneously. Capsaicin was used to activate TRPV1 receptors present on primary afferent fibers and to induce sensitization of dorsal horn neurons, recorded as increase of intracellular calcium changes induced by control dorsal root stimulation. Using this model together with transgenic labeling of specific neuronal populations could be useful in the future, especially for pharmacological testing of new analgesic drugs.

CONTENTS

1   LITERATURE OVERVIEW

1.1 PAIN AND NOCICEPTIVE PATHWAYS

1.1.1 Basic terminology

Pain is a subjective, psychological state that can only be described by verbal or magnitude scaling methods (Merskey and Bogduk, 1994; Breivik et al., 2008; Gélinas et al., 2008). The International Association for the Study of Pain (ISAP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage" (Merskey and Bogduk, 1994; Loeser and Treede, 2008). The ISAP also calls attention to subjectivity of the pain perception stating that if people "regard their experience as pain and if they report it in the same ways as pain caused by tissue damage, it should be accepted as pain".

By contrast, nociception is a physiological phenomena defined as "the neural processes of encoding and processing noxious stimuli" (Loeser and Treede, 2008). As a result, a physiological term nociceptive pain ("pain arising from activation of a nociceptors") was introduced recently (Loeser and Treede, 2008) to differentiate it from a non-nociceptive pain (psychogenic pain or psychalgia) occurring in the absence of tissue damage or pathophysiological cause (Tyrer, 2006).

Pain can be classified from many different points of view (Weiner, 2002; Wall et al., 2006), temporal and physiological being the most common. Temporally, pain is either acute or chronic. Acute pain is presumed to be related to ongoing activation of specific primary afferent neurons functioning as nociceptors – sensory receptors capable of transducing and encoding noxious stimuli. Acute

pain is characterized as normal, predictable, appropriate response to a noxious stimulus or disease process that threatens or produces tissue injury, and that abates following remission of the stimulus or healing of the injury. Acute pain is usually treated with analgesics.

Table 1. Physiological classification of pain (adapted from The Guidelines for a Palliative Approach in Residential Aged Care, 2004).

	Nociceptive			Neuropathic	
	superficial somatic	**deep somatic**	**visceral**	**peripheral**	**central**
Stimulus origin	skin, mucosa of mouth, urethra, etc.	bones, joints, organ capsules	internal organs		
Localized	well	well	poorly	nerve or dermatome distribution	
Referral	no	yes	sometimes	yes	

Chronic pain is associated with a chronic disorder, or pain that persists beyond resolution of an underlying disorder or healing of an injury, and that is often more intense than the underlying process would predict. The lifetime incidence of chronic pain in Western populations is almost 50 % (Clarke, 2000; R., 2000). It should be present for more than six months to be classified as such (Rashiq, 2008); it has possibly unclear pathology and unpredictable prognosis and often needs to be treated multidisciplinary (Ashburn and Staats, 1999).

Physiological classification divides pain into nociceptive and neuropathic types of pain, and can be a mixture of these two types (Tab. 1). Nociceptive pain is divided into somatic (superficial or deep) or visceral. It is detected by

transducers attached to the Aδ and C fiber. Visceral pain is poorly localized and sometimes referred to the body surface, which can be explained by viscero-somatic convergence at the spinal cord level. Somatic pain is well localized and not referred in case of superficial somatic pain. Neuropathic pain (peripheral or central) is defined as a "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system" (Loeser and Treede, 2008). It is typically referred.

1.1.2 Nociceptive pathways

The sequence of nociceptive events, culminating in pain perception (Fig. 1),
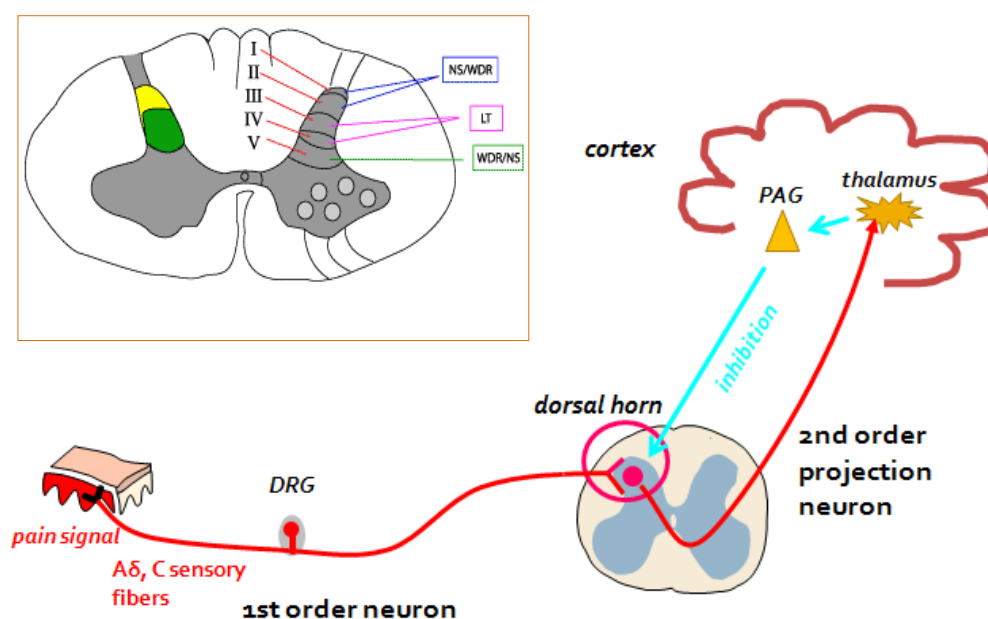
Figure 1. Nociceptive pathways and laminar organization (in rectangle) of the spinal cord with respective location of neuron types receiving noxious (yellow) or innocuous (green) input from C/Aδ or Aβ fibers respectively. NS, nociceptive specific neurons; WDR, wide dynamic range neurons (respond to both innocuous and noxious stimuli); LT, low-threshold neurons (respond to innocuous sensation); DRG, dorsal root ganglion; PAG, periaqueductal grey.

starts with nociceptive stimulus activating nociceptors at first-order afferent neuron nerve ending, where transduction into generator potentials and encoding into trains of action potentials takes place (Kandel et al., 2000).

Nociceptor - "a sensory receptor that is capable of transducing and encoding noxious stimuli" (Loeser and Treede, 2008) - is a peripheral terminal of the primary afferent A delta and C fibers. Aδ fibers are myelinated, fast-conducting (velocity of signal transmission is 12-30ms^{-1}; Millan, 1999), fibers associated with experience of sharp pain. C fibers are slowly conducting (0.5 - 2.0 ms^{-1}; Millan, 1999), small diameter, unmyelinated fibers responsible for delayed, dull and burning pain (Tab. 2). Though non-nociceptive receptors may respond to noxious stimuli, only nociceptors are capable of encoding the stimuli relevant properties like intensity in the painful range or location.

Table 2. Comparison of Aδ and C nerve fibers (Millan, 1999; Wall et al., 2006).

Aδ fiber	C fiber
thermal receptors	polymodal receptors
High threshold mechanical receptors	(mechanical, chemical, thermal)
myelinated	unmyelinated
fast conduction	slower conduction
"first", sharp pain	"second", slower, duller pain
short lasting	prolonged in duration
lamina I and V	lamina II (substantia gelatinosa)

The primary afferent fibers have their somata in the dorsal root ganglion (DRG) and synapse on the central nervous system neurons mainly in superficial laminae of the spinal cord dorsal horn (Fig. 1). Dorsal horn neurons involved in pain transmission are often classified, based on their responses to the peripheral

stimuli, as polymodal or wide dynamic range (responding to both low-intensity and high intensity - normally felt as painful - stimuli) and high threshold nociceptive specific neurons responding just to high intensity stimuli (Wall et al., 2006). Low-threshold neurons respond to low intensity stimuli that are not felt as painful under normal conditions. However, their activation by innocuous stimuli may be perceived as painful under pathological conditions.
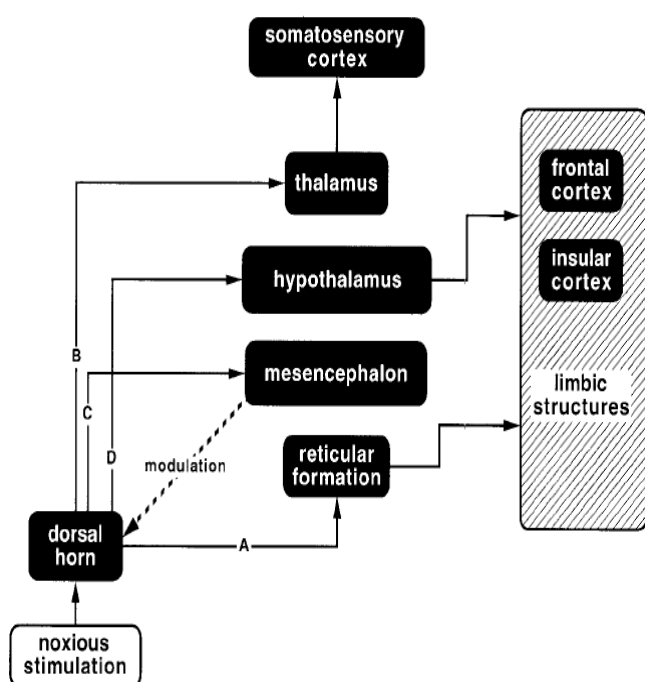
Figure 2. Major pathways of nociceptive transmission for the spinal cord to central structures: spinoreticular (A), spinothalamic (B), spinomesencephalic (C) and spinohypothalamic (D) tracts.

The nociceptive information undergoes significant modulation at the spinal cord level in which both inhibitory and excitatory descending pathways play an important role. From the spinal cord level it is further transmitted by projection (second-order) neurons to the brain (Fig. 1, 2). The highly complex ascending pain projection pathways (Almeida and Lima, 1997) can be divided into two types (Millan, 1999): Monosynaptic pathways project directly to cerebral structures (e.g. spinothalamic tract) whereas polysynaptic pathways possess a relay station

(third-order neurons) of the second-order neurons (e.g. spinoreticular or postsynaptic dorsal column pathways).

Somatosensory cortex is involved in discriminatory aspect of pain and those with association cortex projections are related to affective responses involving both somatic and autonomic motor systems. Many supraspinal control areas (the reticular formation, midbrain, thalamus, hypothalamus, the limbic system of the amygdala and the cingulate cortex, basal ganglia and cerebral cortex) modulate pain perception. Neurons originating from these areas may activate neurons of the descending spinal pathways, which terminate in the dorsal horn of the spinal cord.

1.2 SYNAPTIC TRANSMISSION OF NOCICEPTIVE SIGNALS

Chemically or physically activated primary nociceptive afferents of DRG neurons transmit nociceptive signal to the spinal cord dorsal horn. Upon arrival of action potential, a diverse array of neurotransmitters, neuromodulators and other neuroactive compounds such as inflammatory mediators may be released from the presynaptic central terminals and binds simultaneously to different types of receptors on postsynaptic membrane of second-order neurons as well as on the presynaptic membrane itself (Fig. 3, (Millan, 1999; Willis and Coggeshall, 2004).
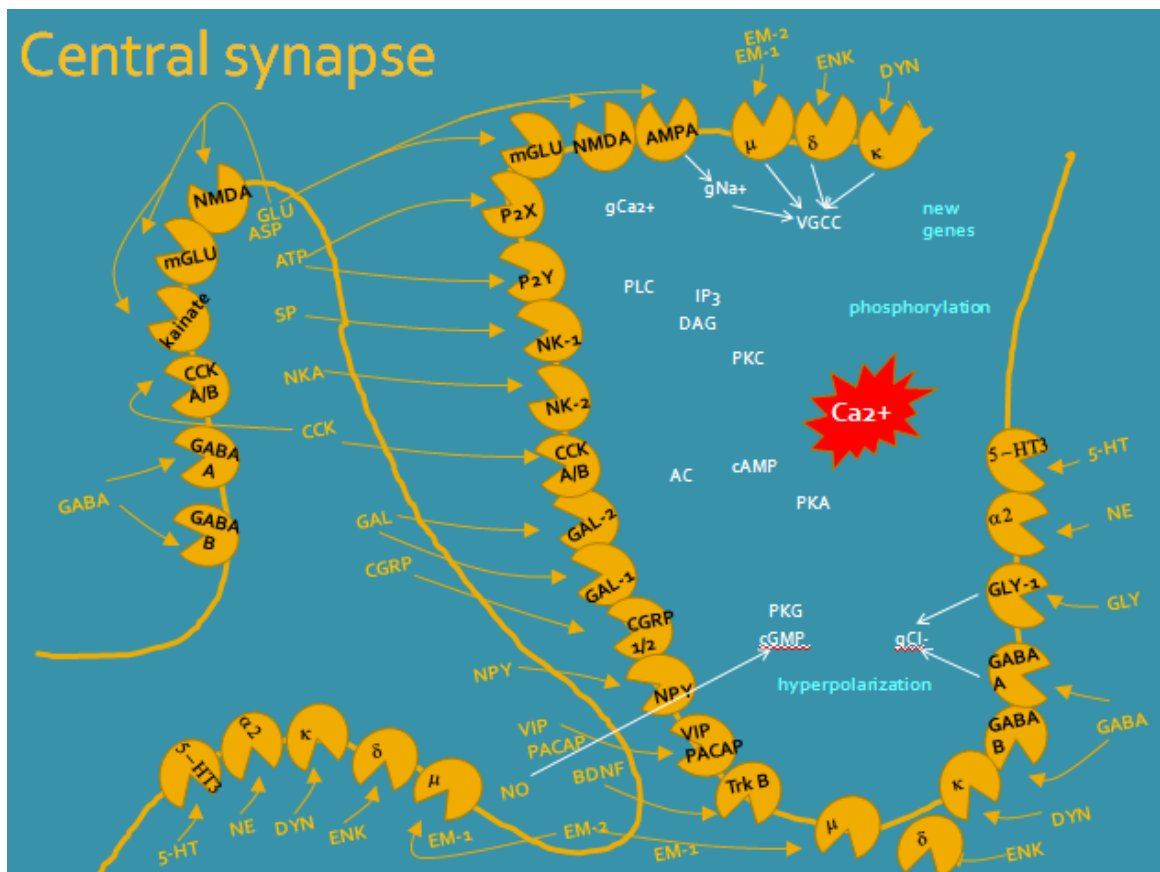
Figure 3. The complexity of nociceptive signaling at the spinal cord dorsal horn synapse.

These compounds can modulate neuronal excitability in a very complex manner. Some of the prominent neurotransmitters/neuromodulators and their synaptic targets are discussed further.

1.2.1 Glutamate receptors

The key neurotransmitter at excitatory synapses is the excitatory amino acid (EAA) L-glutamate (and another EAA L-aspartate). Glutamate acts as an agonist on a large receptor family of glutamate receptors that can be divided in two basic groups: ionotropic receptors and metabotropic receptors.

1.2.1.1 Ionotropic glutamate receptors

Ionotropic glutamate receptors can be divided based on their specific agonists into three basic groups of AMPA, kainate and NMDA receptors. Ionotropic AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid) glutamate receptor/channel consists of four subunits, GluR1-4. The AMPA receptor is an important element of synaptic plasticity involving AMPA receptors phosphorylation and trafficking. GluR subunits have consensus region for phosphorylation by calcium/calmodulin-dependent protein kinase II and protein kinase C (CaMKII, PKC; Barry and Ziff, 2002; Boehm and Malinow, 2005). Structurally similar kainate receptors (named after its specific agonist kainate) are composed of KA1, KA2, and GluR5, 6, and 7 subunits. Their role in basic synaptic transmission is minor compared to the AMPA receptors. However they contribute significantly to synaptic plasticity (Contractor et al., 2000). The kainate receptor may be phosphorylated by cAMP-dependent protein kinase (PKA; Wang et al., 1991; Wang et al., 1993).

The AMPA and kainate receptors' subunit composition defines their properties like permeability. Both receptors are permeable to sodium, potassium and to a much lower extent to calcium. It is known that presence of the GluR2 subunit of the AMPA receptor renders the receptor's impermeability thus guarding the cell against excitotoxicity (Angulo et al., 1997; Kim et al., 2001) and having possibly significant role in synaptic plasticity. The AMPA receptor is the main charge carrier during excitatory postsynaptic potential (EPSP) generation. At the spinal cord neurons the AMPA/kainate receptors are activated by EAA binding and briefly depolarize neuronal membrane (10-20 ms; Antognini et al., 2002), often with action potentials in second-order neurons. The channel activation is regulated by extremely rapid onset of glutamate desensitization, which is – together with action of excitatory amino acid transporters (EAATs) – a neuronal protective mechanism guarding neurons against glutamate-induced excitotoxicity (Yamada and Tang, 1993; Matthews et al., 2003). Diffusion of the neurotransmitter glutamate beyond synaptic cleft after its release was suggested to lasts several hundred microseconds, with concentration peak at 1.1 mM and decay time constant 1.2 ms (Clements et al., 1992). The AMPA receptor's desensitization is lower if specific, often clinically utilized, compounds (e.g. nootropics aniracetam) bind to its allosteric sites (Isaacson and Nicoll, 1991).

The NMDA (N-methyl D-aspartate) receptor is another important ionotropic glutamate receptor. The NMDAR consists of two NR1 subunits each having eight isoforms (1-4 a, 1-4 b) and two NR2 subunits with four different isoforms (A-D) where subunit and isoform composition influences receptor's function. Both subunit types seem to be needed for NMDA receptor's functioning (Cull-Candy et al., 2001). Presence of the genetically related NR3(A-B) subunits seems to inhibit

the channel activity. They bind glycine with high affinity and form excitatory glycine receptors, as they require glycine alone for activation (Chatterton et al., 2002; Awobuluyi et al., 2007). Each subunit has an extensive cytoplasmic domain, which contain residues that can be directly modified by a series of protein kinases and protein phosphatases, as well as residues that interact with a large number of structural, adaptor, and scaffolding proteins (Ultenius et al., 2006) .

The NMDA receptor is a cation channel, permeable to sodium, potassium and small amounts of calcium, characterized by both ligand and voltage gating (though not voltage dependence), requiring co-activation by two ligands, glutamate and glycine. The voltage gating is a result of ion channel block by extracellular magnesium ions and is released during synaptic transmission by increasing AMPA receptor-mediated depolarization (around 0 mV).

This property leads to longer duration depolarization (several hundred milliseconds (Lester et al., 1990) and has been suggested that this channel is a biochemical substrate of Hebbian learning (see chapter 1.3.1. on synaptic plasticity). This way it can act as a coincidence detector for membrane depolarization and synaptic transmission (i.e. only if pre- and postsynaptic cell are simultaneously active will NMDA receptors become unblocked and allow calcium ions to enter the postsynaptic cell). Thus in a way, the NMDA receptor converts an electrical signal into a biochemical signal that can trigger synaptic plasticity (Rao and Sejnowski, 2001).

The NMDA receptors are modulated by many endogenous and exogenous compounds and play a key role in a wide range of physiologic and pathologic

processes including pain where it appears to be responsible for not only amplifying pain, but also playing role in opioid tolerance as well (Popik et al., 2000; Bespalov et al., 2001).

1.2.1.2 Metabotropic glutamate receptors

The second group of glutamate receptors are the metabotropic glutamate receptors, the members of the group C family of G-protein-coupled receptors (GPCRs). The mGluR receptors consist of eight types, which are organized into three groups (Tab.3, Bleakman et al., 1992; Wang and Daw, 1996; Neugebauer, 2001).

Table 3. Metabotropic glutamate receptors groups and effect of activation.

Group	Type	Localization	Effect on synaptic activity
I $G_{q/11}$ (G_s)	**mGlu$_1$** **mGlu$_5$**	**lamina II** **mainly postsynaptic** **on cell bodies**	**excitatory** **↑PLC ↑NMDAR activity**
II $G_{i/0}$	**mGlu$_{2,3}$**	mainly presynaptic	inhibitory ↓AC↓NMDAR activity
III $G_{i/0}$	**mGlu$_{4,6,7,8}$**	mainly presynaptic	inhibitory ↓AC ↓NMDAR activity

The group I having the mGlu1 and mGlu5 members was found largely in CNS on the side of the postsynaptic membrane (Pin, 2009). This group is supposed to play a role in nociception due to its relatively large expression in the pain-related area of spinal cord (Azkue et al., 2003). The mGluR group II consists of mGlu2 and mGlu3 receptor types. Within the brain, mGlu2 receptors are localized

mainly presynaptically and the mGlu3 is localized both pre- and postsynaptically. The mGlu3 receptor is also often found in spinal cord and hippocampus among others.

The mGluRs group III consists of mGlu4, mGlu6, mGlu7 and mGlu8. In connection with pain and synaptic plasticity is important the mGlu7 presence in the spinal cord on the terminals of primary afferent fibers and in the superficial layers of spinal dorsal horn (Willis and Coggeshall, 2004).

Regulation of ionotropic glutamate receptors with metabotropic glutamate receptors can be reciprocal. The group I mGluRs may change excitability of synapse by phosphorylation of NMDA and AMPA/kainate receptors via regulation by various kinases such as PKC, PKA, ERK/MAP, Src, etc. (Chu and Hablitz, 1998, 2000). Together with group III receptors it may inhibit release of substance P - a neuropeptide present in the dorsal root C fiber endings. Further, group I receptors regulate basal neuronal activity via their targeting by synaptic protein Homer (Anwyl, 1999, 2009).

The mGluRs are involved in neurotoxicity and neuroprotection (Azkue et al., 2003; Welsby et al., 2006; Pshenichkin et al., 2008). Like other glutamate receptors, they have been shown to play an important role also in synaptic plasticity (Endoh, 2004; Bonsi et al., 2005). They participate in long-term potentiation/depression (LTP/D) and they are internalized in response to agonist binding (Dale et al., 2002). The group II and group III receptors activation have predominantly inhibitory effect on modulation of synaptic transmission, in contrast to the group I receptors. The mGluRs can directly or indirectly modulate release or action of neuropeptides. Moreover, they are often located outside the active

zone of synapse which suggests that only higher amount of glutamate released during synaptic transmission may activate them. Since it is hypothesized that robust afferent barrage in dorsal root fibers underlies induction of dorsal horn neurons central sensitisation, contribution of the mGluR I receptors in this process may be of particular interest (Fischer et al., 2008). Activation of metabotropic glutamate receptors also modulates activity of various other ion channels (Fagni et al., 2000). High voltage gated calcium channels (VGCC) types N, L, P/Q are inhibited by activity of all of the mGluRs. Yet mGluR I activation may also lead to increased channel opening. In light of inflammatory and neuropathic pain, of particular interest are VGCCs type N (Cohen and Dragovich, 2007). Potassium (K^+) channels, modulated by group I mGluRs, are responsible for increased neuronal excitability due to regulation of frequency of action potential firing. This is done by two mechanisms. By intracellular Ca^{2+} induced K^+ channel after-hyperpolarization (see further after-hyperpolarization induced by calcium released from intracellular stores) and by voltage-dependent slow K^+ channel inactivation.

1.2.2 Neuromodulators

Important role in synaptic plasticity in the CNS in general and in spinal cord in particular play neuromodulators (Bohlen und Halbach and Dermietzel, 2006). Besides ubiquitous mechanism of glutamate and aspartate-driven synaptic transmission at excitatory synapses, many neural systems rely on release of neuropeptides and other neuromodulators such as substance P, calcitonin gene-related peptide (CGRP), and brain-derived neurotrophic factor (BDNF). In the process of nociceptive transmission, the release of neuroactive peptides and activation of metabotropic pathways from C nociceptive afferent neurons add to

the long-term depolarization produced by EAA activation on NMDA receptors and mGluR receptors (De Koninck and Henry, 1991; Batchelor et al., 1997). This eventually may lead to short-term and long-term modulation of synaptic transmission.

Well explored element of the nociceptive synaptic modulation via release of neuroactive peptides, is substance P (Frederickson et al., 1978). Substance P is coexpressed with the excitatory neurotransmitter glutamate in C fiber primary afferents. The endogenous substance P receptor is neurokinin 1 receptor (NK1-receptor), which belongs to the tachykinin receptor sub-family of GPCRs (together with neuropeptides neurokinin A and B - NKA, NKB, Severini et al., 2002). It has been proposed that cytokines and neurotrophic factors can induce NK-1 expression. SP can also induce the cytokines that are capable of inducing NK-1 transcription factors due to its promoter cAMP (and other) sensitive regions related to the cytokine signal transduction pathways (Rameshwar, 1997). TRPV1 receptor agonist capsaicin has been shown to reduce the levels of SP in the spinal cord probably by reducing the number of C fibre nerves (Nagy et al., 1980).

1.2.3 Role of glia in synaptic transmission

The synapse between presynaptic ending and postsynaptic neuron forms together with the perisynaptic glia a more complex signaling structure - a tripartite synapse (Volterra et al., 2002). Many central synapses appear to be finely enwrapped up to the edge of the cleft by the distal processes of astrocytes and actively regulate synaptic transmission by transmitter uptake and release (Hirrlinger et al., 2004).

The astrocytes, the most common glial cells in the CNS, not only serve as a building block of a blood-brain barrier, but also have a range of other actions. They are responsible for the clearance of synaptic neurotransmitters using excitatory amino acid transporters (EAATs) and low-affinity vesicular glutamate transporters (VGluTs). The EAAT terminate the excitatory neurotransmitter signal by uptake of glutamate from the neuronal synapse into the glia, thus also preventing possible glutamate-induced excitotoxicity. The astrocytes also express a range of their own receptors and channels including glutamatergic AMPA/kainate, NMDA, and mGluR receptors. However, structure and properties may differ from the receptors found in the neurons. For instance glial NMDA receptors are weakly (if at all) sensitive to the extracellular magnesium block, which may indicate a predominant expression of the NR3 subunit (Verkhratsky and Kirchhoff, 2007). A fundamental difference between glial and neuronal membranes is that glial cells have a much larger resting conductance, which has been attributed to inward rectifier potassium channels existing in glial cells (Barres, 1991).

Accumulating evidence indicate that such glial receptors may be a target for glutamate released from the presynaptic terminals. Therefore, the existence of a bidirectional flow of information between the neurons and astrocytes during synaptic activity was recently suggested. Importantly, a recent study indicates that direct synaptic connections may exist between neurons and glia, implying that the receptors on glial cells may represent the primary synaptic target of a transmitter released from the neuronal terminals (Bergles et al., 2000). Glial cells are also known to send modulatory inputs to neurons in response to an intrinsic excitability that generates spontaneous Ca2 oscillations (Parri et al., 2001).

Astrocytes signal between each other using calcium. The gap junctions (also known as electrical synapses) between astrocytes allow the messenger molecule IP3 to diffuse from one astrocyte to another. The IP3 activates calcium channels on cellular organelles, releasing calcium into the cytoplasm. This calcium may stimulate the production of more IP3. The net effect is a calcium wave that propagates from cell to cell.

Electrical stimulation of neuronal afferents in hippocampal slices was found to induce elevations in intracellular calcium concentration calcium in the surrounding astrocytes. The glial response was abolished in the presence of tetrodotoxin (TTX) or the mGluR antagonist (MCPG) therefore proving to be a consequence of the synaptic release of glutamate. These data support the view that neurotransmitters released during synaptic activity can also activate astrocytic receptors in the vicinity of synaptic cleft (Vesce et al., 2001).

1.3 MODULATION OF SYNAPTIC TRANSMISSION AT THE SPINAL CORD LEVEL

1.3.1 Synaptic plasticity

In mature nervous system, the synaptic transmission can be modulated, i.e. potentiated or suppressed, in many ways (Fig. 4; Stone, 1991). Modifying synaptic strength, often referred to as synaptic plasticity, is the ability of neural circuits to undergo changes in function or structure organization due to previous
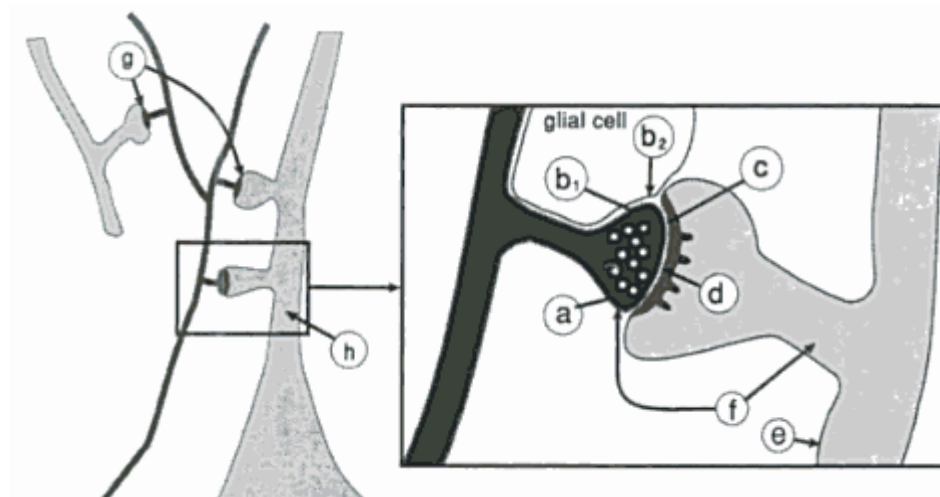
Figure 4. Sites on synaptic contact at which synaptic transmission can be modulated (a) presynaptic terminal with vesicles containing transmitter, (b) sites for reuptake of transmitter 1. into presynaptic terminal, 2. into adjacent glial cell, (c) site of receptors at postsynaptic membrane; (d) site of ionic channels in postsynaptic membrane, (c) neuronal membrane in which conductance changes can occur, (f) sites of possible structural change (e.g. presynaptic knob and postsynaptic dendritic spine, (g) additional synapses, (h) biochemical changes in cytoplasm (adapted from Stone, 1991).

activity. It is thought to underlie both adaptive as well as maladaptive learning processes (Scholz and Woolf, 2002). It is important for normal processes such as memory formation (Wang et al., 2009) and pathophysiological events such as those underlying chronic pain or the synaptic loss in neurodegenerative disorders (Scholz and Woolf, 2002; Bredesen, 2009).

A general rule of synaptic plasticity, known as Hebb's postulate, was first formulated in 1949 by Hebb and experimentally confirmed by Bliss and Lomo (Hebb, 1949; Bliss and Lomo, 1973; Lømo, 2003). It describes a basic mechanism of synaptic plasticity where an increase in synaptic efficacy arises from the presynaptic cell's repeated and persistent stimulation of the postsynaptic cell, and states:

Reverberatory activity in transient assemblies of neurons carries a memory trace that becomes permanently laid down as changes in synaptic weights when a presynaptic neuron repeatedly or persistently takes part in firing the postsynaptic cell.

The rule implies both temporal and spatial constraints on information storage manifested as synaptic plasticity (Froemke and Dan, 2002). As demonstrated on hippocampal preparations, the fundamental factors inducing synaptic plasticity involve (Paulsen and Sejnowski, 2000): temporal order of the synaptic input and the postsynaptic spike within a narrow temporal window (temporally asymmetric Hebbian learning rule); backpropagating action potential serving as a global signal for synaptic plasticity; and a specific temporal pattern of activity — postsynaptic bursting. However, potentiation can occur even in the absence of the postsynaptic somatic spike (Mehta, 2004).

1.3.2 Central sensitization of spinal cord nociceptive neurons

It is acknowledged that in some chronic pain states associated with the presence of pathologically increased sensitivity, synaptic modulation at spinal cord level may play an important role (Woolf and Thompson 1991; Willis 2001).

Table 4. Substances involved in induction of central sensitization.

Neuromediator	Target	Outcome
Glutamate	NMDAR	

Neuropeptides
  Substance P
  NKA
  CGRP
	NK1R
	NK2R
	CGRPR
		Enhanced glutamatergic synaptic transmission.

Prostaglandin E$_2$	EP	Enhanced nociceptor sensitivity.

Pro-inflammatory cytokines
  IL-1β
  TNF-α
	Type 1 IL1
	Types 1 & 2
	TNF
		COX-upregulation.
		Sensitization of nociceptive neurons.

2nd messengers **Nitric Oxide (NO)**	-	Sensitization of STT cells.

A phenomenon called central sensitization, the manifestation of synaptic plasticity at the spinal cord level, was suggested as a possible causal mechanism (Randic et al., 1993; Baranauskas and Nistri, 1998; Ji et al., 2003). Sensitization is a neurophysiological term defined as "increased responsiveness of neurons to their normal input or recruitment of a response to normally subthreshold inputs" (Loeser and Treede, 2008). Interestingly, central sensitization manifests striking similarities with a phenomenon associated with memory formation and retention – long-term potentiation (LTP; Fig. 5; Ji et al., 2003; Lynch, 2004)

Sensitization of nociceptive pathways can be peripheral or central, depending on the part of the nervous system involved. Peripheral sensitization is related to
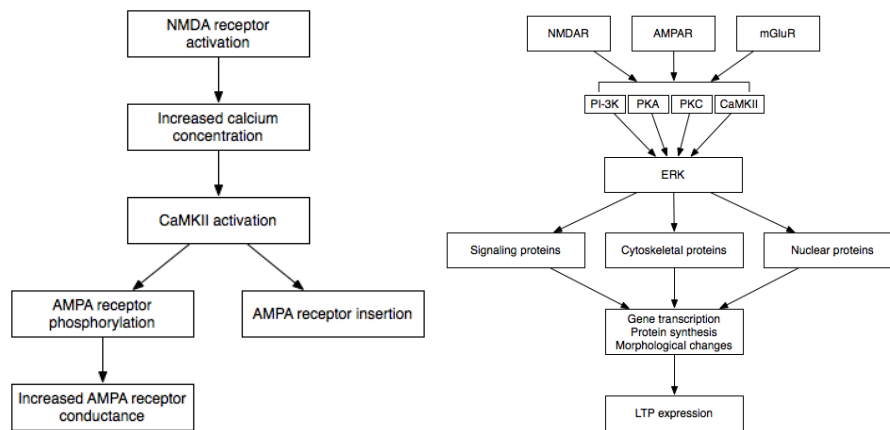
Figure 5. Similarity of the LTP and central sensitization pathway. Adapted from (Lynch, 2004).

the sensitization of peripheral ends of nociceptors located in the skin, muscles, joints and viscera (Millan, 1999). Sensitization arises due to the action of inflammatory chemicals, released around the site of tissue damage or inflammation, which can activate nociceptors directly (ATP, H^+) or indirectly (cyclooxygenase, COX-2, production by activated inflammatory cells). The COX-2 converts arachidonic acid to prostaglandins, which increase the sensitivity of peripheral nociceptor terminals (McCleskey and Gold, 1999; Julius and Basbaum, 2001). COX-2 was shown to be released also in the CNS due to peripheral inflamamtion (Vardeh et al., 2009).

Central sensitization, the subject of this study, is one possible mechanism of post-injury pain hypersensitivity because it has been shown that increased sensitivity after injury is not only attributable to peripheral sensitization (e.g., reduction in threshold of nociceptors) but is also a consequence of increased excitability of dorsal horn neurons (Tab. 4; Woolf, 1983). Central sensitization in the dorsal horn of the spinal cord is characterized by increased spontaneous

activity, enlarged receptive field areas, and increased responses evoked by large- and small-caliber primary afferent fibers. It manifests clinically as phenomena such as hyperalgesia ("increased pain sensitivity") or allodynia ("pain in response to a non-nociceptive stimulus"), (Woolf and Salter, 2000; Scholz and Woolf, 2002; Loeser and Treede, 2008).

Postsynaptic effects of synaptic transmission normally last for several tens of milliseconds (Purves, 2001). At certain conditions, neurotransmission-activated cascade of biochemical events may influence the neuron's future response to stimuli. Although the neurotransmitter (first messenger) becomes inactivated rapidly, the effects of the second messenger may last. These postsynaptic-membrane-depolarizing events converge on a key trigger element of biochemical cascades underlying synaptic plasticity - calcium ions (Balaban et al., 2004). They activate directly or indirectly a range of intracellular effectors, eventually strengthening the synapse and increasing the neuronal excitability (Ghosh and Greenberg, 1995; Baranauskas and Nistri, 1998; Cruzalegui and Bading, 2000).

A prominent component of the biochemical cascade leading to induction of central sensitization is CAMKII. Due to its ability for autophosphorylation, CaMK activity can outlast the intracellular calcium transient that is needed to activate it. In neurons, this property is important for the induction of synaptic plasticity. Pharmacological inhibition of CaMKII blocks the induction of LTP in hippocampus. Upon activation, CaMKII phosphorylates postsynaptic glutamate receptors and thus changes the properties of the synapse.

From temporal point of view, sensitization can be rapid, transcription-independent (short-term synaptic plasticity), or delayed, transcription-dependent (long-term synaptic plasticity).
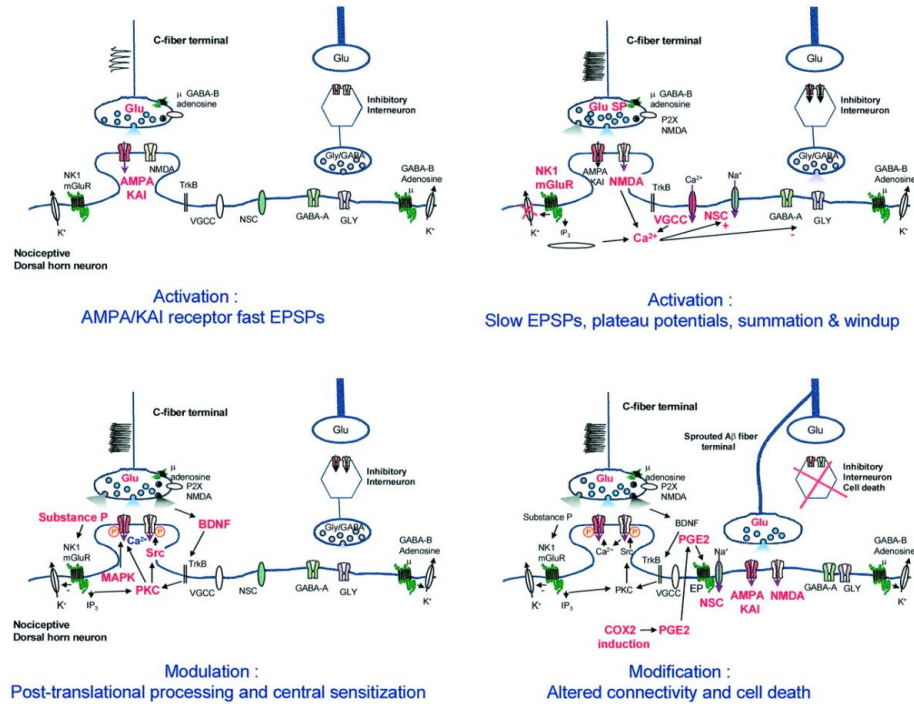
Figure 6. Types of synaptic changes in spinal cord associated with neuronal plasticity (Woolf and Salter, 2000).

The rapid changes of synaptic strength are achieved through changing how well receptors respond to stimulation by altering the length of time they are active. This is associated mainly with phosphorylation and conformational changes in receptors/channels protein molecule of the ionotropic glutamate receptors, (AMPA/kainate, NMDA). Another possible way is the increase in the number of receptors physically present due to receptor cycling between cytoplasm and membrane, or by altering the amount of neurotransmitter (L-glutamate) that is released into the synaptic cleft (Rich and Wenner, 2007). The

sensitization mechanisms can therefore occur both presynaptically (Heinke et al., 2004; Nguyen et al., 2009) and postsynaptically (Ji et al., 2002; Kelly et al., 2005; Wei et al., 2006). For a plastic change to occur postsynaptically and be partially expressed presynaptically, a message must travel from the postsynaptic cell to the presynaptic cell in a retrograde (reverse) direction. Nitric oxide was suggested to be such a retrograde messenger that presumably initiates a cascade of events that leads to a presynaptic component of sensitization (Wu et al., 2001; Ruscheweyh et al., 2006; Zhang et al., 2006; Guan et al., 2007; Schmidtko et al., 2008).

The delayed (transcription-dependent) modulation of synaptic transmission leading to central sensitization is mediated through regulation of protein synthesis in the postsynaptic cell. A neurotransmitter activates a cAMP second messenger, which leads to the binding of a regulatory protein to a DNA strand, which controls the transcription of a gene responsible for protein (eg. new AMPA receptor) synthesis (Woolf and Salter, 2000; Scholz and Woolf, 2002).

A new protein may help to perpetuate the cells activity, or may induce structural changes (Ji et al., 2003). If results of the neural plastic adaptations persists, long-lasting changes in the neuronal structure may also occur (increased dendritic branching and complexity, increased number of synapses per neuron (Julius and Basbaum, 2001; Scholz and Woolf, 2002).

Wind-up is a specific phenomenon related to the hyperexcitability of nociceptors, which may be also implicated in generation of central sensitization (MENDELL and WALL, 1965). It is a homosynaptic activity-dependent plasticity characterized by a progressive increase in firing from the second-order dorsal

horn neurons during a train of repeated low frequency C-fiber or nociceptor stimulation. It is thought to represent an elementary form of central sensitization. The mechanism is NMDAR-mediated (Guirimand et al., 2000). Wind-up results from the temporal summation of slow synaptic potentials (during stimulation, neurotransmitters such as glutamate, substance P, and CGRP elicit slow synaptic potentials lasting several-hundred milliseconds). This produces a cumulative depolarization that leads to removal of the voltage-dependent Mg^{2+} channel blockade in NMDA receptors and entry of calcium ions. Increasing glutamate action progressively increases the firing-response to each individual stimulus.

Wind-up can be registered approximately between 3 and 30 seconds after the last stimulus presentation, the magnitude is decreased with increases in the inter-nociceptive stimulus interval, and increased with increases in both the nociceptive stimulus intensity and the number of nociceptive stimulus presentations, as was shown also in behavioral experiments (Lomas and Picker, 2005).

1.3.3 Experimental techniques used to study central sensitization

1.3.3.1 Capsaicin

Alkaloid capsaicin is a potent substance that causes strong acute pain after intradermal application in humans and is frequently used in pain research (Fig. 7). It is a member of the

Figure 7. Capsaicin: Trans-8-Methyl-N-vanillyl-6-nonenamide.

capsaicinoid family – the derivatives of the o-methoxyphenol, it is a secondary

metabolite and anti-fungal agent of plants belonging to the genus Capsicum (Kim and Chancellor, 2000). Capsaicin is also a neurotoxin having LD50 in rat 8, 190 and 512 mg.kg^{-1} after administration through stomach, hypodermis and skin surface respectively. It irritates quickly mammalian tissues thanks to rapid trans-dermal effect causing sensation of burning upon contact with mucous membranes. However after an initial irritation it produces long and intense analgesics effect.

The effect of capsaicin in nociception is thought to be mediated through activation of TRPV1 receptors that are present in both peripheral and central branches of C-fiber primary afferents. Though TRPV1 receptor is the only known receptor activated by capsaicin, capsaicin's mechanism of action may not be limited to the TRPV1R's binding. Capsaicin was also found to affect the operation of both voltage-gated sodium and calcium channels (Lundbaek et al., 2005) and a recent study suggests that modulation of the excitatory synaptic transmission by capsaicin is not mediated by TRPV1 receptors (Benninger et al., 2008).

Though capsaicin was found to facilitate excitatory synaptic transmission at the spinal cord level (Yang et al., 1998), prolonged exposure of TRPV1 receptor to capsaicin elicits Ca^{2+}-dependent desensitization. Capsaicin reversibly depletes spinal cord presynaptic mediators, namely substance P, leading to reduction in sensation of pain (Gibson et al., 1982). Such depression of synaptic excitation increases with temperature, and is modulated by ATP (Kusudo et al., 2006).

1.3.3.2 TRPV1 receptor

The TRPV1 receptor is a member of the transient receptor potential (TRP) receptor family - the vanilloid receptor subtype 1 (TRPV1; for review see

Tominaga and Tominaga, 2005). It is found both in the peripheral and central nervous system.

The TRPV1 possesses homotetramer structure with multiple regulatory sites that have been proposed to play a definitive role in "fine-tuning" receptor responses to agonists (Fig. 8, Caterina et al., 1997). Further, the receptor physically interacts with a regulatory protein FAF1 (Fas-associated factor 1, known to induce apoptosis), which constitutively regulates, when expressed heterologously, the TRPV1 receptor's response to capsaicin (Kim et al., 2006).
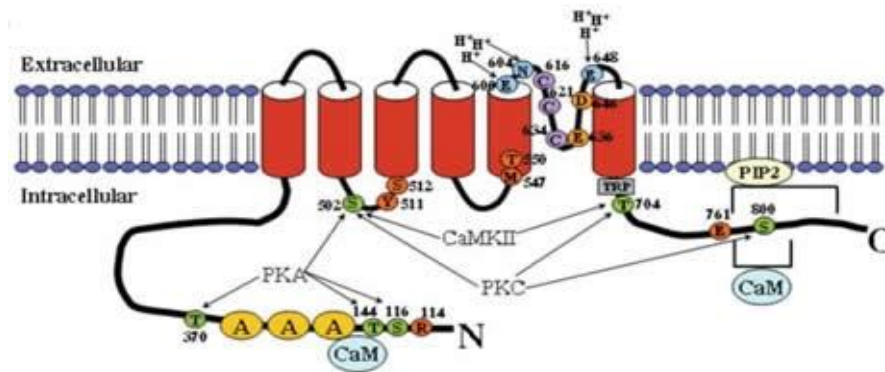
Figure 8. A schematic diagram of the TRPV1 subunit. Six transmembrane domains (red) and a pore loop; vanilloid binding – orange; A - ankyrin repeats (yellow); phosphorylation sites (green); protonatable amino acids in the "P-loop" (blue); cysteine reduction sites in the P-loop (purple); the TRP domain (box; modified from Tominaga and Tominaga, 2005).

The TRPV1 receptor is a lipophilic, nonselective cation channel with moderate selectivity for calcium ($pCa^{2+}:pNa^+=9.6$; fractional calcium current $Pf=6.6 - 9.9$ %; for comparison of Pf values see Tab. 5). It is considered to act as an integrator of various physical and chemical nociceptive stimuli, as it can be

gated by noxious heat (>43 ºC), low pH (protons) and also by endogenous lipids. It is the only member of at least four DRG-expressed TRPV receptors to be activated by both capsaicin and protons (pH < 6.4 at 37 °C). The activation by protons is associated with higher calcium permeability (higher fractional current Pf, see Tab. 5) compared to activation by capsaicin (Samways et al., 2008).

Peripheral TRPV1 receptors and the role of capsaicin in its activation is well explored compared to the central nervous system. In the peripheral nervous system, TRPV1 receptor is found mainly in skin on peripheral terminals of primary neurons, specifically on A delta and C fibers. However, there is an evidence of TRPV1 receptors in spinal cord, raising a question about their pain related function and about an existence of an endogenous ligands (Spicarová and Palecek, 2008). One of the endogenous agonist is thought to be anandamide (Smart et al., 2000; Smart and Jerman, 2000). It was also suggested that TRPV1 receptors in the central nervous system may function as a cannabinoid-gated channel and has been proposed as a target for treatment of pain (Szallasi et al., 1999).

The TRPV1 receptor can act as a proton channel enhancing painful sensation by acidification of nociceptors in inflamed or ischemic tissue (Hellwig et al., 2004). It was shown recently that inflammatory mediators augment the activity of TRPV1 receptor via the action of protein kinase A or protein kinase C, leading to TRPV1 receptor phosphorylation, leading to increase of capsaicin induced currents in the sensory neurons (Premkumar and Ahern, 2000). For instance, pro-inflammatory bradykinin causes hyperalgesia by enhancing TRPV1 sensitivity via signal transduction cascade involving PLCβ, and PKC. PLCβ

hydrolyzes phosphoinositol 4,5-biphosphate (PIP2→IP3+DAG), an endogenous

inhibitor of the TRPV1 receptor, thereby sensitizing the TRPV1 receptor.

1.4 INTRACELLULAR CALCIUM SIGNALING

1.4.1 Role of intracellular calcium

Calcium signalling is a versatile and ingenious system of further information processing at subcellular level of postsynaptic neuron. Neuronal calcium acts as a charge carrier during synaptic transmission and as one of the most important second messenger molecules, with a diverse array of effectors (Berridge et al., 2000). During neuronal excitation and action potential propagation, neurons increase cytoplasmic calcium concentration using two principal sources of calcium: the extracellular space and intracellular calcium stores (ICS). The pathways that lead to Ca^{2+} activation are ligand or voltage-gated ion channels, G protein-regulated pathways, and pathways regulated by receptor-tyrosine kinases.

Low intracellular concentration of calcium 0.1 µmol/l, some four orders of magnitude lower than the extracellular concentration, allows for graded concentration coding (changes of free intracellular calcium concentration) of transmitted signal via rapid increase of intracellular calcium concentration 10-100 fold (for review see Berridge, 1997, 1998). Calcium signals operate on wide temporal and spatial scale (Berridge et al., 2003). In spatial domain, calcium signals can be classified as local, global (intra- and intercellular waves), and those being associated with dynamic calcium changes in intracellular compartments (e.g. intracompartmental signals in endoplasmic reticulum and mitochondria triggering apoptotic processes or activating resident enzymatic cascades (Verkhratsky and Toescu, 2003; Verkhratsky, 2005)).

Complex global Ca^{2+} signals, such as Ca^{2+} oscillations and waves, are constructed by elementary Ca2+ signals that are recruited as basic building blocks due to limited effective range of action of both free (0.1 µm) and buffered (5 µm) calcium (Allbritton et al., 1992).

The elementary calcium signals themselves have been shown to provide local control of many physiological functions including ion channel activity and exocytosis. Local effects often manifests in a spatially restricted region of dendritic spine aparatus. Spines, often with ER's membrane protrusions, limit spatial range of calcium concentration transients, which are highly localized around channel mouth reaching values from 1 to 6 µM in units of ms to peak values 300 µM. This way, spine is a convenient place, where synaptically transmitted information, encoded in calcium transients, can be expressed by plastic changes of the synapse.
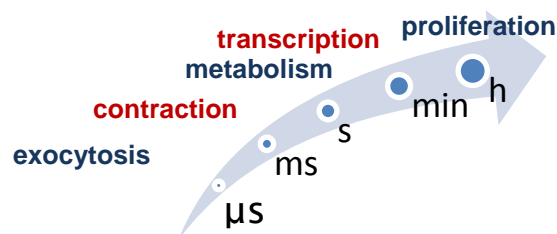
Figure 9. Range of cellular processes in temporal domain triggered by calcium signal.

In a temporal domain (Fig. 9), on a relatively fast time scale (milliseconds), calcium from extra- or intracellular sources directly modulates electrical activity through its control of calcium dependent potassium channels. On a long scale (minutes to days), calcium effects are fundamental to numerous aspects of neuronal development and plasticity including proliferation, secretion,

cytoskeleton management, gene expression, and metabolism (Berridge et al., 2000).

1.4.2 Extracellular routes of calcium entry

Calcium ions move from the extracellular space through plasmatic membrane predominantly by voltage-gated calcium channels (VGCC), which allow electrical activity to moderate calcium concentration. The VGCC are located throughout the neuronal body and processes. This route of entry is largely accountable for the fast somatic calcium transients (Miyakawa et al., 1992) associated with action potential.

In dendrites, an important component of the local calcium concentration elevation form plasmalemmal calcium permeable NMDA and AMPA receptors (Mayer et al., 1987) that are known to be involved in synaptic plasticity. For relative contribution of various plasma membrane proteins to the total elevation of the intracellular calcium concentration see Tab. 5 of fractional calcium currents for several selected receptors/channels (Burnashev et al., 1995; Burnashev, 1996; Garaschuk et al., 1996; Tempia et al., 1996; Burnashev, 1998; Samways et al., 2008).

The fractional calcium current (Pf) is defined as a ratio of the amount of charge carried by calcium ions (Q_{Ca}) and the total charge (Q_T) carried by all permeant ions (Burnashev, 1996, 1998):

$$Pf\,(\%) = \frac{Q_{Ca}}{Q_T} \times 100.$$

Table 5. The fractional calcium currents

receptor / channel	Pf (%)	selected effects
NMDA	8-12	central sensitization, LTP, LTD
AMPA	0.5 - 3.9	
kainate	< 0.2 - 2.0	
$TRPV_1$	6.6 (H_3O^+) - 9.9 (capsaicin)	central sensitization
5-HT3	substantially permeable	
ATP (P_2X)	6.5	putative role in presynaptic neurotransmitter exocytosis
acetylcholine	2.5-4.7	may facilitate spontaeous and evoked glutamate release presynaptically

The values substantially differ depending on subunit composition of the receptor/channel complexes (e.g. presence of AMPA GluR2 subunit) while channel location (somatic, dendritic) having negligible effect (Burnashev et al., 1995; Garaschuk et al., 1996; Tempia et al., 1996). Both non-NMDA receptor channels -the AMPA and kainate - are of the "low-Ca^{2+} permeable"-type and have a fractional Ca^{2+} current that is about twenty times smaller than that of NMDA receptor channels.

Other crucial components of the calcium regulating systems to be found in neuronal plasma membrane are extrusion pumps which oppose the action of calcium channels – the plasma membrane calcium ATPase and the sodium-calcium exchanger (NCX). The (PMCA) extrudes calcium into the extracellular space. It is a low capacity, high affinity (~300-600 nM) system transporting one calcium ion for each ATP molecule used.

The NCX is an antiporter, which removes calcium from cell using energy that is stored in the electrochemical gradient of sodium. The NCX has lower affinity than the PMCA, but it has 50 times greater capacity. The pump's exchange stoichiometry is three sodium ions for each calcium ion. This produces a positive net charge transfer of one proton for each transport cycle resulting in a small depolarization. The direction of the pump may be reversed due to the exchanger's dependence on the sodium concentration gradient. This may happen by either depolarization of the neuron, an increase in internal sodium concentration, or a decrease in external sodium concentration.

Intracellular calcium stores in neurons (see chapter 1.4.3.) are refilled primarily by VGCCs during depolarization. Some neurons possess additional

plasmalemmal mechanism for replenishment of cytosolic calcium – the store operated channels (SOC; for review see (Bolotina, 2004; Parekh, 2008). The SOC are activated by calcium ions from depleted stores (I_{crac}, calcium release-activated calcium current. (Fig. 10) SOC have extremely low conductance, they are voltage independent and activation time in range from 100 ms (lymphocytes) to days (Xenopus oocytes).

Figure 10. Activation of the store operated channel (SOC) via the InsP3/Ca^{2+} signaling pathway.

The putative way the signal about store depletion is transmitted via direct contact due to the colocalization of IP$_3$R and plasma membrane and region restriction of the SOC opening.

1.4.2.1 Intracellular calcium stores

While the influx of extracellular calcium is extensively studied, less information is known about mobilization and supply of intracellular calcium stores. The intracellular calcium stores make up endoplasmic reticulum and, in a smaller extent, mitochondria.

1.4.2.2 Endoplasmic reticulum

Endoplasmic reticulum (ER) is the key neuronal calcium signaling organelle and a dynamic calcium reservoir. The ER integrates extracellular and intracellular (e.g. IP3, Ca^{2+}) input signals in the form of intraluminal free calcium concentration, [Ca^{2+}]l. The [Ca^{2+}]i is determined by a balance between the

calcium flux across the endomembrane into cytoplasm and the intraluminal calcium buffering (Fig. 11).

The ER's calcium is mobilized primarily via the inositol 1,4,5-trisphosphate
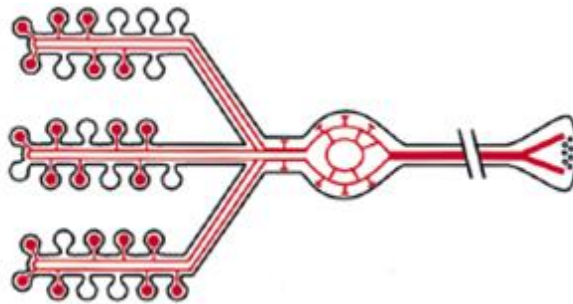
Figure 11: The bimembrane system of a neuron. Endoplasmic reticulum in red (adapted from (Berridge, 1998).

receptor (IP3 receptor) and ryanodine receptor (RYR) releasing calcium into cytoplasm. Both receptor types share structure similarities and are expressed in several isoforms with broad tissue distribution. The third putative calcium release channel was also described; it is activated by sphingosine-1-phosphate and is termed SCaMPER (sphingolipid Ca^{2+} release-mediating protein of the ER).

The InsP3R's activity is controlled by free cytoplasmic Ca and by second messenger IP3. RYR opens directly by free cytoplasmic Ca^{2+} or pharmacological compounds such as caffeine. RYR modulators are cyclic ADP ribose (cADPR) produced by plasmalemmal ADP-ribosyl cyclase and by free calcium. Probability of opening of both channels increases with degree of loading of a ER lumen with calcium. The intraluminal level of calcium is maintained by an activity of SERCA pump. Failure of reticular calcium homeostasis manifests as an unfolded protein

response and endoplasmic reticulum overload response. These forms of stress

responses may trigger apoptotic signals (Verkhratsky, 2005).
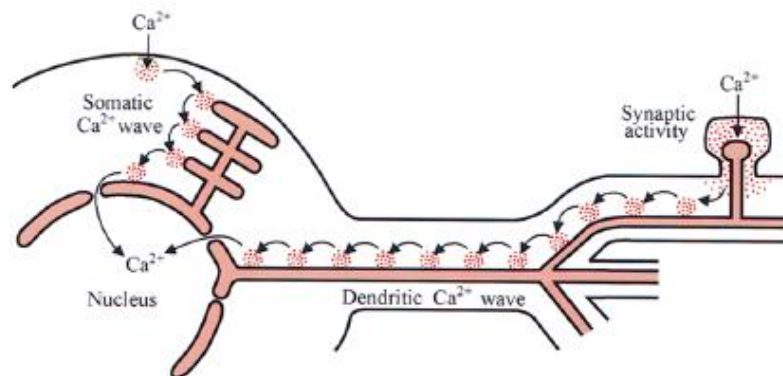
Figure 12. Calcium-induced calcium-release, a regenerative

mechanism of calcium release (Berridge, 1998).

Ins3P receptor underlies local and global calcium fluctuations through

activation of metabotropic receptors either by glutamate or by neuropeptides like

Substance P (inositol-1,4,5-trisphosphate induced calcium release, IICR). The

ER also possesses a unique mechanism of amplification of the calcium signal

entering via cytoplasmic membrane voltage gated calcium channels (VGCC) –

calcium-induced calcium-release (CICR; Fig. 12). The main CICR mediator in

dorsal root ganglion neurons is RYR type 3, calcium sparks from discrete calcium

release units, shows no apparent inactivation in the DRG neurons and

importantly it is a low gain linear function of the trigger calcium concentration

(Tab. 6).

Table 6. Control of CICR in DRG sensory Neurons

CICR-mediator	RYR3
Trigger signal	I_{Ca} at high density
Elemental events	Calcium sparks from discrete CRUs (Ca release unit)
Architecture of control	Hybrid; subsurface CRUs – local control, medullar CRUs forming a common pool system
Peak calcium level	Bell-shaped calcium dependence Fluo-4 ΔF/F signal ≈ 3-4 at -10 mV
Inactivation	No apparent
Ca^{2+} storage capacity	15 rounds of all CRUs firing
Linearity	**Linear function of the trigger calcium concentration**
Gain	**Low: 0.54** (ventricular myocyte: 10-70)
Stability	**Unconditionally stable** Unable of spontaneous calcium waves (ventricular myocytes: stable under normal Ca^{2+} load; instability under Ca^{2+} overload)

1.4.2.3 Mitochondria

Mitochondrion serves as a key neuronal organelle in energetic metabolism, steroid synthesis or apoptosis and its dysfunction has fatal consequences on neuronal integrity. They are abundant within neuronal presynaptic terminals, where they provide energy for sustained neurotransmitter secretion. Mitochondria also possess mechanisms for taking up glutamate that are quite distinct from membrane glutamate transporters (Danbolt, 2001)

Besides these well-explored functions, less is known about its role as a component of intracellular calcium homeostatic system. Although it has long been understood that significant calcium flux occurs across the mitochondrial

membranes, many studies have demonstrated that this flux can have a large impact on the amplitude and time course of calcium transients in neurons (Friel and Tsien 1994; Thayer and Miller 1990; Werth and Thayer 1994). The outer mitochondrial membrane is freely permeable to calcium ions. The control system of calcium ions' flux into and out of the matrix across the inner mitochondrial membrane (for review, see Babcock and Hille 1998; Duchen 1999; Simpson and Russell 1998) involves membrane proteins accountable for calcium supply into the matrix: the uniporters dependent on mitochondrial membrane potential, transporting calcium down its electrochemical gradient. The counter flow of calcium ions back into the cytoplasm is mediated through sodium-calcium exchanger (NCX) on the inner mitochondrial membrane, which is also the primary efflux mechanism thought to operate during normal cellular function. Another pathway for calcium efflux is the permeability transition pore, which conductance is activated at relatively high calcium concentration in mitochondrial matrix, and its opening may be a key to the calcium-mediated cell death.

1.4.3 Calcium binding proteins

Neurons further possess another important component of the calcium signaling and homeostatic toolkit: calcium binding proteins (CaBP). They can be divided into endogenous buffers, which shape the cellular response by regulating concentration of free intracellular calcium, and calcium sensors carrying out the messenger role of Ca^{2+}.
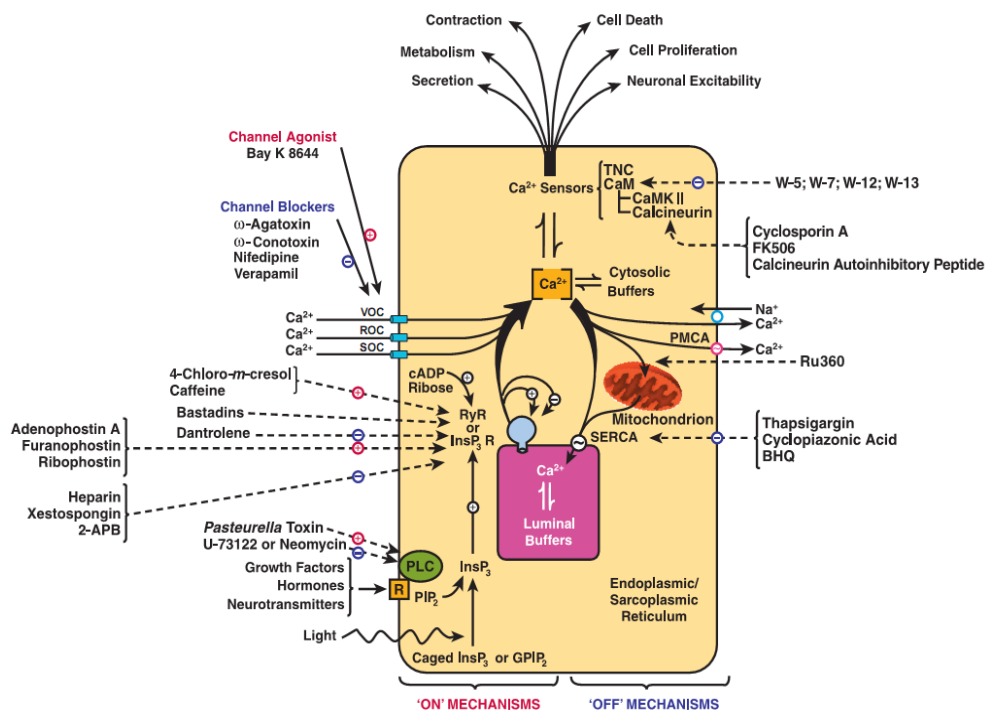
Figure 13. Calcium homeostasis in postsynaptic neuron

and targets of selected pharmacological agents.

1.4.3.1 Endogenous calcium buffers

Cytoplasmic protein molecules binding free calcium ions serve as mobile endogenous buffers, decreasing free calcium concentration and thereby preventing binding of calcium to (activation) other molecules (Fig. 13, 14). These proteins can substantially affect the spatiotemporal characteristics of any

intracellular calcium concentration change, depending on their respective biophysical calcium binding characteristics (Tab. 7).

The fact that more than 95% of Ca^{2+} ions that enter cells under physiological conditions are buffered by endogenous mechanisms is indicative of importance of calcium buffering system for calcium homeostasis (Blaustein, 1988; Neher and Augustine, 1992; Zhou and Neher, 1993).

Table 7. Biophysical characteristics of the slow calcium buffer parvalbumin, fast calcium buffer calretinin) and two common chelators EGTA and BAPTA (Lajtha and Banik, 2007).

	Parvalbumin Kd and k_{on} depends highly on Mg^{2+} concentration	Calretinin	EGTA	BAPTA
Ca^{2+} binding sites	3 (2)	6 (5)	1 (1)	1 (1)
K_d (nM)	150	1500	150	160
k_{on} ($\mu M^{-1}s^{-1}$)	6	100-1000	3-10	100-1000
k_{off} (s^{-1}) = $K_d k_{on}$	0.9	150-1500	0.5-1.5	16-160
$D_{Ca\text{-}buffer}$ ($\mu m^2 s^{-1}$)	43	≈ 25	200	200
$\tau_{capture}$ (ms)	3	0.007-0.07	4-10	0.04-0.4
$d_{capture}$ (μm)	0.4	0.03-0.09	0.7-1	0.07-0.2

It was shown in other neuronal systems that low level of calcium buffers leads to high amplitude short lasting calcium concentration change, while presence of high level of calcium buffers in the cell leads to low amplitude, longer duration calcium transient under the same circumstances (Chard et al. 1993; Palecek et al. 1999). While both fast (calbindin) and slow (parvalbumin) calcium buffers can reduce the calcium transient peak, PV selectively increases the fast component in the calcium transient decay (Chard et al. 1993; Caillard et al. 2000; Lee et al. 2000; Schmidt et al. 2003; Muller et al. 2007). The presence or absence of CBPs acting as calcium buffers in the pre- or postsynaptic part of the

synapse could thus effectively modulate synaptic transmission at the spinal cord level.

The most abundantly expressed calcium buffer in the central nervous system and the focus of attention of this study is a 12 kDa, low molecular weight protein parvalbumin. Parvalbumin is a high affinity calcium ion-binding protein that is structurally and functionally similar to calmodulin and troponin C. It is a member of a protein family with the EF-hand calcium-binding domain.

Studies of PV distribution at the lumbar spinal cord have shown, that it is mostly located in a strongly immune-positive band of small neuronal bodies and neuropil in the inner part of lamina II (Antal et al. 1990; Ren and Ruda 1994) and at the border of laminae II/III (Yamamoto et al.1989). There is also strong PV immunostaining present at the neck of the spinal cord grey matter in the ventro-medial region (lamina VII). Number of PV positive neurons is are present throughout the deeper laminae and also in the ventral horn (Ren and Ruda 1994).
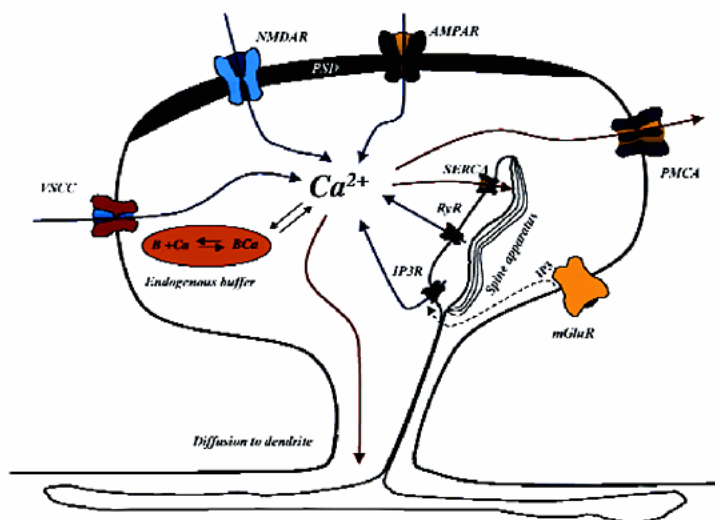
Figure 14. Regulation of free calcium concentration in spine by endogenous. buffer.

Intracellular calcium stores such as endoplasmic reticulum also bind calcium to proteins. Important luminal calcium binding proteins are calsequestrin and calreticulin /Berridge/, characterized by low-affinity (K_d in mM range) and high-capacity (approx. 50

Ca^{2+} ions/molecule). Only small portion of the total calcium pool is in the form of free calcium ions, which target their effectors.

All the aforementioned components (Fig. 13, 14) of the calcium homeostatic system are used as a basis for quantitative models of various complexities. In its most general form, a simplified model of calcium homeostasis (derived from the model of calcium wave) is summarized in the following equation. Pumps, buffers, diffusion, channels all contribute to modify calcium concentration. The equations for each of these mechanisms calculate the change in calcium with respect to time.

$$\frac{d[Ca2+]_{cyt}}{dt} = \frac{1}{V_{cyt}} \left(\phi_{IP3R} + \phi_{RYR} + \phi_{SERCA} + \phi_{PMCA} + \phi_{bufcyt} + \phi diffusion + \phi VGCC, \right.$$

where $[Ca2+]_{cyt}$ is the calcium concentration in cytosol, V_{cyt} is the cytoplasmic volume and ϕ is the flow of calcium through individual components (IP3R: Inositol-3-phosphate receptor; RYR: ryanodine receptor; SERCA: Sarco/Endoplasmic Reticulum Ca^{2+}- ATPase; PMCA: plasma membrane Ca^{2+}- ATPase; buf cyt: cytosolar buffers; VGCC: voltage gated calcium channels (Meyer and Stryer, 1991).

1.4.3.2 Calcium sensors

Induction of the calcium sensitive cellular processes is achieved by binding of free calcium to its effectors. Activation of Ca effectors occurs when free calcium concentration (regulated by set of components of calcium homeostatic system) exceeds a limiting level as happens during neural activity (Fig. 15).
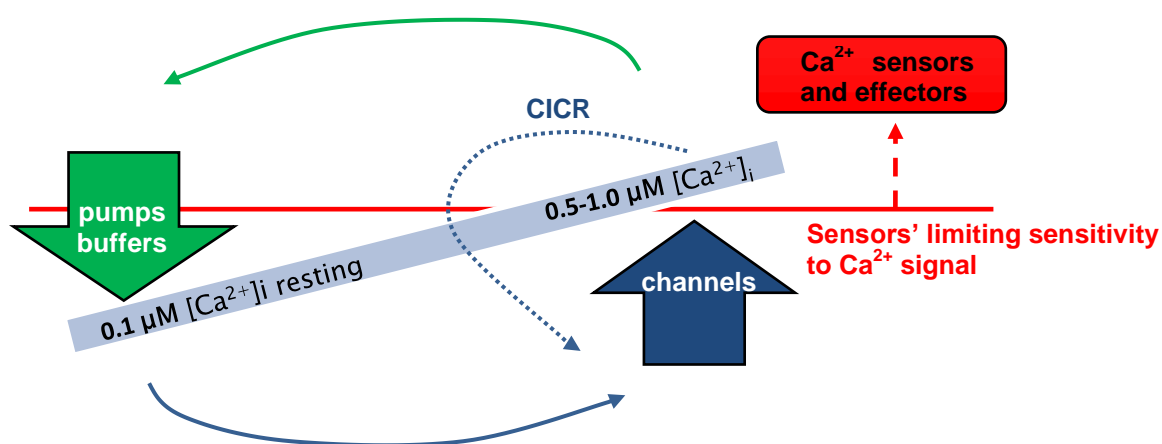
Figure 15. Dynamic equilibrium of free intracellular calcium and shaping of the calcium signal is done by a counterbalance action of calcium mobilizing (blue wide arrow) and removing (green) systems. Calcium concentration exceeding a boundary value activates Ca-effectors that consequently trigger vast array of cellular processes. Thin full arrows depict negative and dashed arrows positive feedback reactions of calcium mobilization and removal. CICR – the calcium-induced calcium-release positive feedback mechanism.

Free cytoplasmic calcium can regulate proteins either by direct recognition of calcium by the protein or binding of calcium in the active site of an enzyme. The long-term effects are mediated by various kinases and phosphatases. One of the most explored interactions of free calcium with a cytoplasmic protein in the process of neural signal transduction is the regulation of calmodulin by formation

of the Ca^{2+}/calmodulin complex (calmodulin has 4 Ca^{2+} binding sites). The complex is an activator or regulator of several enzymes, including calcium-calmodulin dependent protein kinase (CaMK), which plays a role in synaptic plasticity, and adenylate cyclase (AC), which produces cAMP, another important second messenger that activates cAMP-dependent protein kinase (protein kinase A or PKA). Calmodulin itself can regulate other proteins, or be part of a larger protein (e.g. phosphorylase kinase). Calcium is further, together with diacylglycerol (DAG) coactivator of conventional isozymes of protein kinase C (PKC-α,β,γ ;(Mellor and Parker, 1998), which play an important role in a phenomenon unique to the nervous system - synaptic plasticity.

2 AIMS OF THE STUDY

- Analysis of calcium binding proteins expression changes in the lumbar spinal cord dorsal horn and in retrogradely labeled spinal STT neurons after experimentally induced peripheral inflammation in rats using Western blot technique and immunohistochemistry.

- To design calcium imaging setup for measurement of dorsal root stimulation-evoked calcium concentration changes during synaptic transmission in spinal dorsal horn neurons in acute slices using confocal microscope.

- Pharmacological characterization of calcium concentration changes during synaptic activity in dorsal horn neurons. Dependence on the dorsal root stimulation parameters.

- Development of an in vitro model of central sensitization using activation of spinal cord primary afferents with capsaicin.

3 METHODS

56

3.1 MATERIALS

57

Invitrogen, USA	Fura-2 AM
	Fluo-3 AM
	Fluo-4 AM
	Fura Red AM
	OGB-1 AM
	Dextran labeled with Cy2
Sigma-Aldrich, USA	DMSO
	Caffeine
	CNQX
	MK801
	TTX
	Antifoam A
	Pluronic F-127
	DTT
	Iodoacetamide
	Proteases inhibitor P8340
	Sulforhodamine 101
	Anti-calbinding, -parvalbumin, -calretinin antibody
	BSA (bovine serum albumin)
	DPX synthetic resin
Tocris Bioscience, UK	CPCCOEt
	L-AP3
	R,S-MCPG
	AIDA
	MPEP
	Nitrendipine
	DHPG

LY367385

Jackson ImmunoResearch Laboratories, USA	Secondary antibody peroxidase conjugated
	Biotinylated antibody peroxidase conjugated
	Donkey serum

All other common laboratory chemicals were purchased from Lachema, Czech Rep. or Sigma-Aldrich, Czech Rep.

All chemicals were of p.a. purity.

3.2 SOLUTIONS AND BUFFERS

Buffer for Homogenization I (HB; pH 7.5)

- 0.25 M sucrose (or 0.9 % NaCl to get rid of viscosity)
- 1.0 mM EDTA
- 20 mM Tris-HCl
- 3.0 mM $MgCl_2$
- 0.02 % NaN_3
- H_2O
-

Buffer for Homogenization II (HB; pH 7.5)

- 30 mM Tris-HCl
- H_2O

Separating Gel (10 ml, 15 % of acrylamide)

- A: 30 % acrylamide / 0.8 % bis-acrylamide (5 ml ≈ 3.0 g acrylamide)
- B: Tris-Cl / SDS; pH 8.45
- C: H2O (0.6 ml)
- D: Glycerol (1,3 g)
- E: APS 40 µl 10% (w/v)
- F: TEMED 4.0 µl
-

Stacking Gel (8.3 ml, 4 % of acrylamide)

- A: 30 % acrylamide / 0.8 % bis-acrylamide (1.1 ml)
- B: Tris-Cl / SDS, pH 8.45 (2.1 ml)
- C: H_2O 5.2 ml
- E: 10 % (w/v) APS 45 µl
- F: TEMED 4.5 µl

Solution A: 30 % acrylamide/0.8 % bis-acrylamide (50ml, 4 °C)

- 15.0 g acrylamide
- 0.4 g N,N'-methylene-bis-acrylamide
- H_2O to 50ml

Solution B: Tris-Cl/SDS (pH 8.45; 100 ml, 4 °C)

- 36.4 g Tris Base in 60 ml H_2O ($\approx$ 3.0 M Tris-Cl)
- 12 M HCl (pH adjustment)
- H_2O to 100 ml
- SDS 0,3 g ($\approx$ 0.3 % SDS)

Solution E: 10 % (w/v) APS

Dissolve 50 mg APS in 500 µl H_2O. Prepare fresh.

4X Tris-Cl/SDS (10 ml; pH 8.0)

- 0.60 g Tris base in 4 ml H_2O.
- 12 M HCl (pH adjustment)
- H_2O to 10 ml total volume
- 0.04 g SDS.

Tricine Sample Buffer, 2x (10 ml, 4°C)

- 2 ml 4x Tris-Cl/SDS ($\approx$ 0,1M)
- 3.2 g glycerol
- 0.8 g SDS (recrystallization optional)
- 2 mg Coomassie blue G-250
- H2O to 10 ml

Running Buffer 10X (cathode buffer; 400 ml)

- 100 mM Tris pH 8.25 ($\approx$ 60,56 g Tris base)
- 100 mM Tricine (89.60 g Tricine)
- 0.1 % SDS
- 5 g SDS
- H_2O to 400ml, pH 8.25 /no adjustment needed/

Dilute 10X stock solution 1:9 with H_2O before use.

Towbin Buffer (1000 ml)

- 3.03 g Tris base (25 mM)
- 14.4 g glycine (192 mM),
- 20% (v/v) methanol (pH 8.3)

Dissolve 3.03 g Tris base and 14.4 g glycine in 500 ml H2O. Add 200 ml of methanol; add ddH_2O to 1000 ml.

Note: the pH will range from pH 8.1 to 8.5 depending on the quality of the Tris, glycine, methanol, and H2O, no SDS for low molecular weight proteins. Prepare fresh.

Tris-buffered saline (TBS; pH 7.5, 5000 ml)

- 12.1 g Tris base (≈20 mM Tris-Cl)
- 146.2 g NaCl (≈500 mM NaCl)
- 12 M HCl (pH adjustment)
- H_2O to 5000 ml

TTBS Wash Solution (pH 7.5, 4012 ml)

- 0.05% Tween 20 (pH 7.5) in TBS

Add 12 ml Tween 20 (1:5 w/w in ddH_2O) to 4000 ml TBS.

Blocking Buffer (10 ml)

- 2 % BSA-TBS

Add 0.2 g BSA to 10 ml TBS. Stir to dissolve. Prepare fresh.

Antibody-diluting Buffer (50 ml)

- 1 % BSA-TTBS

Add 0.5 g BSA to 50 ml TTBS. Stir to dissolve. Prepare fresh.

Note: Non-azide solution if using HRP conjugates.

PBS (1000 ml)

- 8.0 g NaCl
- 0.20 g KCl
- 1.44 g Na_2HPO_4

- 0.24 g KH_2PO_4
- 800 ml H2O

Coomassie Stain Solution (1000 ml)

- 1 g Coomassie Blue G-250
- 400 ml methanol
- 100 ml acetic acid
- 500 ml H2O

Destain Solution

- 400 ml methanol
- 100 ml acetic acid
- 500 ml H2O

Fluo-4 AM Stock Solution

- 1 mM Fluo-4 AM (in DMSO)
- 20% Pluronic acid (w/v, in DMSO)

All aqueous solutions were prepared with redistilled water.

3.3 ANALYSIS OF PROTEIN EXPRESSION

3.3.1 Experimental Arthritis

The experimental arthritis was induced by unilateral intra-articular injection of 3 % mixture of kaolin and carrageenan in physiological saline solution under ether anesthesia. The animals were left to recover in their home cages for 24 to 28 hours before the transcardial perfusion. Arthritis was not induced in a control group of animals.

3.3.2 Immunohistochemistry

The immunohistochemical analysis of spinal cord calcium-binding protein expression was performed on population of spinal cord dorsal horn neurons of arthritic adult male rats (250 – 350 g) and on subpopulation of fluorescently labeled STT neurons using retrograde tracer (Palecek et al., 2003). 7-10 days after the retrograde tracer injection the rats were deeply anesthetized with sodium pentobarbital (70 mg/kg i.p.) and transcardially perfused with heparinized physiological saline solution followed by an ice cold solution of 4% paraformaldehyde. The fixed spinal cords and brains were removed and placed overnight in the 4% paraformaldehyde solution. Spinal cord segments were marked in the ventral part with a fine pin on one side. The spinal segments L3-4 were cryo-protected in 30 % sucrose solution and then serially sectioned at 30μm thickness by cryocut (Leica Microsystems, Germany). The serial sections were placed in three vials so that slices in each one were separated by at least 60 μm (2 slices). Each of the vials was then used for immunostaining with a different specific antibody to calbindin, parvalbumin (monoclonal) or calretinin

(polyclonal). Slices were washed in phosphate buffered saline (PBS), incubated in 3 % donkey serum and incubated overnight at room temperature with primary antibody under constant slow motion. On the next day, slices were washed, incubated with Texas Red corresponding secondary antibody, washed, dried and mounted with synthetic resin DPX. In control experiments no positive stain was observed when primary antibody was omitted in the process. The histological slides were analyzed under a fluorescent microscope (Olympus AX, Olympus, Japan) with appropriate filters for Cy2 and Texas Red fluorescent dyes.

A percentage of labeled STT neurons in the L3-4 spinal segments expressing CBP was calculated and averaged in the experimental group. A paired t-test was used for evaluation of statistical differences between the ipsilateral and contralateral (control) sides.

3.3.3 Spinal cord tissue processing

For confirmation of arthritis-induced changes in parvalbumin expression levels, Tricine SDS-PAGE and western blot technique were used. Animals were deeply anesthetized with sodium pentobarbital (80 mg/kg intraperitoneally) and laminectomy was performed. Dissected lumbar spinal cord was rapidly immersed in ice-cold physiological solution and hemisected. Left and right side spinal cord parts (L3-5) were weighed and homogenized with hand-held pellet pestle in 30 mM Tris-HCl buffer (w/v 1:10) containing 0.1 µl of inhibitor of proteases per 1 mg of tissue. Prepared samples were let to sit on the ice for 10 min and centrifuged at 5000 rpm for 10 min at 4 °C. Supernatant was removed and centrifuged again at 13 000 rpm for 20 min. Aliquot was taken for spectrophotometric total protein concentration quantitation.

3.3.4 Quantification of the total protein concentration

The protein concentration for each sample was determined according to method of Bradford (Bradford, 1976) using Bio-Rad Protein Assay Kit (Eppendorf, Germany) and Eppendorf BioPhotometer according to the manufacturer's recommendations. Stock solution of IgG glycerol/water (1.43mg/ml) was prepared and aliquots were stored at -80 °C. 1.5 ml of Bio-Rad dye reagent was diluted in 6 ml of water (reagent/water - 1:4. Overall, 10 test tubes was prepared (3 samples per each spinal cord hemisection (dx, sin), 1 blank sample, and 3 standards). 10 microliters of sample buffer (HB) was placed in "blank" test tube, 3 x 10 microliters of sample, and 3 x 10 microliters of standards was placed in 3 clean dry test tubes. Standards were prepared as follows: Standard 1:10 microliters of IgG glycerol stock solution; Standard 2:7.5 microliters of IgG + 2.5 microliters of ddH_2O; Standard 3:5 microliters of IgG + 5 microliters of ddH_2O.

0.5 ml of diluted dye reagent was added to each of the 10 tubes and stirred (without excess foaming) or mixed several times by gentle inversion of the test tube. After a period of from 5 minutes to one hour, absorbance (A_{595}) of standards and samples versus absorbance of the reagent blank was measured using at least 60 microliters of the sample in a cuvette. Measurement was repeated five times in successive steps. Resulting A_{595} values of spinal cord samples were plotted against concentration of standards. Unknowns were read from the regression curve.

3.3.5 SDS Electrophoresis of low molecular weight proteins

3.3.5.1 Preparation of Gels

Parvalbumin, protein samples and molecular mass standards (Bio-Rad) were resolved on 12% gels using Tricine-SDS-PAGE, which is a preferred electrophoretic system for the resolution of proteins smaller than 30 kDa (Schägger and von Jagow, 1987). Glass plates of electrophoretic apparatus were carefully washed using distilled water and acetone. APS (solution E) and TEMED (solution D) were added to separating gel solution, gently swirled to mix. Separating gel was poured immediately between apparatus glasses 5.5 cm from the bottom of a shorter glass. Separating gel surface was covered with 0.5 cm thick layer of ethanol to isolate the surface from air oxygen, deactivator of polymerization. After the resolving gel polymerized, ethanol was removed and gel surface washed with the distilled water and dried using a thick chromatographic paper. The stacking gel was prepared and used immediately after adding APS (solution E) and TEMED (solution E) component. The stacking gel was poured by pipette onto the polymerized resolving gel to fill up the shorter glass. Immediately after pouring of the stacking gel the well form was put inside the gel and allowed 2 hours to polymerize. The well form was later removed and any unpolymerized acrylamide flushed out before sample loading. Gel was put into the electrophoretic apparatus, allowing equilibrating in electrophoretic (running) buffer for a while.

3.3.5.2 Preparation of samples and standards

In order to avoid proteins' modification, a reduction and alkylation of sample proteins was performed before proceeding to sample application to the

electrophoretic setup. A *cracking buffer* was prepared by mixing sample buffer (pH 8.0) with 0.5 M water solution of dithiothreitol (DTT) in the 100:1 volume ratio.

Subsequently, one volume of sample was added to one volume of the cracking buffer, stirred, and boiled for 3 min. in the Eppendorf tube in the block thermostat set to 100 °C. The tube was stirred again and allow to cool down at room temparature. After 20 min. period, sample was alkylated by incubating 1 min. at 100 °C with 1/20 volume (based on cracking buffer) of 0.25 iodoacetamide.

Then the sample was applied to gel or stored at 4 °C before use. Together with samples, a polypeptide kaleidoscopic molecular weight standard was loaded in one well (5 µl per well) to monitor electrophoresis in the real time. In order to identify luminescence signal of a protein of interest with help of western blot technique, biotinylated standard was used. The standard was heated at 100°C for 3 min, stirred and 10 µl was loaded to the gel (1 µl of stock solution of standard + 9 µl of the sample loading buffer). Every heating and stirring step was followed by 2 min-long centrifugation at 5 000.

3.3.5.3 Running electrophoresis

The prepared samples from left (sin) and right (dx) spinal cord parts were loaded to the gel at the amount of 10 µl per well alternatively (sin/dx/sin). The same amount caleidoscopic and biotinylated standard was used.

Electrophoresis had run at 85 V constant-voltage mode ($\approx$ 25 to 35 mA) for approx. 2 hours at 4 °C until blue bands reached the bottom of the gel.

3.3.5.4 Western blot

Proteins were then transferred to a 0.2 μm nitrocellulose membrane with blotting apparatus (Bio-Rad), at 15V overnight. Western blots were developed using technique based on method described by Towbin (Towbin et al., 1992).

Mouse monoclonal anti-parvalbumin was used as primary antibody at a dilution of 1:2000. The secondary antibody conjugated to horseradish peroxidase and/or biotinylated antibody followed by streptavidin peroxidase (Jackson Immuno Research Laboratories, Inc., USA) were used at a dilution of 1:6000, 1:32000 and 1:20000 respectively. The proteins were visualized with an ECL kit (Bio-Rad) and data were captured using Fuji LAS-1000 Imaging System CCD Camera. Quantitative analysis of immunoblots was performed using the image analysis program ImageJ (ver. 1.35h).

3.4 Calcium imaging study of spinal cord synaptic transmission

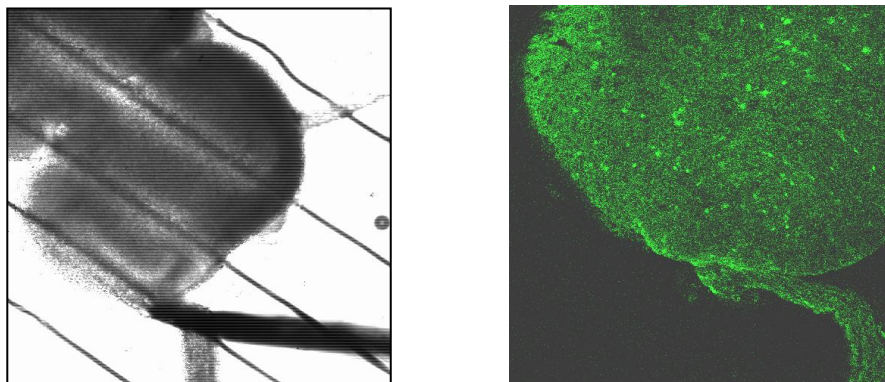### 3.4.1 Preparation of acute spinal cord slices

Figure 16. Acute spinal cord slice in the recording chamber with glass suction electrode approaching at the dorsal root of the spinal cord (left) and dorsal horn neurons in the slice labeled with fluorescent calcium sensitive probe Fluo-4 (right).

Transverse slices (300-400 μm; Fig. 16) with dorsal roots attached were prepared from lumbosacral enlargements of P 12-25 Wistar rat spinal cords as described in detail elsewhere (Palecek et al., 1999). In short, quickly dissected spinal cord of anaesthetized rat (sodium pentobarbital 50 mg/kg i.p.) was placed in ice-cold high-Mg2+/low-Ca2+ dissection artificial cerebrospinal fluid (dis-ACSF), consisting of (in mM) 95.0 NaCl, 1.8 KCl, 1.2 KH2PO4, 0.5 CaCl2.2H20, 7.0 MgSO4.7H2O, 26.0 NaHCO3, 25.0 D-glucose, 50.0 sucrose; pH 7.4. Dura mater was removed carefully so that spinal roots were left attached. Agar block-embedded lumbar enlargement was glued on the microtome stage chamber (Leica VT1000, Germany) filled with a gassed ice-cold dis-ACSF solution and sliced using sapphire blade. The slices with dorsal roots attached were transferred into a 31°C-warm dis-ACSF solution for 30 min to facilitate neurons' recovery.

All experiments were approved by the local Institutional Animal Care and Use Committee and were consistent with the guidelines of the International Association for the Study of Pain.

3.4.2 Fluorescent dye loading and calcium imaging

The method used for bulk loading of the spinal cord slice with esterified form of fluorescent dyes was similar to those described previously (Stosiek et al., 2003). Neurons in spinal cord slices were loaded with calcium sensitive dye Fluo-4 (Invitrogen, USA), which was selected for its excitation spectrum spectra matches well with the Argon laser 488 nm wavelength, providing high quantum yields.

The slices were incubated with Fluo-4 acetoxymethyl ester (10 µM) in the dis-ACSF medium containing 0.02 % Pluronic F127 (Sigma-Aldrich, MO, USA) at room temperature (24–26 °C, pH 7.4) for 60 min. The stained slices were then rinsed and kept in normal ACSF medium at room temperature (in mM: 127.0 NaCl, 1.8 KCl, 1.2 KH2PO4, 2.4 CaCl2.2H20, 1.3 MgSO4.7H2O, 26.0 NaHCO3, 25.0 D-glucose; pH 7.4, 302 mOsm).

For the imaging, slices were placed on a glass bottom of a submerged-type recording chamber, which was on a stage of an inverted laser scanning confocal microscope (TCS SP2 AOBS, Leica Microsystems, Germany) and continuously perfused with the normal ACSF (2-3 ml/min) at room temperature. The slice was maintained in the recording chamber under nylon mesh in order to avoid motion artifacts caused by the ACSF perfusion. Before recording, the slice was kept untouched for 15 min in the chamber to avoid possible tissue compression under

the causing z-axis motion. During this period, recording parameters of the confocal microscope, laser, and the electrical stimulator were adjusted.

The fluorescent dye Fluo-4 was excited using a 488/20mW Ar laser (Leica Microsystems, Germany) and the emitted light was filtered using a band-pass filter (498-600 nm). Confocal fluorescence images (512 x 330 pixels) were obtained using an oil immersion objective lens (HC PLAPO 20x, NA 0.70, ImmCorr CS; Leica Microsystems, Germany). Fluorescence signals in the spinal cord neurons were monitored at 293 ms frame rate.

Subsequent electrical stimulation of the dorsal root evoked calcium/fluorescence transients that were clearly visible (good signal to noise ratio) and not saturated due to technical reasons. Initially, several types of control measurements were carried out in order to visualize synaptic transmission reliably.

3.4.3 In vivo neuron/glia differentiation

Neuronal and glial cells were differentiated using two different methods. The first one takes advantage of the glial cells Ca-response in a low-K+ saline (Dallwig and Deitmer, 2002) and the second one is based on in vivo astroglial marker sulforhodamine 101 (SR101) staining (Nimmerjahn et. al, 2004).

When the low K+ test was used, extracellular solution with following composition was applied on the slice for 1 min when calcium concentration changes were recorded: 128.6 NaCl, 0.2 KCl, 1.2 KH2PO4, 2.4 CaCl2.2H20, 1.3 MgSO4.7H2O, 26.0 NaHCO3, 25.0 D-glucose; pH 7.4, 302 mOsm; in mM). When in vivo glial-specific marker SR101 was used, the slice was incubated with

approx. 1 µl of 1 mM SR101 stock solution for approximately 20 min. and subsequently rinsed by fresh normal ACSF. A quality of staining and rinsing was visually controlled by acquiring a single image every 5 min until sufficient signal to noise ratio was achieved.

3.4.4 Pharmacological experiments

The final concentrations of drugs added to the bath solution during pharmacological interventions were as follows: 1 µM TTX (tetrodotoxin), 45 µM CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) , 45 µM MK801 (dizocilpine), 20 mM caffeine (caffeine substituted corresponding amount of NaCl in the ACSF solution in order to maintain constant osmolality).

3.4.5 Control stimulation protocol

A synaptic activity in the superficial spinal cord dorsal horn neurons was
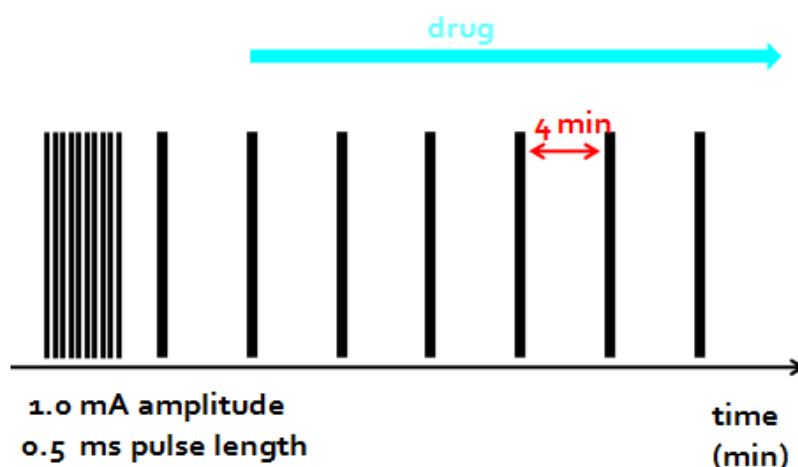

Figure 17. Stimulation pulses were applied in regular 4 min intervals in order to allow for synapse recovery. Each train of stimuli lasted for 1 second and consisted of 10 pulses of 0.5 ms length. Averaged fluorescence intensity changes in neuronal soma region from 3 control stimulations were compared to fluorescence intensity after drugs administration or after capsaicin (0.4 µM) or high frequency (100 Hz, 100 pulses total, 20p-400ms-20p burst) sensitization protocol.

evoked by stimulation of dorsal root through glass suction electrode. The stimulation protocol consisted of individual pulse trains applied in regular 4 min intervals in order to allow synapse recovery. Each train of the stimuli lasted for 1 second and consisted of 10 pulses of 0.5 ms duration and 1 mA current intensity. In some experiments 0.1 ms duration of the stimulus was used for preferential activation of dorsal root A-fibers (Fig. 17).

3.4.6 Data analysis

The fluorescence intensity changes were recorded using LCS software and stored for offline image analysis with ImageJ software (Rasband W.S., MD, USA). The recorded 12-bit images underwent basic processing including background subtraction using rolling-ball algorithm, rigid-body-transformation registration method for minimizing motion artifacts, and correction for bleaching by calculating fluorescence decay constant k over the course of experiment using monoexponential curve fit. Particular ROIs covering the cell soma were selected using Time Series Analyzer ImageJ plug-in (Balaji J., Cornell Uni., NY, USA). Further signal processing using Savitzky-Golay or Fast Fourier Transform filters, as well as graph plotting was performed using Microcal Origin Pro 8.0 software (MA, USA).

The Fluo-4 fluorescence intensity change, representing change in $[Ca2+]i$ in neuronal soma region as a consequence of neuronal activity was expressed as a fluorescence change relative to a basal fluorescence intensity ($\Delta F/F0$; in %) before the stimulation, in order to avoid error caused by uneven dye loading and dye loss during experiment. The basal intensity value was calculated for each neuron by averaging 10 frames captured prior to the initiation of the electrical

stimulation. An effect of drugs administration on the stimulation-evoked intracellular calcium concentration changes was assessed by comparing ΔF/F0 peak value prior and after the drug administration. The reference peak ΔF/F0 value was calculated as an averaged value obtained from 3 control stimulations

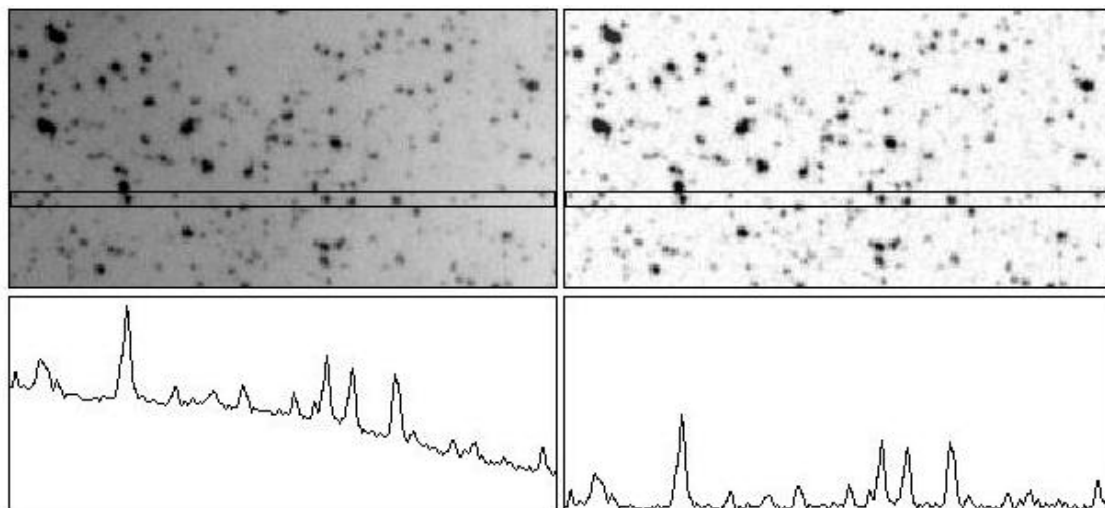**Figure 18. Application of the rolling ball algorithm (adapted from ImageJ online documentation http://rsb.info.nih.gov/ij/).**

before the drug was applied. The time series filters were chosen considering preservation of the original shape of the ΔF/F0 curve. The rolling-ball algorithm (Fig. 18; based on the "rolling ball" algorithm described in Stanley Sternberg's article, "Biomedical Image Processing", IEEE Computer, January 1983) removes smooth continuous backgrounds. The algorithm (since ImageJ v1.39f) uses an approximation of a paraboloid of rotation. In practice, 8-bit or RGB images should be at least as large as the radius of the largest object in the image that is not part of the background. For 16-bit and 32-bit images with pixel value ranges different from 0-255, the radius should be inversely proportional to the pixel value range (i.e. 0.2 to 5.0 for typical 16-bit images. We used the value 50 of the *Rolling Ball Radius,* that is the radius of curvature of the paraboloid.

The rigid-body transformation algorithm, used to for image registration (alignment), performs translations and rotations but maintains the size of the original image. It is implemented in the StackReg plugin, which accepts a stack of images as input and automatically aligns each image to the selected slice.

The fluorescence decay constant was computed by selecting all image as ROI for calculation of /b in log file/. We assumed monoexponential decay, though in some cases bleaching is a biexponential process.

Further signal processing used to reduce noise was Savitzky-Golay Smoothing Filter procedure (Savitzky, 1964) or Threshold filter, as well as graph plotting was performed using Microcal Origin Pro 8.0 software. Time-domain method of smoothing like Savitzky-Golay filter is based on least squares polynomial fitting across a moving window within the data. The filter is designed to preserve the higher moments within time-domain spectral peak data, which are usually 'flattened' by other adjacent averaging techniques like moving averages. It effectively smoothes data sets with uniform X-spacing. This method was chosen to preserve the original shape of the $\Delta F/F0$ curve and the degree of the underlying polynomial was set to value of X.

The second employed procedure – the Threshold Filter uses Fast Fourier Transform (FFT) algorithm to eliminate noise corresponding to frequency components that are below a specified threshold level in an active data plot. The threshold level is set interactively by adjusting movable threshold level line in the frequency component spectrum of the analyzed time series (Fig. 19).
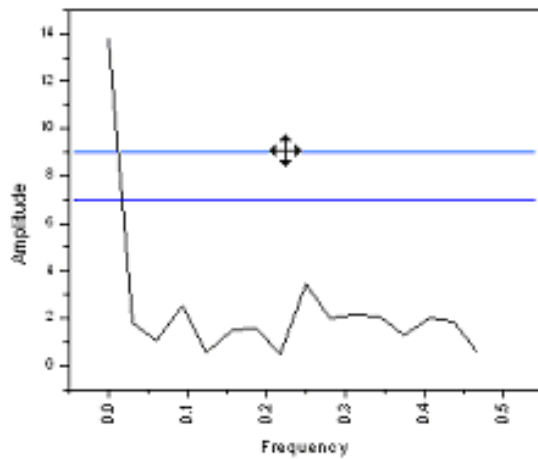
Figure 19. The frequency component spectrum with a moveable threshold level line. Threshold filter based on FFT procedure used to filter out low frequencies in an active data set (Microcal Origin Pro ver.8 documentation).

All data are presented as a mean ± standard error of the mean (SEM). For statistical analysis, t-test (Welsh's t-test is used when variance in groups differs) or Repeated Measures ANOVA were used. The hypotheses were tested for significance at 0.1 %, 1 %, and 5 % level.

4 RESULTS

4.1 CHANGES IN CA-BINDING PROTEIN EXPRESSION IN A MODEL OF ARTHRITIS

4.1.1 Changes in parvalbumin expression in spinal cord dorsal horn

In this study, the changes in parvalbumin expression in ipsilateral and contralateral sides of the spinal cord in rats with experimental unilateral knee joint arthritis were analyzed. The level of parvalbumin expression was assessed using two immunological techniques – Western blot of hemisected spinal cord and

Figure 20. Off-line densitometric analysis of the L5 spinal cord segments performed using AIDA software package. Two symmetrical areas in the dorsal horn and ventromedial region were used for the analysis.

densitometric analysis on immunohistochemically stained fixed slices from two dorsal horn regions with high-density immunostaining (substantia gelatinosa and ventromedial part of the dorsal horn).

For the immunohistochemical densitometric analysis, histological sections from ipsilateral and contralateral sides of the spinal cord lumbar enlargement (L3-L5) in 5 animals was analyzed (Fig. 20). The parvalbumin expression in the spinal cord tissue ipsilaterally to the arthritis was significantly reduced to 68 ± 12% (SEM, $p<0.05$, paired t-test) when compared to the control side. We found

no significant side-to-side difference in the parvalbumin concentration between left and right spinal cord side in the control animals (n=3).

The densitometric analysis showed that the area of immunopositivity in the symmetrical parts of superficial dorsal horn was smaller on the arthritic side when compared to the side contralateral to side of the arthritis. The sum of signal intensity in this area and the intensity/area quotient was also lower (Fig. 21 A).
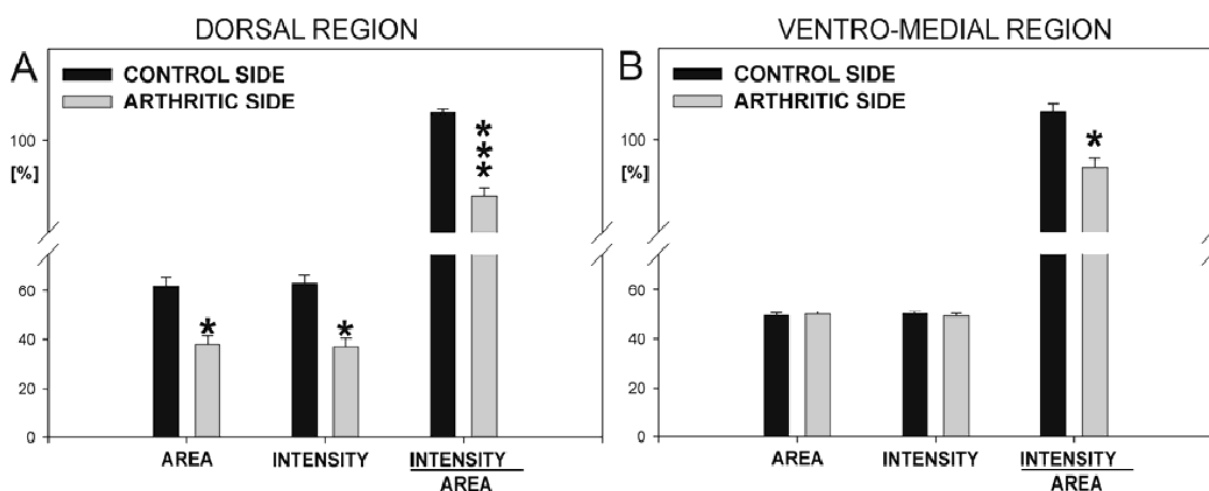

Figure 21: Densitometric analysis of the parvalbumin expression in the dorsal (A) and ventro-medial (B) region of the spinal cord; * p<0.05, *** p<0.001. Comparison of area, intensity, and intensity/area quotient of parvalbumin expression on the control and arthritic side; the sum of the positive areas on both sides represents 100%.

There was no significant difference in the area and intensity of staining in the ventro-medial part of the dorsal horn; however, the quotient of the intensity/area was significantly reduced on the arthritic side (Fig. 21 B).

The Western blot technique was used to confirm the results of the densitometric analysis. In order to separate low molecular weight proteins such as parvalbumin (12 kDa) on gel, we used modification of the standard SDS

electrophoresis: the Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine SDS-PAGE). In the tricine system developed by Schägger and von Jagow, tricine replaces glycine in the running buffer. This results in more efficient stacking and destacking of low molecular weight proteins and higher resolution of smaller peptides ((Schägger and von Jagow, 1987). For better visual control over timing of the protein electrophoretic transfer, we used Bio-Rad's Caleidoscopic polypeptide standards.

In order to avoid gel electrophoresis-induced protein modifications (cysteine reacting with mercaptoethanol of free acrylamide forming intermolecular disulfide bonds or glutamic acid carboxy residues methylation; Haebel et al., 1998) spinal cord samples were subjected to DTT reduction and iodoacetamide alkylation procedures (Lane, 1978). Before loading on gel, homogenates from control and experimental spinal cord sides were spectrophotometrically compared for total protein amount (Fig. 22). For transfer of proteins resolved on gel, the nitrocellulose membrane gave sufficient results. The optimal transfer time was determined using two nitrocellulose membranes on each other and monitoring the transfer of the kaleidoscopic polypeptide standard.
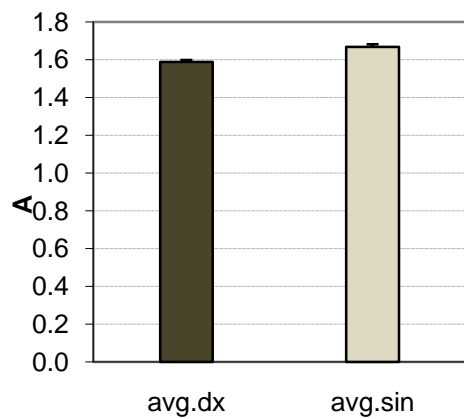
Figure 22. Comparison of absorbance (A) of samples from the control (avg.dx) and arthritic (avg.sin) site as determined by Bradford's Total Protein Concentration Assay. The averaged total protein content in both samples was not significantly different (n=5, p<0.05).
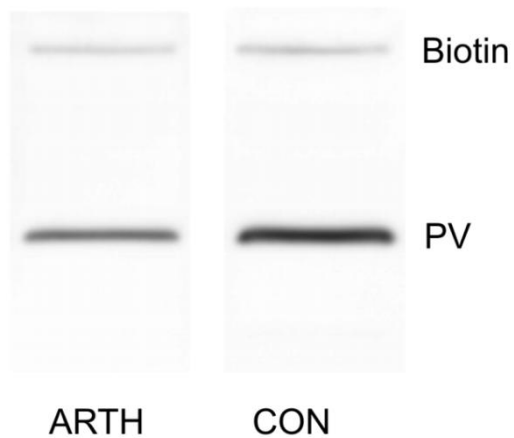

Figure 23. Example of parvalbumin Western blot from ipsilateral (ARTH) and contralateral (CON) spinal cord tissue from animals with unilateral arthritis visualized using cooled CCD camera. Endogenous biotin was used as an internal control.

A cooled CCD camera, which captures a digital image of the nitrocellulose membrane was used to acquire luminescence signal from proteins subjected to the chemiluminescent detection method. The image was analyzed by densitometry, which evaluated the relative amount of protein staining and quantified the results in terms of optical density. Results provided by this method confirmed the results of densitometric analysis on frozen spinal cord sections. In the arthritic area, the expression of parvalbumin was markedly decreased compared to the control side of the spinal cord (Fig. 23).

4.1.2 Changes in Ca-binding protein expression in STT neurons

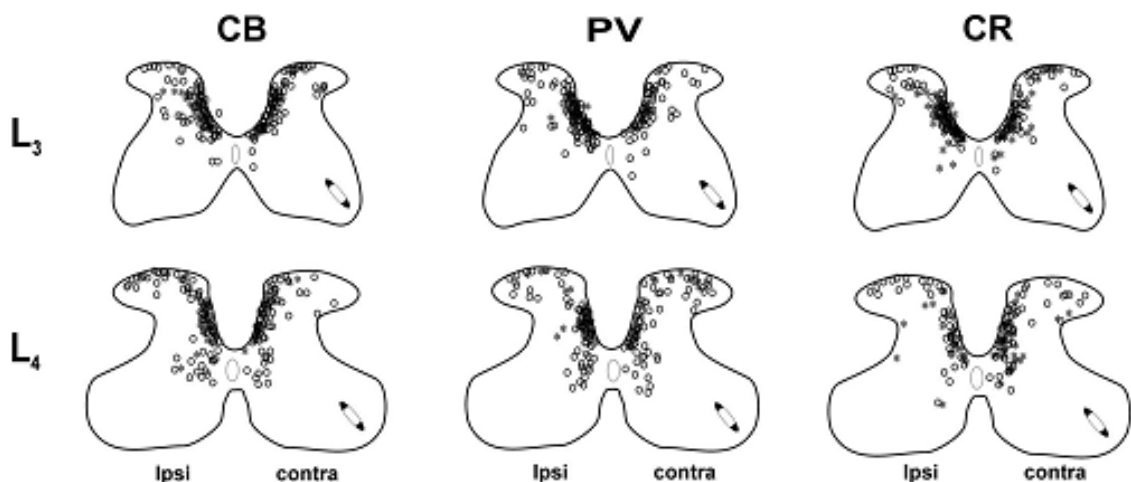**Figure 24. Location of the STT cells was marked on a schematic spinal cord section drawing. Majority of the cells were found in the medial dorsal horn grey matter.**

The retrogradely labeled STT neurons were located in the medial dorsal horn grey matter, while a smaller population was found in the superficial laminae (Fig. 24). Number of these neurons was found to be colocalized with calcium binding
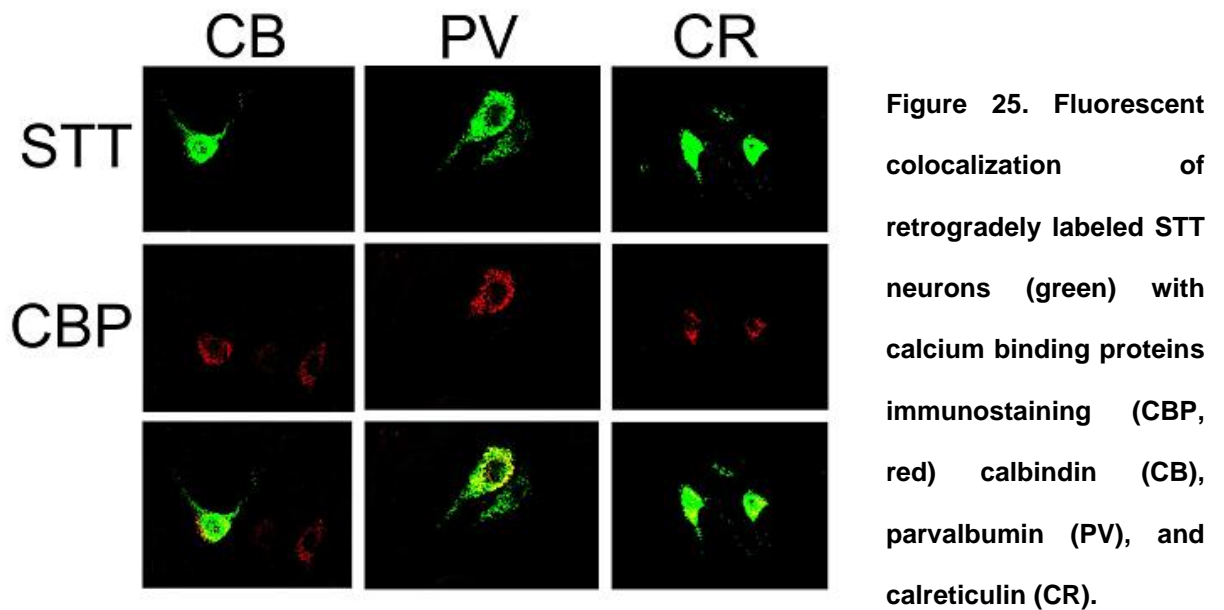
Figure 25. Fluorescent colocalization of retrogradely labeled STT neurons (green) with calcium binding proteins immunostaining (CBP, red) calbindin (CB), parvalbumin (PV), and calreticulin (CR).

protein immunostaining (Fig. 25). On average there were 42 retrogradely labeled STT neurons for each CBP in the histological slides from the L3-4 segments in each animal. Altogether 377, 374 and 358 STT neurons in the segments L3-L4 were evaluated for presence of CB, PV, and CR, respectively (n=9).

On the contralateral (control to the induced arthritis) spinal cord side, anti-CBP antibody labeling revealed 11 %, 9 % and 47 % colocalization of the STT neurons and neurons expressing calbindin, parvalbumin, and calreticulin respectively (Fig. 26). Similar results were found in the group of control, intact animal (n=3; CB 7%, PV 13%, CR 57%). On the experimental (ipsilateral) side, the STT/CBP colocalization was found to be significantly increased for CB (23 %) and PV (25%) compared to the contralateral side 24 hours after arthritis induction (Fig. 26). The number of CR expressing STT neurons (50%) did not change significantly.

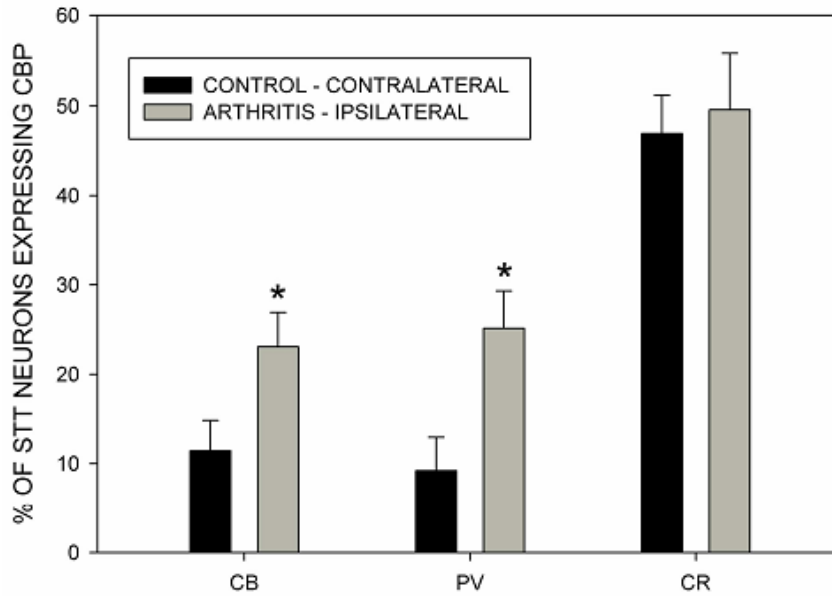**Figure 26. The percentage of STT neurons positively immunostained for calbindin (CB), parvalbumin (PV) and calretinin (CR) 24 hours after arthritis induction ipsilaterally and contralaterally to the inflamed knee joint. There was a significantly higher number of STT neurons positively stained for CB and PV, while the number of STT neurons stained for CR did not change. (* p<0.05, paired t-test).**

4.2 DORSAL ROOT STIMULATION-EVOKED SYNAPTIC ACTIVITY INDUCED CA TRANSIENTS IN SPINAL CORD SLICES

Extracellular recording of neuronal activity in the spinal cord of anesthetized rats previously demonstrated that central sensitization manifests by increased number of action potentials. It is also known that the amount of calcium entering into cytoplasm during neuronal activity is related to number of action potentials. We worked with these assumptions to design experiments using calcium imaging of spinal cord dorsal horn neurons.
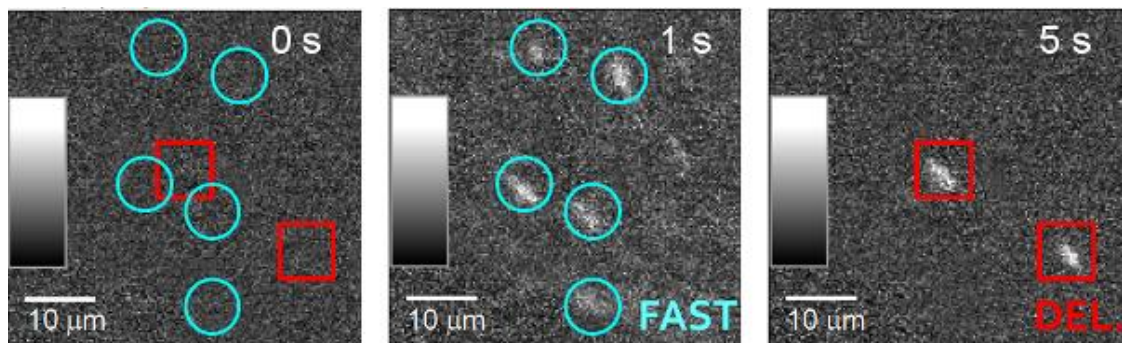
Figure 27. Calcium transients in the dorsal horn. The three images show [Ca2+]i changes in the spinal cord superficial dorsal horn cells at a different time period: Before the dorsal root stimulation (0 s), at the end of the 1 s pulse train when fast calcium transients occurred (1 s) and 5 s after the stimulation when the [Ca2+]i,D changes were close to their peak level. The fast and delayed calcium transients in this slice occurred in different cells.

Changes in neuronal free calcium concentration ([Ca2+]i) were recorded in cells in the superficial dorsal horn area. Control stimulation of the dorsal root (0.5 ms, 10 Hz, 1 s, 1 mA) usually evoked two temporally distinct changes of the $[Ca^{2+}]i$ in the spinal cord dorsal horn cells (Fig. 27). The number of recorded cells activated by the stimulation differed in each slice depending on the vitality of the

Figure 28. Two types of temporally distinct profiles of intracellular calcium concentration changes recorded in dorsal horn cells as a result of spinal cord dorsal horn stimulation (triangle: FAST, square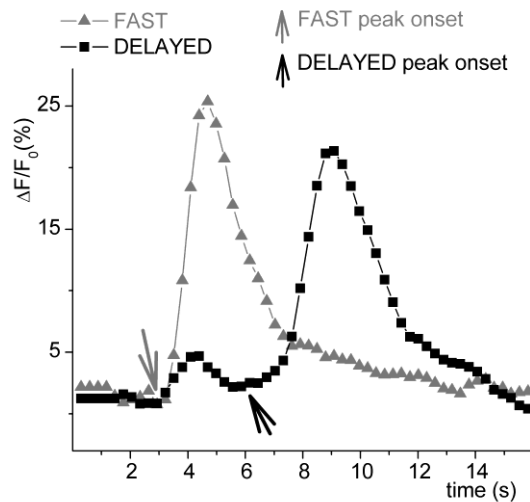: DELAYED response). Fluorescence responses of both cell populations recorded during single 15 s scan are superimposed to emphasize the temporal differences of the induced calcium concentration changes between the groups. The arrows mark the onset of the calcium concentration change, while stimulation started at 3 s.
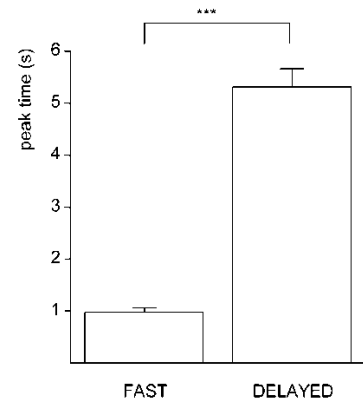
Figure 29. The mean time to peak of the calcium concentration change in the group of neurons with fast and delayed response was 1.0 ± 0.1 s (n=54, N=5 slices) and 5.3 ± 0.3 s (n=45, N=8 slices) respectively (weighted avg., Welsch's t-test, p<0.001).

cells stained with the Fluo-4 dye and the position of the stimulating electrode in the dorsal root. When the cells recorded under the control conditions were analyzed, populations of cells with fast ($[Ca2+]i,F$) and delayed ($[Ca2+]i,D$) response to the dorsal root electrical stimulation were identified. On the superimposed examples of the fast and delayed $[Ca2+]i$ recordings in two different cells (Fig. 28) it is evident that the $[Ca2+]i,F$ transient started with the beginning of the stimulus train and peaked at the end of the stimulation.

The cell with the delayed response showed a small [Ca2+]i increase also during the stimulation but a much larger [Ca2+]i increase came several seconds after the stimulus termination. On average the [Ca2+]i,F responses had maximal amplitude at the end of the stimulation (1.0 ± 0.1 s, n=54). The onset of the
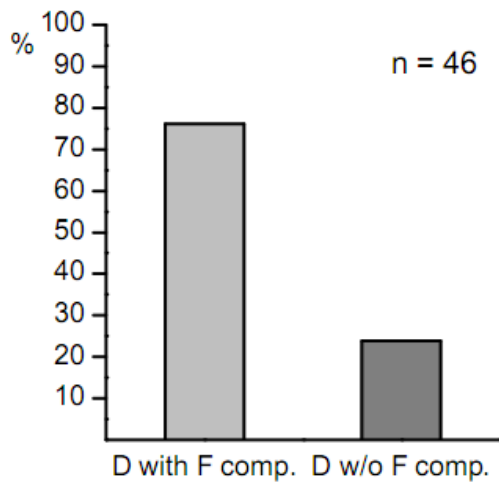
Figure 30. The delayed calcium transient with and without the fast component. Upper figure shows a relative proportion of each group. Lower figure is a histogram of the relative amplitude of the fast component.
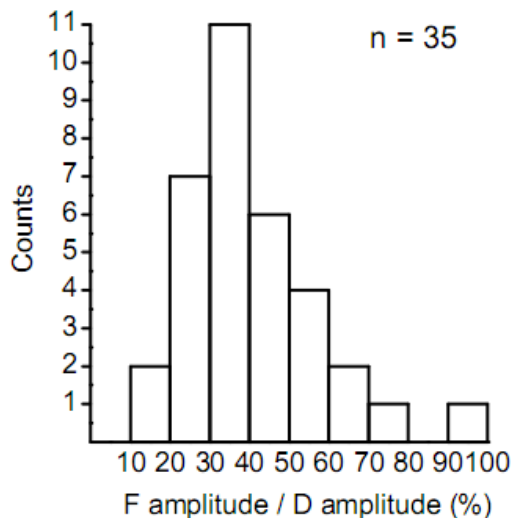
[Ca2+]i,D response in the second population of cells started on average at 2.8 ± 0.3 s and peaked at 5.3 ± 0.3 s (n=45) after the beginning of the stimulation (Fig. 29). The amplitude ($\Delta F/F_0$) of the calcium transients in the [Ca2+]i,F and [Ca2+]i,D population was 40.5 ± 6.2 % and 36.5 ± 23.3 % respectively.

In 77% (n=35) of the $[Ca2+]i,D$ cells it was possible to distinguish a low-amplitude ($\Delta F/F_0$ = 12.6 ± 1.0 %) peak component that corresponded temporally to the $[Ca2+]i,F$ response. In this population of cells with both the fast and the delayed component present, the amplitude of the fast component represented on average 40.6 ± 2.8 % of the delayed peak amplitude (Fig. 30).

4.3 THE SPECIFICITY OF NEURONAL ACTIVATION

To rule out a possibility that the recorded [Ca2+i] changes were in glial cells, two methods to differentiate between neurons and glia were employed.

First, staining with in vivo glial specific marker Sulforhodamine 101 (SR101) was used to differentiate between neurons and glia. This method was used previously both on *in vivo* and *in vitro* cortex preparations (Ren et al., 2000; Nimmerjahn et al., 2004). Initially, we labeled a living spinal cord slice with SR101 and found no overlap between SR-101 and Fluo-4 labeled profiles (Fig. 31 and 32). This suggested that the Fluo-4 labelled cells were preferentially neurons.
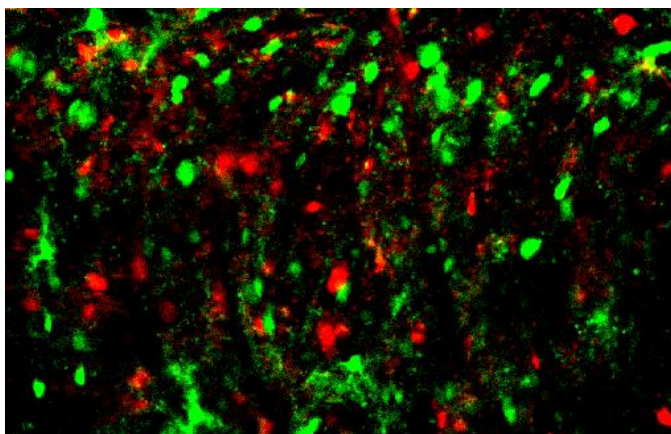
Figure 31. The recording area of the spinal cord slice labeled with Fluo-4 calcium sensitive probe and SR-101 glial specific marker.
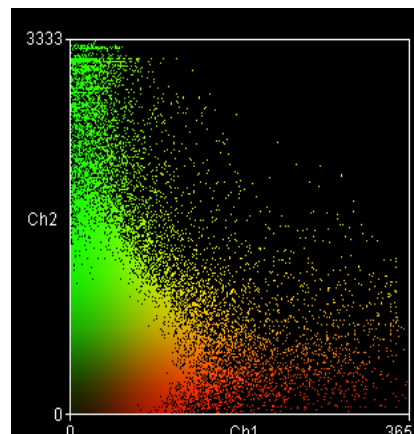
Figure 32. A colocalization diagram of the area of the spinal cord as shown in Fig. 28.

However Fluo-labeled cells are only slightly visible before stimulation, therefore the image showing strong green signal might arise from death cells. In order to rule out this possibility, we have further analyzed only colocalization

between the SR-101 labeling and those cells, which responded to the electrical stimulation with a change in fluorescence. As a result, none of the 49 cells which responded to the stimulation (29 fast, 20 delayed) was colocalized with the *in vivo* glial-specific marker SR101 (Fig. 33).
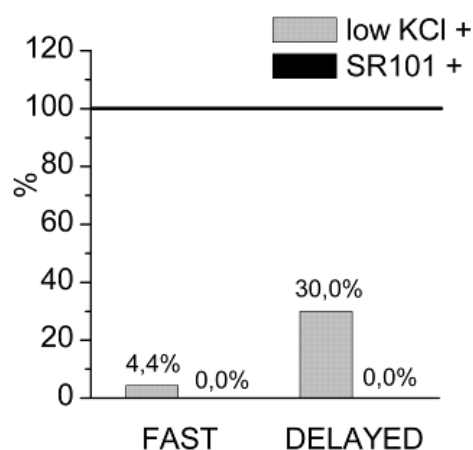
Figure 33. Fluo-4/SR101 colocalization and an effect of K+ withdrawal. The graph shows a relative fraction of Ca transients due to K+ withdrawal (light bar) in a group of previously identified fast and delayed responses (corresponding to 100% baseline). Black bar represents Fluo-4/SR101 colocalization in the same group of cells.

The second method used a substitution of the normal recording solution with ACSF containing 0.2 mM KCl. The application of low-KCl ACSF solution is known to evoke calcium concentration changes in astrocytes due to calcium influx through barium-sensitive calcium channels, but not in neurons (Dallwig et al., 2000; Dallwig and Deitmer, 2002).

Out of the 29 cells with previously identified fast response, only 4.4 % show intracellular calcium transient upon the low-KCl ACSF substitution (low KCl+). The colocalization analysis of the cell population responding to the electrical stimulation of dorsal root with delay revealed a different colocalization pattern:

30% of the [Ca2+i]i,D cells showed responses evoked by the low-KCl solution, in

contrast to the [Ca2+i]i,F group of cells.

4.4 REPRODUCIBILITY AND STABILITY OF THE FAST AND DELAYED CALCIUM TRANSIENTS
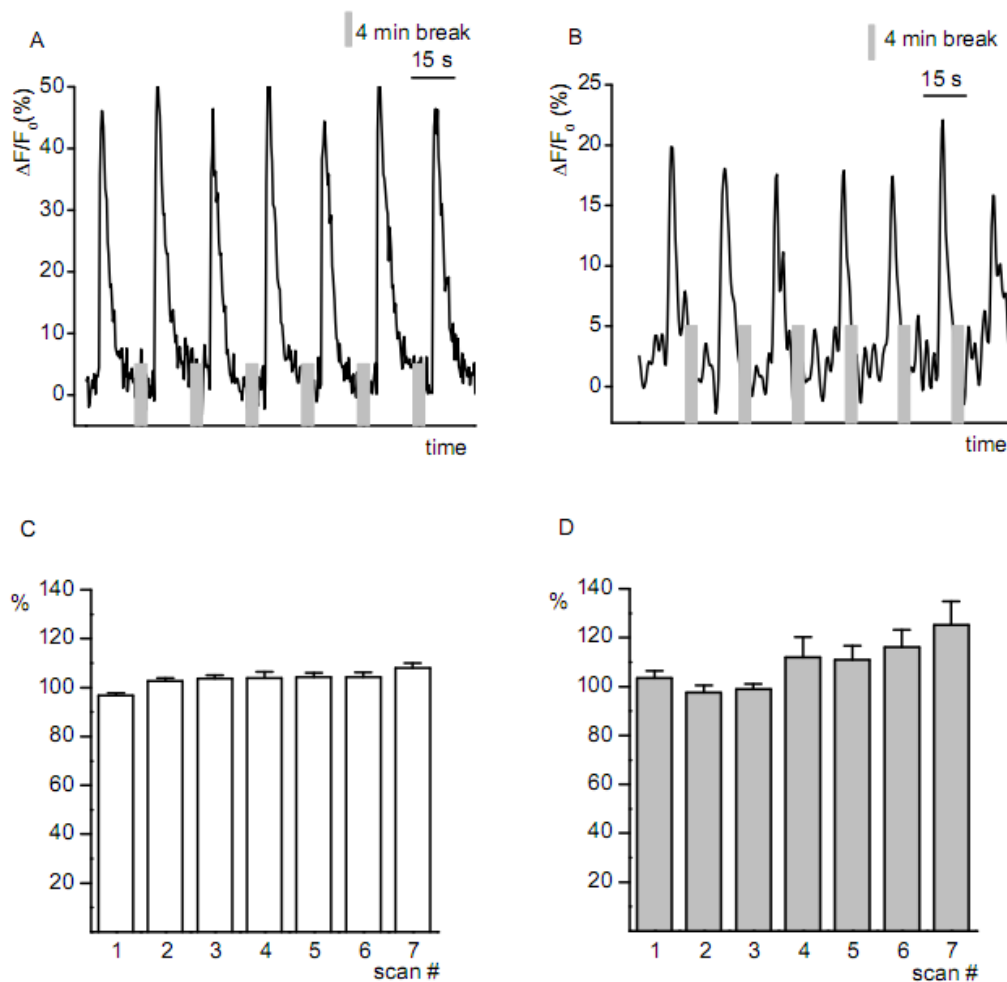
Figure 34. Synaptically evoked calcium transients in a $[Ca2+]i,F$ (A) and $[Ca2+]i,D$ (B) neuron. A 15 s-long recordings of the neuronal responses to individual stimulations were separated by 4 min. intervals depicted here by grey rectangles. Averaged responses of the neuronal populations with the fast calcium peak onset (C, n=61) and with the delayed calcium peak onset (D, n = 46) were calculated.

A reliable interpretation of the drug application effects for this in vitro model experiments depended fundamentally on the stability of the synaptically evoked $[Ca2+]i$ changes during the course of the experiments. Therefore $[Ca2+]i$

changes in the populations of cells with the fast and delayed responses, evoked by consecutive control stimulations with 4 min intervals between the stimulations, were analyzed under control conditions. The stimulation-evoked responses were stable over the testing period of approximately 40 min (Fig. 34). No significant change in the peak fluorescence intensity, representing intracellular calcium concentration change, was observed in either the fast (n=61) or the delayed (n=46) responses. The averaged responses with the $[Ca2+]i,D$ response showed mild increase in fluorescence in time, though no statistically significant trend was identified.

4.5 PHARMACOLOGICAL CHARACTERIZATION

4.5.1 Sodium channel blocker tetrodotoxin

The sodium channel blocker tetrodotoxin (TTX) was used to block action
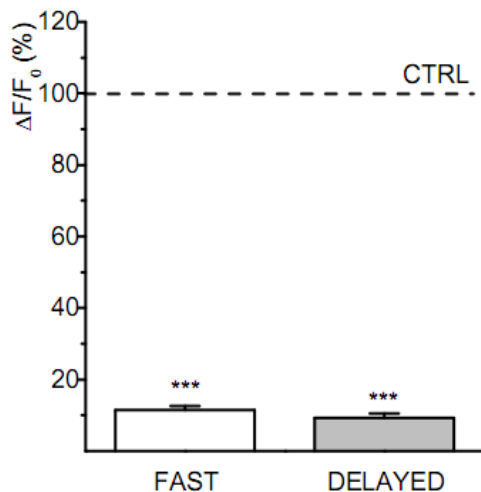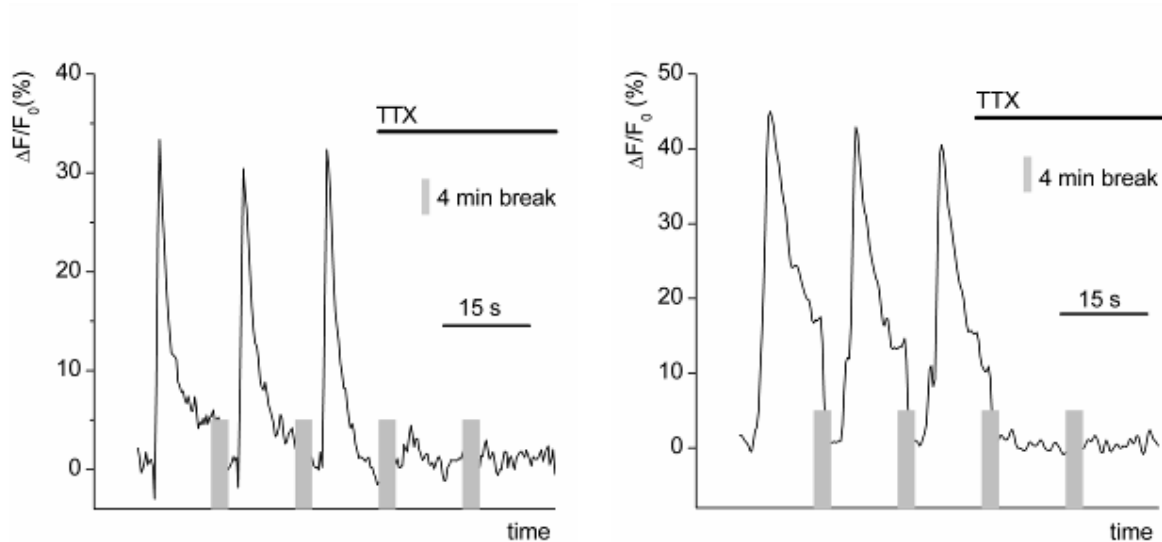
Figure 35. Sodium channel blocker tetrodotoxin. The application of sodium channel blocker tetrodotoxin (TTX) blocked both the fast (11.4 +/- 1.2% of the control, n=36, p<0.001) and the delayed (9.2 +/- 1.3% of the control, n=17, p<0.001) intracellular calcium concentration changes evoked by the stimulation of the dorsal root. Upper figures show a typical effect of the TTX application on the synaptically evoked fast and delayed calcium transients, respectively.

potentials in the dorsal root slice preparation and to test for presence of any direct stimulation of the cells by possible current spread from the glass suction

electrode-sealed dorsal root. Stimulation of the dorsal root evoked action potential that lead to synaptic transmission between the central branch of the of the dorsal root ganglion neuron and a dendrite of a postsynaptic neuron located in the spinal cord superficial laminae.

The 1 μM TTX bath application rapidly abolished both the fast and the delayed fluorescence changes in the dorsal horn spinal cord neurons. The effect of the TTX application was observed almost instantaneously and the remnants of the responses were difficult to differentiate from the background noise after the TTX application. The numerical value of the signal amplitude present at time corresponding to the original response peak represented on average 11.4 ± 1.2 % of the fast and 9.2 ± 1.3% of the delayed control responses (Fig. 35).

4.5.2 Ionotropic glutamate receptor antagonists and ICS depletor caffeine

The AMPA receptor antagonist CNQX and a non-competitive NMDA receptor antagonist MK801 coapplication was used to investigate the role of ionotropic glutamate receptors in the origin of the fast and delayed calcium transients. The AMPA/NMDA antagonist coapplication suppressed considerably stimulation evoked somatic $[Ca2+]i$ increase in the group of the fast-responding neurons (Fig. 36). On average the peak of the $[Ca2+]i,F$ responses was reduced to 34.7 ± 6.6 % of the control preapplication level (n = 44, p < 0.001). The peak amplitudes of the delayed responses $[Ca2+]i,D$ recorded in the same slices were not affected substantially after the antagonists perfusion. The averaged value of the $[Ca2+]i,D$ peak was 92.6 ± 13.4 % of the control level (n=18).
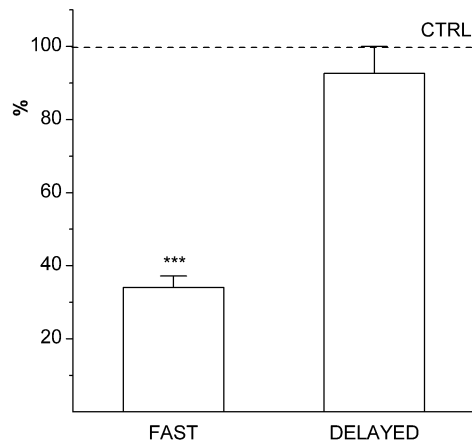
Figure 36. The coapplication of glutamate receptor antagonists CNQX and MK801 decreased calcium concentration change in the fast-responding cells (34.7+/-6.6 % of the control, p<0.001, n=44), whereas the cells reacting with a delay were not significantly affected (92.6+/-13.4 % of the control, p<0.05, n=18, C).

The contribution of intracellular calcium stores (endoplasmic reticulum) to free calcium concentration increase in the neuronal cytoplasm was investigated using bath application of intracellular calcium stores depletor caffeine. Intracellular calcium stores depletor caffeine (20 mM).

Caffeine addition to the perfusing ACSF solution induced increase in the somatic peak of the [Ca2+]i,F response, that is clearly visible in the example recording (Fig. 37, 38). On average caffeine application increased the peak amplitude of the [Ca2+]i,F response to 168 ± 18.8 % of the control pre-application value. The delayed [Ca2+]i,D responses were almost completely abolished by the caffeine application (Fig. 39).

In one of the experiments, caffeine administration was followed by CNQX/MK801 coapplication (Fig. 37, 38). As a result, the caffeine-potentiated fast [Ca2+]i,F peaks were virtually abolished (12.1 ± 2.3 %, n = 7) by the ionotropic glutamate antagonists application. In some of the $[Ca^{2+}]i,D$ responses there was also revealed a visible peak corresponding to the fast response once the delayed peak response was suppressed by the caffeine application. This fast

peak component was abolished upon CNQX/MK801 coapplication (10.7 ± 2.9 %, n = 6).
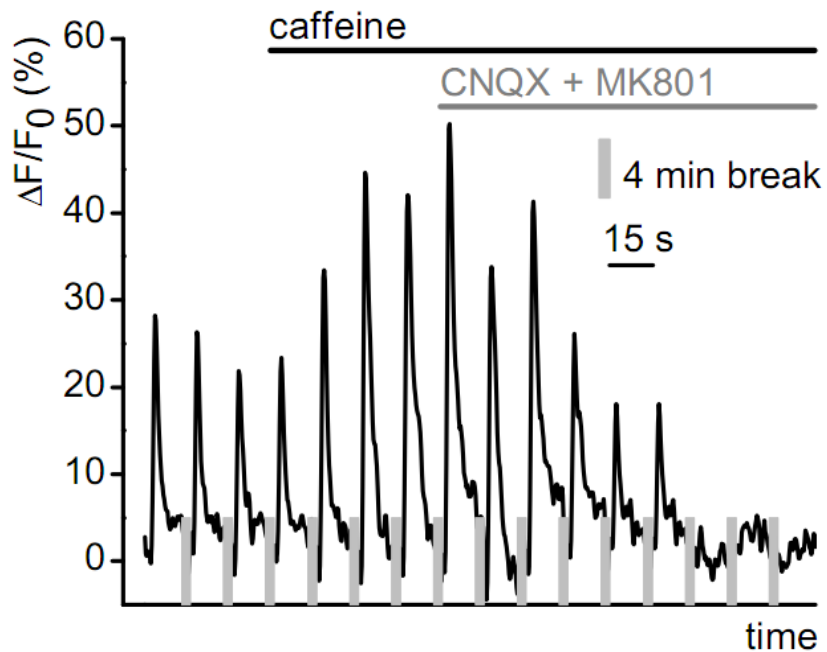
Figure 37. An example a neuron with the fast calcium transients. The application of caffeine potentiated the responses evoked by dorsal root stimulation, which were then completely abolished upon subsequent CNQX/MK801 administration. The graph shows several consecutive 15 s-long scans interrupted by 4 min. inter-scan intervals.
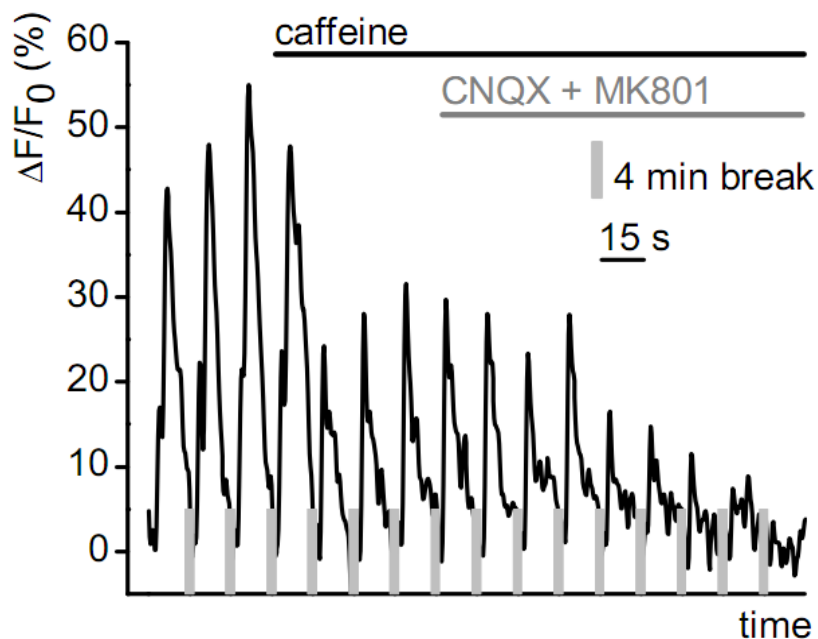
Figure 38. An example a recording from neuron with the delayed calcium transients. The application of caffeine abolished the evoked delayed responses. The fast component of the delayed response was potentiated after the caffeine application and completely abolished upon subsequent CNQX/MK801 administration. The graph shows several consecutive 15 s-long scans interrupted by 4 min. inter-scan intervals.
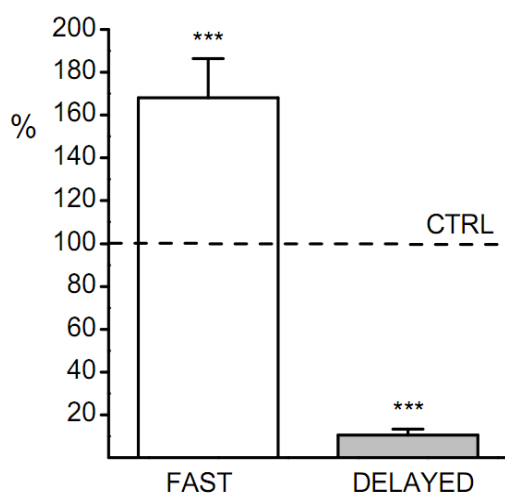
Figure 39. The application of 20 mM caffeine increased the fast, synaptically evoked, [Ca2+i] changes to 168.7 ± 18.8 % of the control value (n=44, N=4 slices, p<0.001) and substantially diminished the delayed responses to 18.3 ± 4.3 % of the control value (n=24, N=4 slices, p<0.001).

4.6 CALCIUM WAVE PROPAGATION
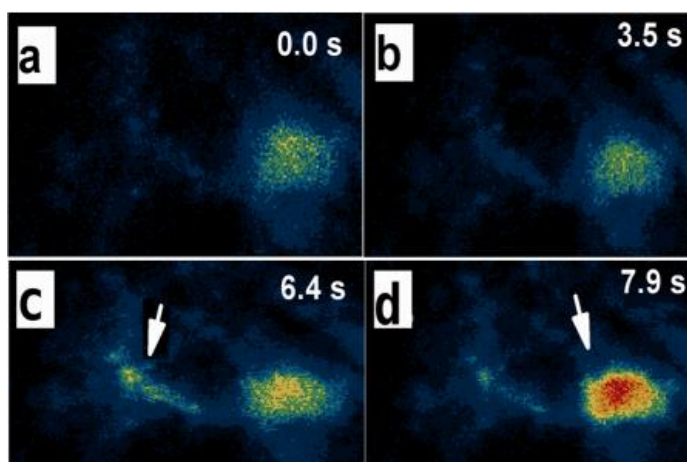
Figure 40. Dynamics of the fluorescence signal change in four different periods of time (0.0, 3.5, 6.4, and 7.9 seconds after the initiation of the stimulation) in different regions of a single neuron. White arrows point at the location of regional fluorescence extremes. Standard pseudo-color look-up table is used to illustrate fluorescence intensity.
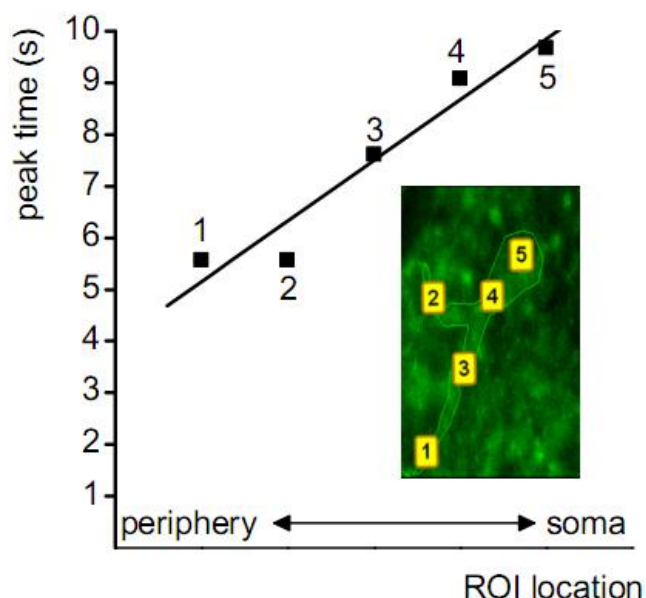
Figure 41. The confocal image of another neuron with synaptically evoked delayed calcium response (the neuron's outline is highlighted). The ROIs were chosen in different parts of the cytoplasm to demonstrate the spatiotemporal dimension of the calcium wave propagation.

In some of the neurons with the delayed response it was visible on the recordings that the somatic $[Ca^{2+}]_{i,D}$ response is in fact result of a calcium wave propagated from dendrites. To further demonstrate this and the involvement of intracellular calcium stores in the process of the delayed calcium transient, a single $[Ca2+]_{i,D}$ neuron was scanned at 100 ms frame rate and with increased resolution due to the confocal scanner zooming. A slow calcium wave

propagation was recorded and is demonstrated with the selection of several regions of interest (ROI) in different parts of the neuron in order to analyze the spatiotemporal dimension of the delayed $[Ca2+]i,D$ change in the neuron's cytoplasm (Fig. 40, 41). A ROI´s distance from the neuronal soma was plotted on the ordinal scale against the time point of a calcium wave front reaching the ROI. The data show calcium wave propagating from the dendritic to the somatic region. Moreover, a fast response component was observed on the calcium curve in each of the selected ROIs.

4.7 DIFFERENTIAL DORSAL ROOT STIMULATION

It is known that activation of unmyelinated nerve fibers by electrical current is dependent on the stimulus duration. Electrical stimulation of a sciatic nerve with 0.1 ms pulse duration preferentially activates myelinated A-fiber types, whereas using 0.5 ms pulse duration leads to recruitment of both C- and A-fiber types (Fig. 42; Allen et al., 1999).
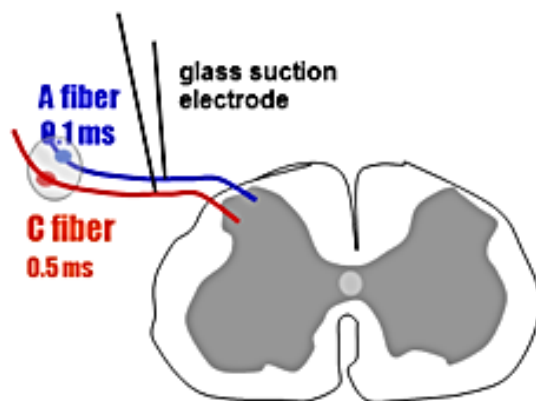
Figure 42. Drawing of the glass suction electrode placed in the dorsal root containing A and C primary afferents. The 0.1 ms stimulation pulse duration leads to preferential recruitment of the fast conducting A fiber types whereas 0.5 ms pulse activates both A and C fiber types.

In our experiments we have used this difference to presumably activate the myelinated afferents in the dorsal root with 0.1ms pulse duration and myelinated and unmyelinated fibers with 0.5 ms duration of the stimulation pulse in our control stimulation protocol under the same current amplitude in one preparation. The 0.1 ms stimulation led to a significantly higher proportion of the $[Ca2+]i,F$ responses per slice on average (n_F=20, n_D=5), while the 0.5 ms stimulus evoked

more $[Ca2+]_{i,D}$ responses per slice on average (n_F=21, n_D=10; values are rounded to the nearest integer; 6 slices in total; pair sample t-test, $p<0.05$). The increased proportion of the delayed responses with the 0.5 ms duration of the stimulus is also evident from the decreased Fast/Delayed response ratio (Fig. 43).
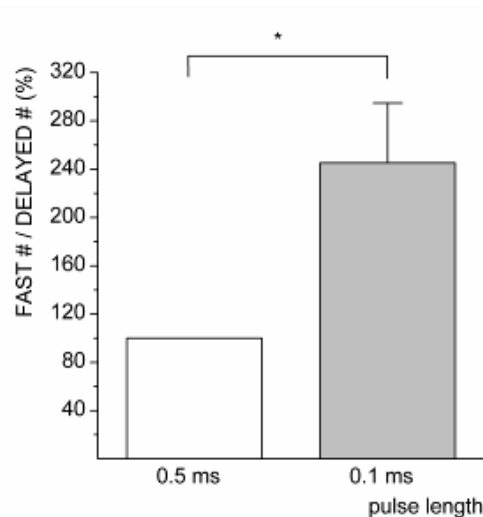
Figure 43. Stimulation with the 0.5 ms long pulse significantly increased the proportion of neurons showing delayed Ca response - i.e. lowers the F/D ratio (at 0.1ms, n=20 and n=5 cells per slice on average for fast and delayed response, respectively; at 0.05 ms, n=21 and n=10 cells per slice on average; numbers are rounded to the integer; 6 slices in total; pair sample t-test, p<0.05).

4.8 IN VITRO MODEL OF CENTRAL SENSITIZATION

4.8.1 Number of stimulation pulses

Sensitization of synaptic transmission in our model is characterized by increased number of action potentials evoked by control stimulation following sensitization protocol. The increased number of action potentials was in our experiments recorded as an increase of intracellular calcium concentration. In
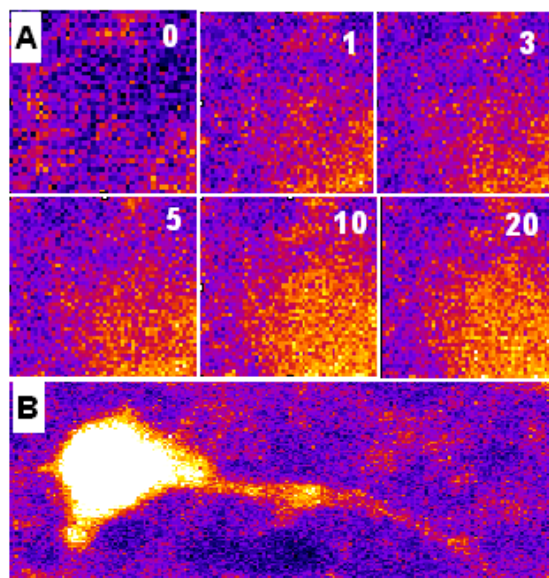
Figure 44. (A) A dependence of fluorescence signal change on an increasing number of stimulation pulses (0 – before stimulation, 1, 3, 5, 10, 20 pulses respectively). (B) A picture of a whole body of the tested neuron subjected to experimentation.

order to register the increased calcium influx by this method a sufficient room for sensitization-induced increase in fluorescence must be present. Therefore analysis of the fluorescence dependence on the number of stimulation pulses was performed. Fig. 44 shows a confocal image of a single neuron and qualitative changes in stimulation-evoked fluorescence intensity in relation to the number of electrical stimulation pulses used. The pseudocolor image demonstrates increase in calcium influx as a result of increasing number of applied stimulation pulses (1, 3, 5, 10, 20 pulses respectively). Responses to the

10 and 20 pulses are qualitatively indistinguishable therefore quantitative method of analysis follows.

The quantitative description of the fluorescence/ number of pulses correlation shows graphs in Fig. 45.
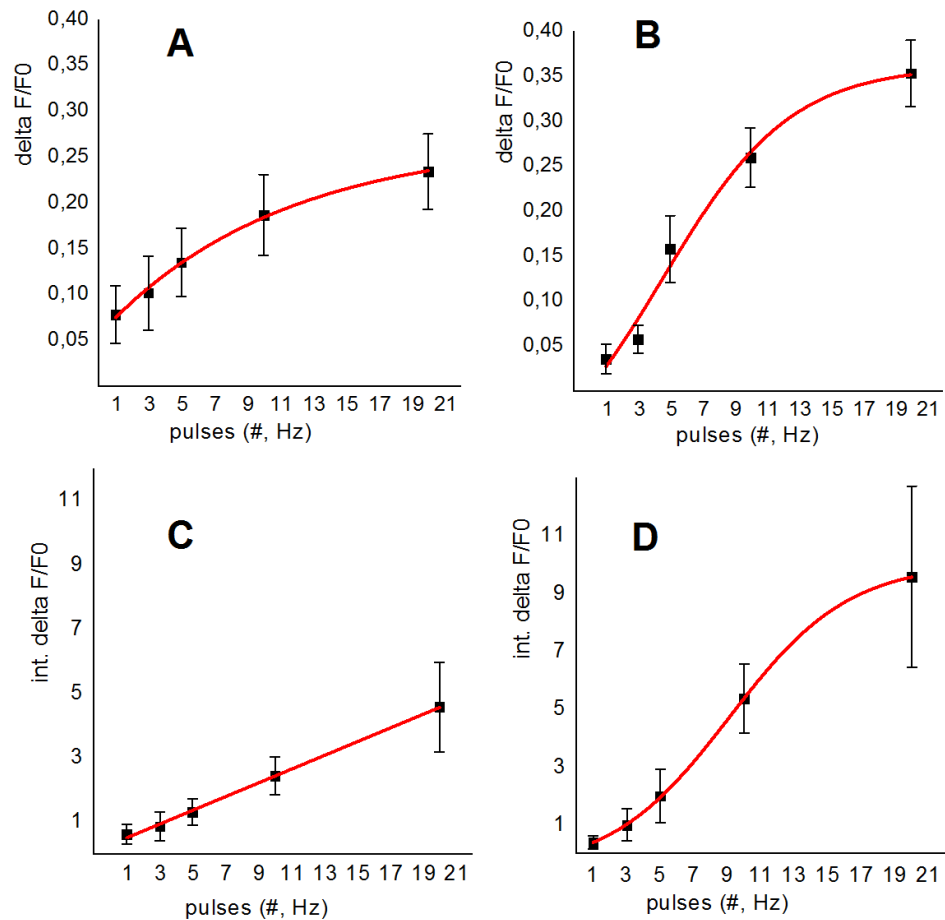
Figure 45. The dependence of fluorescence intensity of the calcium transient on number of stimulation pulses. 1, 3, 5, 10, and 20 stimulation pulses were applied to the spinal cord dorsal root. Fast (A, C) and delayed (B, D) calcium transients were analyzed based on fluorescence extreme value (ΔF/F0; A, B) or area under curve value (integral ΔF/F0; C, D).

We used two methods for the analysis of the fluorescence intensity change: calculation of the area under curve and the curve extreme. The reason for this is a possibility that, due to relatively low scanning frequency (293 ms per frame), calcium homeostatic mechanisms may flatten the calcium curve peaks which might be partly avoided by area-under-curve analysis.

The quantitative analysis revealed that both the fast and the delayed responses increase with increasing number of stimulation pulses. The fluorescence increase in the range of 1 to 20 pulses is non-linear with one exception – the fast transient analyzed using area-under curve method (Fig. 45C). The curves of the other analyzed fluorescence/pulse # dependencies tend to plateau and follow exponential (Fig. 45AB) or dose-response relationship (Fig. 45D).

4.8.2 Capsaicin-induced sensitization
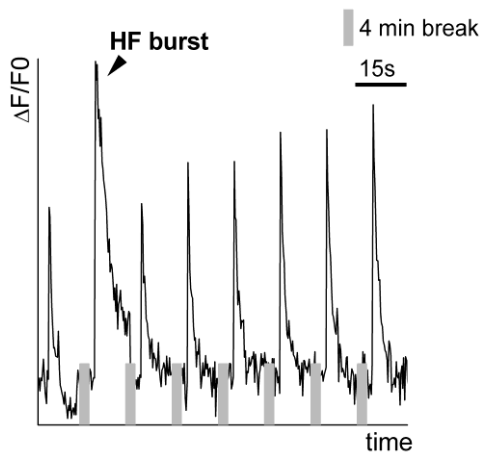
Figure 46. An example of a neuron potentiated after high-frequency burst application.
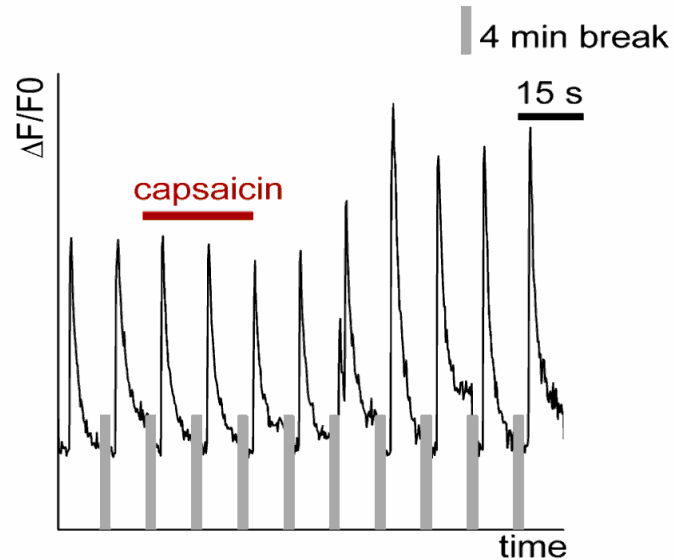
Figure 47. An example of the potentiation of the synaptic efficacy following capsaicin application.

Several experimental protocols to induce potentiation of synaptic transmission were used in various central nervous system preparations. A method used very frequently to induce LTP that was also used in spinal cord preparations is administration of high frequency stimulation. We have tested this experimental approach during preliminary experiments when we stimulated the dorsal root with a high-frequency electric burst. Despite being able to record potentiation of the synaptic transmission (Fig. 46), this method was not as efficient as application of capsaicin, which was given a priority in the following sensitization experiments.
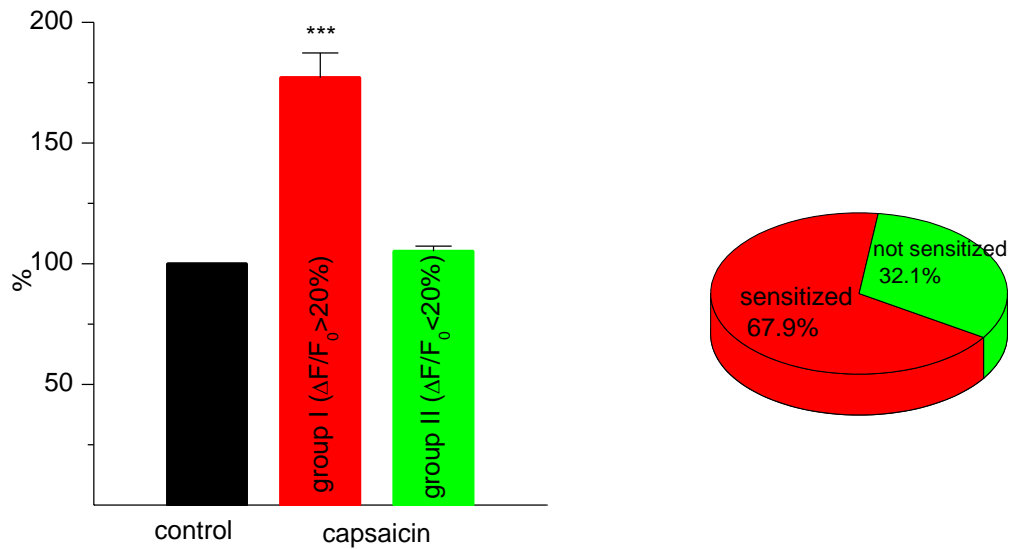
Figure 48. The average capsaicin-induced potentiation was 77,1% (left, p<0.001).The sensitization level was defined arbitrarily as a 20% increase of the stimulation induced fluorescence response above the control level before the capsaicin application. Out of 53 capsaicin-treated neurons were 67,9% sensitized (pie chart).

We have found that the capsaicin application causes increase in the fluorescence induced by the dorsal root stimulation. An example recording in a single neuron is shown in Fig. 47. The sensitization threshold was defined arbitrarily as at least 20 % increase in the stimulation-induced fluorescence response above the control level (before the capsaicin application). The average capsaicin-induced sensitization was 177.1 % of the control response and out of the 53 capsaicin-treated neurons 67.9 % were sensitized (Fig. 48).

5   DISCUSSION

5.1 CHANGES IN CA-BINDING PROTEIN EXPRESSION IN A MODEL OF ARTHRITIS

5.1.1 Changes in parvalbumin expression in spinal cord dorsal horn

In our study, carried out using densitometric analysis of immunohistological sections and Western blot techniques in control and arthritic rats, we have shown that peripheral inflammation leads to decreased expression of parvalbumin in the neuropil of the superficial dorsal horn ipsilateral to the arthritis and we assume that some of these changes could occur in GABAergic interneurons.

The presence of parvalbumin and other calcium-binding proteins in the spinal dorsal horn was described in several morphological studies (Yamamoto et al., 1989; Antal et al., 1990; Zhang et al., 1990; Ren and Ruda, 1994). Parvalbumin is located in all layers of the spinal cord; mainly in the inner part of lamina II (Antal et al., 1990; Ren and Ruda, 1994) or along the lamina II/III border (Yamamoto et al., 1989), which is in good agreement with the present study. It was found in the postsynaptic as well as presynaptic terminals in the inner part of lamina II (Antal et al., 1990), which indicates a possible important role for parvalbumin in modulation of sensory inputs both at presynaptic and postsynaptic levels.

Parvalbumin acts in neurons as a mobile endogenous calcium buffer that affects spatiotemporal characteristics of calcium transients (Neher and Augustine, 1992; Palecek et al., 1999) and is involved in modulation of synaptic transmission. It decreases intracellular free calcium concentration and ensures the residual calcium clearance after intracellular calcium transient (Atluri and

Regehr, 1996). It is known to accelerate the initial decay of the calcium transient (Chard et al., 1993; Caillard et al., 2000; Lee et al., 2000a; Lee et al., 2000b; Schmidt et al., 2003; Müller et al., 2007). The impact on synaptic transmission was observed in the interneuron/Purkinje cell synapse, which was converted from a depressing into a facilitating one by elimination of parvalbumin from the presynaptic ending (Caillard et al., 2000; Collin et al., 2005). Experiments performed on the Calyx of Held synapse showed that parvalbumin accelerated the decay of the calcium transient and short term facilitation (Müller et al., 2007) and similar results were also shown in rat dorsal root ganglion neurons (Chard et al., 1993).

Several studies have shown that unilateral peripheral inflammation can induce bilateral changes in protein expression in the spinal cord level as a consequence of peripheral inflammation. In most of the studies, the effect was much more pronounced on the side ipsilateral to the unilateral inflammation (Traub et al., 1994; Zhang et al., 2002). These changes may be dependent on the exact model of inflammation used (complete Freund's adjuvant vs. carageenan) and the time when the changes were evaluated after the induction of inflammation. In our experiments we did not observe any obvious changes on the contralateral side when compared to the control animals. In densitometric studies it is usually difficult to compare density of staining in different tissue slices, unless a very significant change in staining is present. Therefore we did not attempt to do densitometric comparisons between the control and experimental animals.

Immunohistochemical studies showed that a transient decrease of parvalbumin immunoreactivity was described in cultured cortical neurons as a

result of application of an NMDA receptor antagonist specifically in parvalbumin interneurons (Kinney et al., 2006). Marked reduction of the parvalbumin expression was observed also in the hippocampal interneurons (De Jong et al., 1993) In a region associated with pain transmission (Willis and Coggeshall, 2004), 43% of the neurons in substantia gelatinosa are presumably GABAergic inhibitory interneurons (Todd and Sullivan, 1990) and parvalbumin-containing neurons are thought to represent a subpopulation of these GABAergic neurons (see Baimbridge et al., 1992 for a review). At the spinal cord level, it was shown that 5-21% of the GABA immunopositive neurons in the substantia gelatinosa region coexpressed parvalbumin (Antal et al., 1991). In the population of parvalbumin immunopositive neurons in this spinal cord dorsal horn region (laminae II + III), that are colocalized with GABAergic neurons expressing green fluorescent protein in transgenic mice (Hantman et al., 2004), is much higher (75%, Laing et al., 1994; 60-70%, Antal et al., 1991). These data suggest that a majority of the PV immunopositive neurons in this region of the spinal cord dorsal horn may be GABAergic inhibitory interneurons. Based on this evidence it seems plausible to suggest that some of the changes in PV expression in the substantia gelatinosa region seen in our study were due to reduced expression of PV in GABAergic interneurons.

We suppose that reduction of the parvalbumin content in the presynaptic terminals of the GABAergic interneurons increases residual calcium level during high frequency bursts of activity and potentiate facilitation and increased transmitter release. This would lead to increased potency of inhibitory circuits at the spinal cord level under the peripheral inflammatory conditions. These results

suggest that parvalbumin downregulation can play an important role in modulation of nociceptive transmission.

5.1.2 Changes in calcium binding protein expression in STT neurons

The immunohistochemical technique and model of experimental arthritis were subsequently used to analyze expression of slow Ca buffer parvalbumin and two fast Ca buffers calbindin and calreticulin in retrogradely labeled STT neurons in the spinal cord. About half of the labeled STT neurons stained for calreticulin, 11 % for calbindin, and 9 % for parvalbumin. In animals with the knee arthritis, the number of the CB and PV positive STT neurons on the ipsilateral side significantly increased (23%, 25%) while the number of CR positive STT neurons did not change. The only related study exploring STT neurons calcium binding protein immunoreactivity (in monkeys) reported higher calbindin levels likely due to species differences (Craig et al., 2002).

Considering the suggested role of parvalbumin in potentiation of GABAergic synaptic transmission (chapter 5.1.1), the increase of PV expression in the STT projection neurons may attenuate STT neurons sensitivity to afferent input and thus reduce nociceptive transmission to supraspinal centers. Both of these mechanisms could contribute to the modulation of spinal neuronal activity and counteract the excessive neuronal activity coming from the periphery under inflammatory conditions.

Since calbindin is known to be relatively resistant to the neurotoxic effect of glutamate (D'Orlando et al., 2001, 2002), the increased expression be explained by protection against glutamate-induced excitotoxicity as a result of sustained synaptic activity arising from the inflamed periphery (Schaible & Schmidt 1985;

Westlund et al., 1992; Sorkin et al., 1992). In hippocampal neurons increased expression of CB suppressed posttetanic potentiation (Chard et al., 1995). Increased expression of CB in STT neurons could counteract their sensitization following the development of knee arthritis (Dougherty et. al. 1992).

Calretinin expression in STT neurons was not significantly altered in our experimental model; however its high expression suggests an important role in the spinal sensory function. A dense calretinin staining was previously found in the superficial laminae and in the lateral spinal and cervical nuclei (Ren et al., 1993). Some recent studies indicate neuromodulatory (Schurmans et al., 1997) and neuroprotective (Henzi et al., 2009) functions, and a possible role of calretinin in the induction of central sensitization through its coexpression with the Kv 4.3 alpha subunit of voltage-gated K(+) channels (Huang et al., 2005).

5.2 CALCIUM IMAGING STUDY OF SPINAL CORD SYNAPTIC TRANSMISSION

In this project we have used calcium imaging technique on acute spinal cord slices to study activity of the superficial spinal dorsal horn neurons activated by electrical stimulation of the dorsal root. We have dissected synaptically evoked intracellular calcium concentration changes based on their temporal and pharmacological characteristics and explored a possibility to use this in vitro model as a tool in pain research.

5.2.1 *Synaptically-evoked calcium transients in the spinal cord dorsal horn*

Stimulation of the dorsal root evoked two temporally distinct calcium transients in the body of the dorsal horn cells: The fast $[Ca^{2+}]i$ transient, synchronized with electrical stimulation and immediately following the stimulus, and the delayed $[Ca^{2+}]i$ increase, which was preceded in most cases with a fast component of lower amplitude.

It is generally accepted that the majority of the depolarization-induced calcium influx can be ascribed to the opening of the voltage-gated calcium channels during action potential propagation (Miyakawa et al., 1992). For instance, experiments investigating the role of the VGCC in the spinal cord nociceptive transmission found that the calcium influx due to the postsynaptic membrane depolarization is substantially blocked by application of an unspecific VGCC antagonist Cd^{2+} (Heinke et al., 2004). As the predominant channel involved in both the pre- and postsynaptic signaling was indentified the N-type voltage-gated

calcium channel. Accordingly, as action potentials are generated in the course of the dorsal root electrical stimulation, the VGCC-mediated calcium influx should coincide with the electrical pulse duration. These characteristics apply well to the fast calcium transients recorded in our experiments.

However, in contrast to the fast $[Ca^{2+}]i$ transients, the delayed $[Ca^{2+}]i$ responses were not previously seen in the spinal cord preparations, therefore a more detailed analysis followed.

5.2.2 Identification of the cell types associated with the calcium transient

The most abundant cell types in the nervous system are glial cells, which were previously found to show calcium response to the synaptic activity. It is known that from four types of glial cells inhering in the spinal cord gray matter (Chvatal et al., 1995) the astrocytes are most likely to be morphologically mistaken for neurons. For this reason, we have considered a possibility that calcium transients arise from the activation of glia rather than neurons.

Two methods, the SR101 labeling and low-KCl ACSF glia activation technique, was used to differentiate in vivo between neurons and glia in the group of cells responding to electrically evoked synaptic transmission with calcium transients. The SR101 labeling method was based on the finding that in neocortical preparations the SR101 stains specifically protoplasmic astrocytes (Nimmerjahn et al., 2004) while calcium sensitive dye OGB-1 is taken up by both neurons and glial cells. None of the fast or delayed calcium responses to the electrical stimulation were colocalized with the in vivo glial-specific marker SR101, suggesting that the recorded responses were of neuronal origin.

The method using testing application of low KCl solution takes advantage of the fact that only glial cells respond with calcium transient to low-KCl saline application. This response is as a result of activation of barium sensitive calcium channels (Dallwig and Deitmer, 2002). In our experiments, only 4.4 % of the previously identified cells with fast transients responded to the low-KCl ACSF substitution with an increase in intracellular fluorescence intensity. These results taken together with the results obtained with the SR101 method suggest that the fast calcium transients occurred in neurons.

In the population of cells with the delayed $[Ca^{2+}]i$ transient, we have identified a 29%-fraction of cells, which reacted positively to the low-KCl ACSF application. Though SR101 labeling implies that none of the recorded cells responding to the stimulation were astrocytes, the low-KCl method suggests that at least a fraction of the cells, showing delay in [Ca2+i] increase, could be of glial origin. The difference in identification of glial cells between the low-KCl and SR101 methods might be explained by the possibility that the cells were of glial origin other than astrocytes, by coincident spontaneous release of calcium from intracellular stores in neurons during low-KCl application or more likely by the fact that spinal cord preparation differs from experiments carried out on cortex.

Taken together results of both the SR101 method and the low-KCl method, we can conclude that we cannot exclude the possibility that some of the delayed responses occurred in the glial cells while the majority was present in the neurons.

5.2.3 Reproducibility and stability of the fast and delayed calcium transients

Correct interpretation of results and analysis of pharmacological experiments requires stable control recording. In case of our experiments, it corresponds to the capability to repeatedly induce calcium transients with low amplitude variability and ideally with no significant changes during the time series of the evoked fluorescence responses. Artificial trends are often introduced due to dye bleaching and due to long periods of laser illumination (i.e. a decrease in fluorescence intensity in time) or due to dye leakage (decrease in dye concentration over time or phototoxicity (Eggeling et al., 1998, Stosiek et al., 2003). In our experiments destruction of the fluorophore was mostly avoided by low power of the Argon laser used for the fluorophore excitation. The remaining photobleaching was compensated by mathematical correction using monoexponential fit (Hoebe et al., 2007). Further, it is known that repeated synaptic activation leads to synaptic fatigue as a result of train of electric pulses (Tancredi et al., 2000). We have successfully avoided the synaptic depression by introducing at least 3 min time period between the consequent stimulations.

By testing reproducibility and stability of the synaptically evoked time-dependent fast and delayed [Ca2+i] transients in the course of control experiments we have found that no significant changes in the amplitudes of both fast and delayed [Ca^{2+}] responses were observed. The data shown that electrical stimulation itself, applied in 3 to 4 min intervals, does not interfere with potential

effects of the drugs under examination and the model can be used as a tool to monitor changes in synaptic activity.

5.2.4 Pharmacological characterization

5.2.4.1 Sodium-channel blocker and ionotropic glutamate receptor antagonists

Both the fast and delayed responses to the electrical stimulation were completely abolished by sodium-channel blocker tetrodotoxin (TTX). TTX a sodium channel blocker prevents action potentials. Our results thus show that the postsynaptic neuronal calcium transients were caused by action potential generation rather than direct VGCC channels opening. Direct activation of VGCC channels could be observed, if stimulation electrode was placed directly into the slice when following stimulation the subsequent somatic calcium transient showed peak plato. Similar plato is also observed when vitality of the neurons is severely compromised, therefore this type of responses were excluded from the evaluation (Limbrick et al., 1995).

Application of the AMPA receptor antagonist CNQX with the non-competitive NMDA receptor antagonist MK801 inhibited the evoked calcium transients in our experiments. We assume that this was due to inhibition of postsynaptic glutamate receptors activated under control conditions by glutamate released from central terminals of the stimulated dorsal root nerve fibers. In this manner, application of these antagonists prevented glutamate induced depolarization of postsynaptic membrane, generation and propagation of action potential in the postsynaptic neuron thereby preventing voltage-gated calcium channels-mediated calcium influx.

The antagonists (CNQX and MK801) of the ionotropic glutamate receptors suppressed the fast $[Ca^{2+}]i$ transients significantly. To the contrary, the delayed $[Ca^{2+}]i$ responses were not significantly affected by the combined ionotropic receptor antagonists' application, suggesting other source of intracellular calcium increase such as calcium release from intracellular calcium stores.

5.2.4.2 *Intracellular calcium stores depletor caffeine*

Several other studies showed delayed calcium increases similar to those in our experiments in visual cortical preparations (Miyakawa et al., 1992; Nakamura et al., 1999; Zhou and Ross, 2002; Larkum et al., 2003). These results suggested involvement of the intracellular calcium stores in the process of cytoplasmic calcium elevation in response to the synaptic activity. We have tested the possible involvement of calcium stores in mediation of the delayed responses in our experiments with application of caffeine. The 20 mM caffeine potentiated the stimulation-evoked fast $[Ca^{2+]i}$ transients but simultaneously suppressed significantly the delayed $[Ca^{2+}]i$ responses suggesting the involvement of calcium release from calcium stores.

Xanthenes like caffeine activate ryanodine receptor (RYR) by increasing probability of the channel opening, thus potentiating their sensitivity to calcium as a native ligand. This leads to store replenishment via calcium-induced calcium-release (CICR) mechanism (Shmigol et al., 1996). A lasting milimolar caffeine concentration acts as an endoplasmic reticulum depletor by sensitizing ryanodine receptors to calcium insomuch that basal free calcium concentration becomes activatory. This also effectively inhibits calcium buffering function of the endoplasmic reticulum and probably constitutes the main factor of the fast

calcium response potentiation, due to increased accumulation of free calcium in cytoplasm after calcium influx through the VGCC during the stimulation.

The suppression of the delayed calcium response after the caffeine application and consequent depletion of the endoplasmic reticulum, points at an intracellular origin of this delayed free cytoplasmic $[Ca^{2+}]i$ increase. This is further supported by experiments in which a single neuron was scanned with high magnification. In these experiments propagating calcium wave from dendrites into neuronal body was shown, similar to previously reported experiments (Jaffe and Brown, 1994). The wave propagation from the dendritic area into the neuronal soma is consistent with the origin of the delayed response due to calcium release from intracellular stores after dendritic activation due to synaptically released neurotransmitters and/or neuromodulators.

A similar two-phase delayed Ca transient was previously evoked nonsynaptically by intrasomatic current injection where intracellular calcium stores were activated by calcium entering through Verapamil-sensitive VGCC and the second phase of the delayed transient was substantially suppressed by combined ryanodine and IP3 receptor antagonists' application (Kato et al., 1999). Furthermore, experiments investigating synaptically evoked calcium transients also registered delayed $[Ca^{2+}]i$ change with essentially the same characteristics as were demonstrated in our experiments (Miller et al., 1996; Nakamura et al., 1999; Connor and Pozzo-Miller, 2003; Larkum et al., 2003; Waters et al., 2003).

Experiments carried out on spinal cord slices using 100 Hz stimulation and fura-2 indicator showed, in agreement with our findings, that the first calcium peak is largely of ionotropic origin with a intracellular calcium stores' calcium

contribution (Luo et al., 2008). These experiments didn't demonstrate the delayed calcium release present in our experiments, which might be due to combination of use of the probe with low quantum yield and 100 Hz stimulation in the described experiments. 100Hz stimulation in our experiments led to merge of the fast and the delayed peak, which might explain the 46 % suppression of the somatic transient after IP3 receptor antagonist application in the published experiments (Luo et al., 2008). The incomplete blockade of the delayed response in their experiments may be caused by an overlap of a potentiated fast $[Ca^{2+}]i$ curve decay-phase with the delayed calcium peak.

Calcium mobilized in presynaptic endings by IP3 released from intracellular calcium stores can be important also in nociceptive synaptic transmission due to modulation TRPV1 receptor (the same pattern of TRPV1 receptor's activity modulation manifests a G protein-coupled receptor – the chemokine receptor CCR2 upregulated following nerve injury; White et al., 2007). Calcium that enters into cytoplasm through TRPV1 receptor can influence the function of the TRPV1 receptor itself (by negative feedback) as well as the function of other channels (e.g. downregulation of VGCC), enhance glutamate release and NO production (Samways et al., 2008). Evidence suggest that TRP homologs are related to store-operated channels (SOCs; Parekh, 2008). This way TRPV1 receptor acts as a store operated channel. This is consistent with a finding that intracellular calcium mobilization as a result of, for instance, mGluRs, substance P, or other neuroactive peptide activation can lead to anandamide production beyond normal physiological levels and activate TRPV1-dependent Ca^{2+} influx (van der Stelt et al., 2005). In respect to the nociceptive transmission and modulation, calcium-activated endogenous anandamide may act as a calcium signal amplifier

contributing to the central sensitization. This hypothesis is based on the fact that robust stimulation of nociceptive pathways associated with central sensitization induction is supposed to be linked to intracellular calcium stores mobilization (see next chapter 5.2.5 on differential dorsal root stimulation). However an effect of the opposite direction should be taken into account when considering involvement of calcium mobilized from intracellular stores. That is the calcium activated potassium channel conductance which leads to the slow afterhyperpolarization of the postsynaptic membrane (Fig. 49)
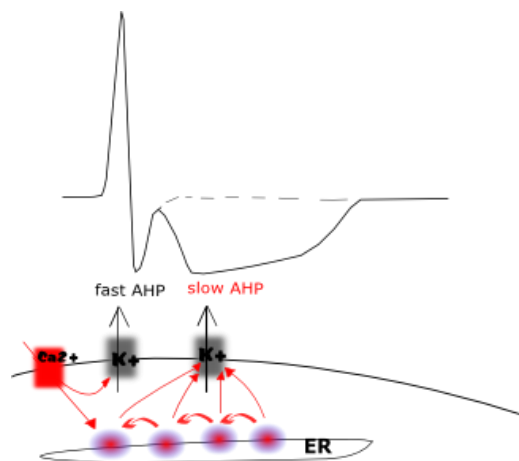
Figure 49. Release of calcium from intraluminal space increases Ca^{2+} activated K^{+} channel conductance and leads to hyperpolarization.

5.2.5 Differential dorsal root stimulation

It was found that stimulation with 0.5 ms pulse duration, capable of both A and C fibers recruitment, led to the increased number of delayed [Ca^{2+}]i transients compared to stimulation with A-fiber-specific 0.1 ms pulse. The number of evoked fast transients was not affected by the two stimulation protocols.

It was shown previously that C fibers are activated by more intensive stimuli than A fibers (Purves, 2001). In our experiments different duration of the stimulation pulse was used to activate C+A fibers (0.5 ms) and A fibers (0.1 ms) while keeping the rest of the stimulation parameters constant. An increased proportion of neurons with the delayed calcium response was detected when the 0.5 ms stimulation pulse was used, suggesting a link between stimulation of C nerve fibers and stimulation induced calcium release from internal stores. The possible mechanism is G_q protein-mediated inositol-1,4,5-trisphosphate (IP_3) synthesis (Womack et al., 1988) and internal calcium stores activation. The GPCRs involved could be metabotropic glutamate receptors (mGluRs), activated by additional glutamate released by higher intensity stimulation or specific glutamate release at C fiber innervated synapses. C fiber stimulation was shown to release specific neuropeptides such as substance P and neurokinin A in the spinal cord that act on their neurokinin receptors. The SP release increases with the firing frequency of C fibers. Moreover, sensitization of IP3R and RYR by increased influx of extracellular calcium due to action of C fiber-released neuromodulators and neurotrophins like CGRP and BDNF elevates PKC levels and increases excitability (Woolf and Salter, 2000).

These results suggest that intracellular calcium stores were activated via metabotropic pathways in our experiments and may play an important role in pain transmission at the spinal cord level.

5.2.6 In vitro model of central sensitization

A patch clamp technique is a common neurophysiological technique used to study intracellular mechanisms of synaptic modulation in living cells. Despite its

robustness, it is not convenient when study of neuronal populations in their natural environment is of primary concern. Limiting can be a the dialysis of the cell content during the whole-cell recording setup or a delayed action of ionophores and decreased current resolution at the perforated patch clamp recordings (Mathias et al., 1990) . Moreover it is impossible to record from more than several cells at the same time in a tissue slice.
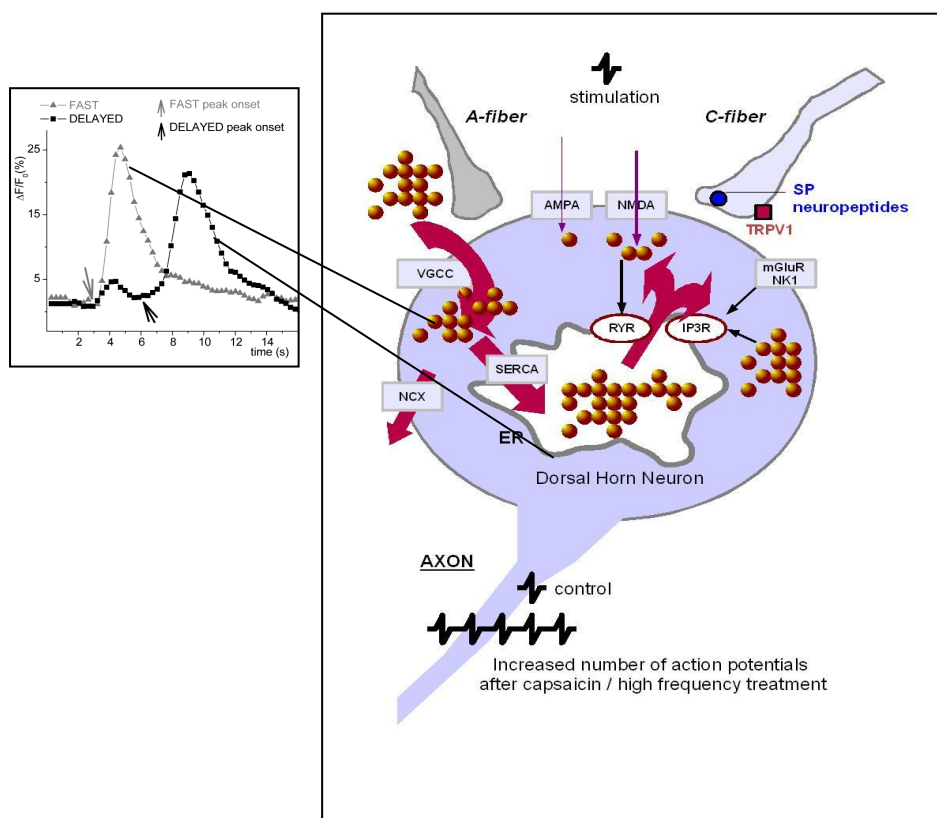
Figure 50: Graphical summary of the main results. The small figure shows the two types of calcium responses as recorded during our experiments and the large drawing illustrates the accountable mechanisms for each type of the response - the fast Ca-transient as a result of the VGCC channels opening and the delayed Ca-transient as result of the intracellular calcium stores mobilization. In the larger picture there is also demonstrated how synaptic transmission modulation leads to increased number of action potential.

To overcome the restraints associated with this traditional method, employment of optical methods together with bulk loaded fluorescent probes and in vitro acute tissue slice preparations were recently used (Ikeda et al., 2000; Ikeda et al., 2003) to study intracellular biochemical processes. This experimental approach was tested on several central nervous system preparations previously, especially in hippocampal and neocortical pyramidal neurons (Zhou and Ross, 2002; Larkum et al., 2003). As the activity of neurons can be measured in a large population of neurons at the same time, it may be also useful for drug testing with potential pharmacological significance in pain research.

The voltage-gated calcium channels (VGCC) in neuronal plasma membrane are the principal source of fast somatic calcium transients during action potentials membrane depolarization. In our experiments we have tested a hypothesis that measurement of the intracellular calcium concentration changes by fluorescent probe is a reliable representation of the action potential-mediated synaptic activity in neurons. The qualitative analysis of the stimulation efficiency (dependence of the fluorescence change on the number of applied stimulation pulses; 1, 3, 5, 10 and 20) showed that there is a strong correlation between those two quantities. This is in good agreement with previously published work where membrane depolarization was induced by direct current application in patch clamp experiment (Balaban et al., 2004).

Further analysis revealed that both the fast and the delayed responses increased with the increasing number of stimulation pulses. However, the fluorescence increase in the range of 1 to 20 pulses is non-linear with one exception – the fast transient analyzed using the area-under curve method. The

curves of the other analyzed fluorescence/pulse number dependencies tended to plateau and follow exponential relationship. For this reason, analysis of the capsaicin-induced sensitization was performed using 3 stimulation pulses. This was well under the saturation level and also avoided possible consequences of single action potential propagation failure when only one action stimulation pulse would be applied. Experiments performed previously on primary cultured hippocampal neurons using electrical field stimulation and Fluo-4 AM probe measured in soma showed a linear dependence between the number of action potentials and the calculated peak internal calcium release (Jacobs and Meyer, 1997) The described experiments showed an all-or-none type dependence of the on the applied electric field strength and pulse width.

The plateau of the fluorescence intensity and the corresponding calcium transient amplitude with increasing number of stimulation pulses with the same stimulation frequency may be due to a number of mechanisms. The most likely is that the intracellular calcium homeostatic mechanisms, described in the introduction, were able to reduce the intracellular calcium concentration associated with the action potentials more effectively with the longer duration of the stimulus series. With the low stimulation frequency of 10 Hz, these calcium buffering and extrusion mechanism were able to reduce the further calcium influx and thus additional action potentials did not evoke any further significant intracellular calcium increase. Other possible mechanism of the calcium response saturation would include synaptic depression, when additional electrical stimuli and propagating action potentials in the dorsal root afferent fiber did not evoke glutamate release at the synapse. Also calcium-dependent inactivation of the VGCC channels could play a role. However, these factors are less likely as

primary afferents in the peripheral nerve and consequently in the dorsal root are able to transmit significantly higher frequencies than 10 Hz under physiological conditions.

Previous in vitro models of spinal cord sensitization have used high frequency (100Hz) burst to induce synaptic potentiation. While this approach was shown to be quite effective, it did not give as good results in our preliminary experiments. We have thus decided to use capsaicin, an activator of TRPV1 receptors present at primary afferent nociceptive fibers in the dorsal root/spinal cord. It was shown previously that capsaicin can induce robust increase of EPSCs in dorsal horn neurons (Spicarova and Palecek, 2009). Capsaicin was also used repeatedly to activate nociceptors in the periphery (skin) and to induce sensitization of dorsal horn and spinothalamic neurons (Palecek et al., 2003)

In our experiments capsaicin was administered on the spinal cord slice to induce central sensitization, likely due to activation of the presynaptic TRPV1 receptors. It was crucial to keep the capsaicin concentration relatively low, as it was shown previously that high concentration of capsaicin can lead to TRPV1 receptors desensitization and also to nerve fiber destruction (Pospisilova and Palecek, 2006). Experiments on spinal cord slices showed that 2 µM capsaicin concentration induced reduction of C-fiber evoked EPSC (Baccei et al., 2003).

In our experiments, the capsaicin application induced a significant increase of the control stimulation evoked calcium transients (on average 177.1 % of the control response) in a subpopulation of 67.9 % of the 53 capsaicin-treated neurons. We assume that this calcium transient increase was due to increase in number of action potentials fired by the recorded neurons. This would correspond

to the increased number of action potentials recorded in spinothalamic neurons after peripheral activation of cutaneous nociceptors with capsaicin (Dougherty and Willis, 1992; Palecek et al., 2003). The increased number of action potentials evoked by the control stimulation after capsaicin application can be thus also interpreted as increased synaptic efficacy. The potentiation of the responses lasted at least 30 min. after the capsaicin application. We suppose that this model could be used as an additional tool for pharmacological experiments investigating mechanisms of central sensitization.

6 CONCLUSIONS

- Expression of calcium buffering protein parvalbumin is decreased in the dorsal horn after peripheral inflammation.

- Expression of calbindin and parvalbumin was increased in STT neurons as a result of peripheral inflammation. Calretinin expression level is high in STT neurons and remains unchanged after peripheral inflammation. Calcium buffering proteins may play an important role in calcium homeostasis a modulation of nociceptive transmission in dorsal horn neurons.

- Preparation of acute spinal cord slices and loading cells with calcium sensitive Fluo-4 indicator enabled recordings of intracellular calcium concentration changes in dorsal horn neurons.

- Stimulation (10Hz, 1s, 0.5 ms pulse length) of primary afferents in the dorsal root evoked increase of the calcium concentration in number of dorsal horn neurons. These neurons showed two types of responses (fast and delayed) measured as the onset of the evoked calcium transient.

- The fast intracellular calcium changes were most likely due to calcium influx through voltage dependent calcium channels during action potential firing of the neurons induced by the stimulation. This process was dependent on activation of AMPA/NMDA receptors.

- The delayed responses were due to calcium release from intracellular stores and were diminished by caffeine application. This calcium change

was not affected by AMPA/NMDA antagonists and was most likely mediated by metabotropic receptors.

- Higher proportion of neurons showed delayed calcium response following the stimulation with 0.5 ms pulse duration, suggesting that activation of C-fiber afferents preferentially leads to the delayed calcium concentration changes.

- Intracellular calcium concentration changes mediated by metabotropic receptors and induced by calcium release from internal stores may play an important role in the modulation of synaptic transmission at the spinal cord level.

- Capsaicin application induced significant potentiation of the calcium transients induced in the dorsal horn neurons by dorsal root stimulation.

- Calcium imaging in population of neurons might be a useful tool for pharmacological tests of drugs with potential antinociceptive effects.

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8 LIST OF ABBREVIATIONS

$[Ca^{2+}]i$	Intracellular calcium concentration
5-HT 3	5-hydroxytryptamine (serotonin) receptor 3
AC	Adenylate cyclase
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
cADPR	Cyclic ADP Ribose
CaMKII	Ca2+/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CB	Calbindin
CBP	Calcium-binding proteins
CGRP	Calcitonin gene related peptide
CICR	Ca-induced Ca release
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CR	Calretinin
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglion
DTT	Dithiothreitol

EAA	Excitatory amino acid
EAATs	Excitatory amino acid transporters
ER	Endoplasmic reticulum
ERK/MAP	Extracellular-signal-regulated kinase/Mitogen-activated protein kinase
FAF1	Fas-associated factor 1
FFT	Fast Furier transform
GPCR	G protein-coupled receptor
IASP	International Association for the Study of Pain
IICR	Inositol-1,4,5-trisphosphate-induced calcium release
IP_3	Inositol 1,4,5-trisphosphate
LD50	Median lethal dose
LTP/D	Long-term potentiation/depression
MCPG	α-Methyl-4-carboxyphenylglycine
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
MK801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten--5,10-imine maleate
NCX	Sodium-Calcium exchanger
NK	Neurokinin
NMDA	N-methyl D-aspartate
PBS	Phosphate buffered saline (PBS)
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMCA	Plasma membrane Ca2+ ATPase
PV	Parvalbumin

ROI	Region of interest
RYR	Ryanodine receptor
SCaMPER	Sphingolipid Ca2+ release-mediating protein of the ER
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca2+-ATPase
SOC	Store-operated channel
SP	Substance P
SR101	Sulforhodamine 101
STT	Spinothalamic tract
TBS	Tris-buffered saline
TEMED	N, N, N', N'-tetramethylethylenediamine
TNF-α	Tumor necrosis factor-alpha
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channels
VGluTs	Vesicular glutamate transporters