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DIPLOMARBEIT

Titel der Diplomarbeit

Cytokines mediate glial - neuronal crosstalk - Effects of IL-1ß on synaptic transmission

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Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

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I. Zusammenfassung

Schmerz ist eine lebenserhaltende biologische Funktion des Organismus, die den Körper sowohl vor potentiell gefährdenden, als auch vor bereits bestehenden Gewebeschäden warnt. Schmerz wird über die Aktivierung von Schmerzrezeptoren, sogenannten Nozizeptoren ausgelöst. Nozizeptoren sind spezialisierte Nervenendigungen, die auf starke mechanische, chemische und physikalische Reize reagieren. Die nozizeptive Information wird dann über schwach myelinisierte Aδ- und nicht myelinisierte C-Fasern in das Hinterhorn des Rückenmarks und in weiterer Folge in das Gehirn weitergeleitet, wo der Schmerz erstmals wahrgenommen wird. In den oberflächlichen Laminae I/II des Rückenmarks kann es an der ersten synaptischen Umschaltstelle zwischen afferenten Neuronen und verschiedenen nachgeschalteten Rückenmarksneuronen zur Modulation der nozizeptiven Information kommen. Unter pathologischen Bedingungen, wie peripheren Nervläsionen oder Entzündungen kann die nozizeptive Information moduliert werden. Hier kommt es zu einer veränderten Übertragung der nozizeptiven Information, die einer Verstärkung der Schmerzempfindlichkeit (Hyperalgesie) zugrunde liegen kann. Ein zelluläres Modell der Hyperalgesie ist die Langzeitpotenzierung (LTP) zwischen primär afferenten C-Fasern und nachgeschalteten Rückenmarksneuronen. Im Rückenmark nimmt sie eine Rolle als spinaler Schmerzverstärker ein, da es auf zellulärer Ebene zur längerfristigen Verstärkung der synaptischen Übertragung kommt. Im Zentrum der Beobachtungen standen zunächst lange Zeit neuronale Prozesse. Die Beteiligung nicht neuronaler Zellen an der synaptischen Plastizität wurde erst kürzlich untersucht. Neue Ergebnisse der letzten Jahre sprechen aber für die Bedeutung des am häufigst vorkommenden Zelltyps im zentralen Nervensystem: Gliazellen. Die Frage, die sich nun stellte, war, ob Gliazellen einen wesentlichen Einfluss auf die veränderte synaptische Übertragung bei einer spinalen LTP nehmen.

Transversalschnitte mit intakten dorsalen Wurzeln juveniler Ratten wurden angefertigt. Die dorsalen Wurzeln wurden mit einer Suction-Elektrode stimuliert und Ganzzellableitungen in Lamina I Neuronen des dorsalen Horns durchgeführt. Im Rahmen der Ganzzellableitungen wurden erregende postsynaptische Ströme (EPSCs) gemessen. Es wurde gezeigt, dass Gliazellen und das von Gliazellen sekretierte Zytokin IL-1ß die synaptische Übertragungsstärke beeinflussen können. Gliazellen scheinen, den aufkommenden Daten zufolge, eine wichtige Rolle in der synaptischen Plastizität zu spielen.

II. Abstract

Pain is described as an unpleasant sensory and emotional experience which protects the body from damaging or potentially damaging situations, by activation of nociceptors. Nociceptors react in response to noxious mechanical, chemical, and physical stimuli. The nociceptive information is then transmitted from primary afferent Aδ- or C-fibers to the spinal dorsal horn. Already at the first synapse, between nociceptors and spinal dorsal horn neurons, nociceptive information can be modulated.

Under pathological conditions like peripheral nerve injury and inflammation, nociceptive information can be modulated, and may be associated with an increased pain perception (hyperalgesia). A cellular model of hyperalgesia is long-term potentiation (LTP) between primary afferents and nociceptive-projection neurons in spinal dorsal horn. Neuronal plasticity was long thought to be entirely controlled by neurons.

Over the last years, glia have emerged as important contributors to pathological and chronic pain conditions. Question arises, whether glia cells crucially influence synaptic plasticity in pain pathways.

Transversal spinal cord slices with dorsal roots attached were dissected from rats. Dorsal roots were stimulated and excitatory postsynaptic currents (EPSCs) were recorded in spinal dorsal horn neurons. We showed that glia cells responded to a conditioning stimulus which also induced potentiation of EPSC- amplitudes in spinal dorsal horn neurons. We further demonsrated that the cytokine IL-1ß, secreted by glia cells, can increase synaptic strength. These findings suggest involvement of IL-1ß in induction and maintenance of synaptic plasticity between primary afferents and spinal dorsal horn neurons. Taken together, glia cells seem to be potential modulators of nociceptive transmission, and therefore key contributors to the development of pathological and chronic pain.

1. Introduction

In the nociceptive system pain is induced upon activation of nociceptors. These are sensory nerve endings of primary afferent Aδ- or C-fibers that react to potentially damaging stimuli. These fibers contact spinal neurons some of which transmit the nociceptive information to the brain. Aδ-fibers are thinly myelinated axons which have moderate conduction velocities. Aδ-fibers are associated with acute, sharp pain. C-fibers are unmeylinated and have the smallest diameters and conduction velocities of all primary afferent fibers. They contribute to the second phase of pain perception, mediating long lasting, burning pain. The synapse between primary afferent Aδ- or C-fibers and spinal laminae I/II neurons is the first synapse where nociceptive information can be modified (**Fig. 1**).

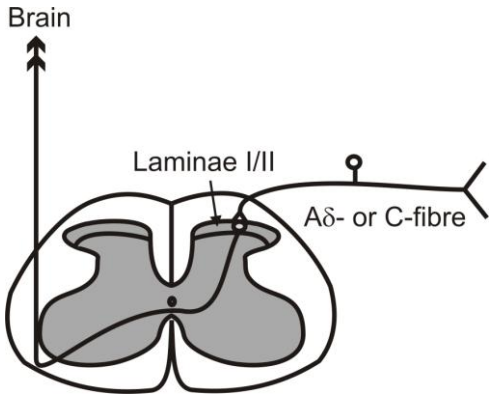


Figure 1: The nociceptive system: Primary afferent fibers convey nociceptive information from periphery to spinal dorsal horn neurons (mostly laminae I/II neurons) which further project to supraspinal centres. At the first synaptic connection in laminae I/II, between primary afferent fibers and laminae I/II neurons, nociceptive information can be modulated for the first time.

Spinal mechanisms leading to the modulation of the nociceptive information include e.g. potentiation of synaptic strength, reduced synaptic inhibition or changes in intrinsic properties (Sandkühler, 2009;Woolf and Salter, 2000). Enhanced neuronal responsiveness can lead to facilitation of spinal nociception and further to an increased pain perception (hyperalgesia), following peripheral

nerve injury (Bennett and Xie, 1988) or inflammation (Lee et al., 2004). A proposed cellular model for hyperalgesia is the long-term potentiation (LTP) between primary afferent nociceptive fibers and spinal lamina I dorsal horn neurons (Ikeda et al., 2003; Ikeda et al., 2006). LTP is the enhanced state of neurotransmission, which arises after a conditioning stimulus, such as high frequency stimulation (HFS) of primary afferent fibres. Upon strong stimulation high amounts of the neurotransmitter glutamate are released. Glutamate then binds to the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), an ionotropic receptor for glutamate which mediates fast synaptic transmission. Following activation of AMPA receptors, Na^+ flows into the postsynaptic neuron, resulting in a depolarization. Enhancement of synaptic transmission can be triggered by activation of the voltage-dependent, glutamatergic *N*-methyl *D*-aspartate receptor (NMDAR). NMDARs are ionotropic glutamate receptors which are also voltage-dependent, as a result of an ion channel block by extracellular Mg^{2+}-ions. The activation of NMDA receptors causes, next to Na^+, an influx of Ca^{2+}. The rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) triggers a cascade of cellular signalling events which are necessary for the induction and sustained expression of LTP (Ikeda et al., 2003; Ikeda et al., 2006). It has been shown, that the magnitude of $[Ca^{2+}]_i$ is correlated with the magnitude of LTP in vitro (Ikeda et al., 2003). Recent studies showed that NMDAR opening may also be enhanced by NK1 receptor activation (Lieberman and Mody, 1998). At least two different stages of LTP can be distinguished depending upon its duration and signal transduction pathways involved. Early phase LTP is independent of novel protein synthesis and lasts from half an hour to 3 hours. Early phase LTP mainly includes phosphorylation of receptors/ion channels, or regulatory proteins, or cell-surface expression of channels in primary afferents and dorsal horn neurons (Sandkühler, 2009; Woolf and Salter, 2000). Late phase LTP lasts longer than 3h and requires protein synthesis. Long lasting modifications are caused by altered gene expression, loss of inhibitory interneurons and establishment of novel excitatory synaptic connections

(Sandkühler, 2009; Woolf and Salter, 2000). Signalling pathways in induction and maintenance of LTP involve activation of protein kinase C, mitogen-activated protein kinase, protein kinase A, calcium/calmodulin-dependent protein kinase II, nitric oxide synthase, inositol triphosphate receptors, and ephrin-EphB2 receptor tyrosine kinase (Ikeda et al., 2006; Ikeda et al., 2003; Song et al., 2008; Xin et al., 2006; Yang et al., 2004; Zhang et al., 2005). As reviewed above, LTP can be induced between primary afferent fibers and spinal dorsal horn neurons and may contribute to hyperalgesia caused by trauma, neuropathy, or inflammation (Sandkühler, 2007).

The emerging literature implicates a role for glial cells and inflammatory cytokines in the genesis or maintenance of neuropathic and chronic pain (Watkins et al., 2007; Watkins and Maier, 2005). Under basal conditions, microglia and astrocytes do not appear to be important regulators of pain transmission in the nociceptive system. This conclusion is based on the fact that drugs that inhibit glial function do not influence responses to heat or mechanical stimuli in naive animals (Ledeboer et al., 2005; Meller et al., 1994).

The role of glial cells in the spinal dorsal horn changes, following peripheral nerve injury (PNI) (Raghavendra et al., 2004; Tsuda et al., 2005; Watkins et al., 2001; Zhuang et al., 2006). Upon peripheral nerve injury microglia and astrocytes become activated and undergo cell transition from bystander/surveillance states to enhanced response states. Enhanced response states include morphological and molecular changes in activated microglia and astrocytes (Tsuda et al., 2005; Zhuang et al., 2006). After stimulation, primary afferent fibers release neurotransmitters at their spinal terminals, which activate their cognate receptors on postsynaptic dorsal horn neurons and glial cells. Neurons and glial cells share the same types of ionotropic and metabotropic receptors which enable cross-talk between neurons and glia (Haydon, 2001; Watkins et al., 2008). Specific features of microglial and astrocytic responses are phosphorylation of mitogen activated-protein kinase (MAPK) p38 and c-Jun N-terminal kinases (JNK) leading to the

production of multiple inflammatory mediators, such as cytokines IL-1ß, TNF-α, IL-6, chemokines, neurotrophic factors, proteases and prostaglandins. They modulate neuronal activity and contribute to nociceptive processing (**Fig. 2**) (DeLeo et al., 2004; Ji and Strichartz, 2004; Watkins et al., 2001; Watkins and Maier, 2003).

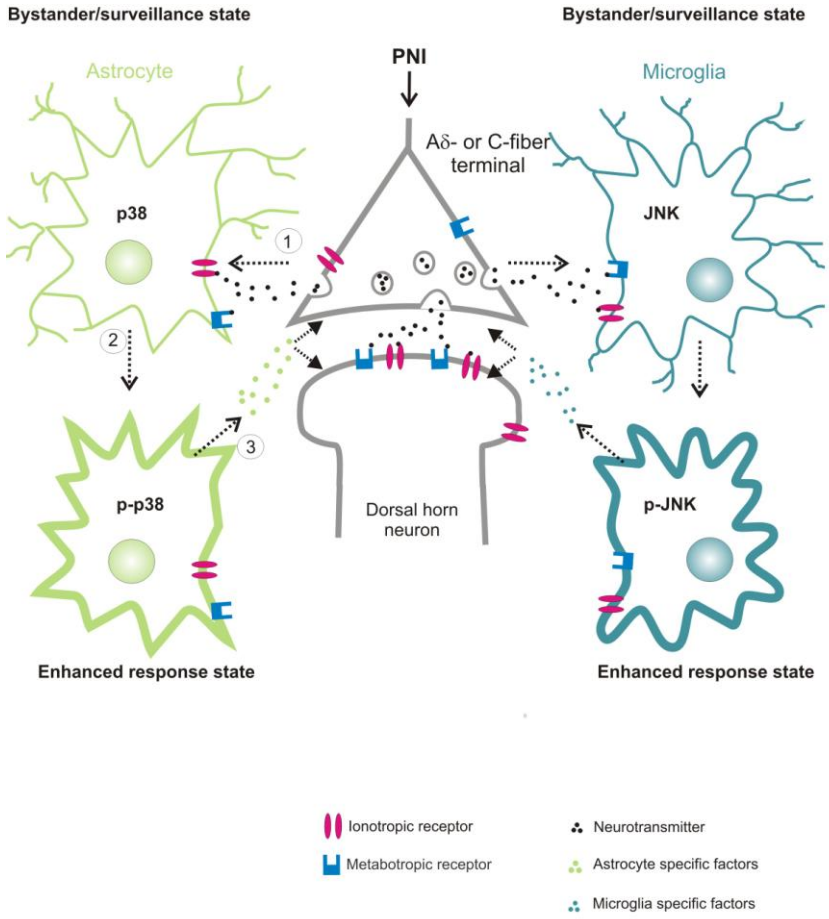


Figure 2: Activation of microglia and astrocytes following PNI. After PNI or tissue injury, primary afferent fibers release pain-related neurotransmitters, and activate ionotropic and metabotropic receptors on spinal dorsal horn neurons, microglia and astrocytes (**1**). Activation of these receptors induces cell transition from bystander/surveillance state to enhanced response state (**2**). Responses include morphological and molecular changes in microglia and astrocytes. Activation of microglia and astrocytes is associated with a hypertrophic, amoeboid shape and phosphorylation of MAPK p38 in microglia and JNK in astrocytes, leading to the production and release of various proinflammatory mediators (**3**). Glial-neuronal crosstalk modulates synaptic strength and contributes to nociceptive processing.

Astrocytes are the most abundant glial cell types within the central nervous system (CNS). Their processes form part of the blood brain barrier and they are also in close proximity to the synaptic cleft. Astrocytes communicate with each other via changes in $[Ca^{2+}]_i$. They respond to various neurotransmitters, such as glutamate with transient elevations of $[Ca^{2+}]_i$. Ca^{2+} is released from intracellular stores after activation of metabotropic glutamate receptors (mGluRs) (Pasti et al., 1997). The signal transduction pathway mediated by mGluRs activates phospholipase C which leads to the generation of inositol 1,4,5-trisphosphate (IP_3) formed by hydrolysis of phosphatidylinositol 4,5 bisphosphate. IP_3 is reported to trigger release of $[Ca^{2+}]_i$ from IP_3-sensitive $[Ca^{2+}]_i$-stores (Berridge, 1993) and subsequent release of gliotransmitters e.g. excitatory amino acids (EEA), ATP, cytokines, chemokines and prostaglandins (Volterra and Meldolesi, 2005). Gliotransmitters can either act on receptors localized on glial cells themselves, or they can activate neuronal receptors. Increasing evidence suggests that gliotransmitters released by activated astrocytes can modulate neuronal excitability and synaptic transmission (Santello and Volterra, 2009). Cytokines like IL-1ß and TNF-α have been described as pain-related amplifiers in the nociceptive system (Milligan and Watkins, 2009; Watkins et al., 1995). TNF-α application is associated with an increasing surface expression of AMPA receptors and increase of EPSC-amplitudes (Beattie et al., 2002). Like TNF-α, IL-1ß, another proinflammatory cytokine expressed in glial cells, is thought to contribute to the induction of neuropathic and inflammatory pain. It facilitates perception of noxious stimuli at the spinal level by phosphorylation of the NMDA receptor subunit NR-1 (Zhang et al., 2008). Otherwise, application of IL-1R antagonist alleviated inflammatory hyperalgesia and also prevented phosphorylation of NR-1 subunit in rats (Zhang et al., 2008). Studies also showed that spinal LTP required activation of NMDA receptors (Ikeda et al., 2003).

Microglia are the resident macrophages of the brain and spinal cord and constitute 5 - 10% of the total glial cell population. Similar to astrocytes, microglia are also capable of releasing pro-inflammatory cytokines upon activation and changing their morphology, from a ramified into a hypertrophic, amoeboid shape (Clark et al., 2010; Tsuda et al., 2005). Studies showed that activation of microglia, induced surface-expression of $P2X_4$ and phosphorylation of p38 MAPK, correlated with an increased pain-perception to noxious stimuli (hyperalgesia) and pain-perception to normally innocuous stimuli (allodynia) following peripheral nerve injury (PNI) (Tsuda et al., 2003; Tsuda et al., 2004). Recent findings suppose a specific role of microglia in the early-phase development of neuropathic pain. IL-1ß which is thought to be processed by different metalloproteases (Kawasaki et al., 2008a), is reported to induce phosphorylation of p38 MAPK, thereby enhancing IL-1ß production, leading to increased pain sensitivity (Sung et al., 2005). These data suggested that cytokines mediate glial-neuronal crosstalk and that IL-1ß could have a crucial role in the modulation of synaptic transmission.

The hypothesis was tested, whether glial cells contribute to the induction of spinal LTP (**Fig. 3**) and whether the proinflammatory cytokine IL-1ß can modulate synaptic tansmission.

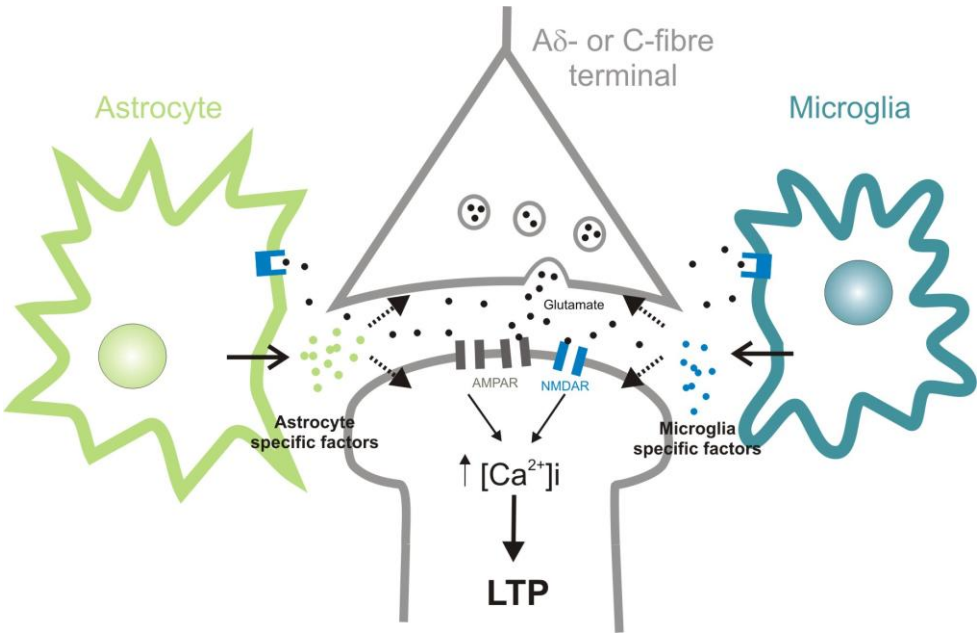


Figure 3: Synaptic strength regulated by pre- and postsynapse, and glial release of proinflammatory mediators. Rise in $[Ca^{2+}]_i$ in the postsynaptic cell induces LTP of synaptic strength. Mechanisms for synaptic plasticity in the spinal dorsal horn include phosphorylation of receptor/ion channels, receptor trafficking, induced gene expression, de novo protein synthesis and establishment of novel excitatory synaptic connections.

We tested effects of IL-1ß on C-fiber evoked AMPAR and NMDAR mediated EPSCs respectively, and also on spontaneous EPSCs (sEPSCs). sEPSCs reflect the local global input of EPSCs following spontaneous release of all presynaptic terminals. Frequencies and amplitudes of sEPSCs were determined to study changes on receptor conductance and open probability.

LTP of C-fiber evoked EPSC amplitude was induced following HFS in lamina I neurons (Ikeda et al., 2003;Ikeda et al., 2006) (**Fig. 5**).

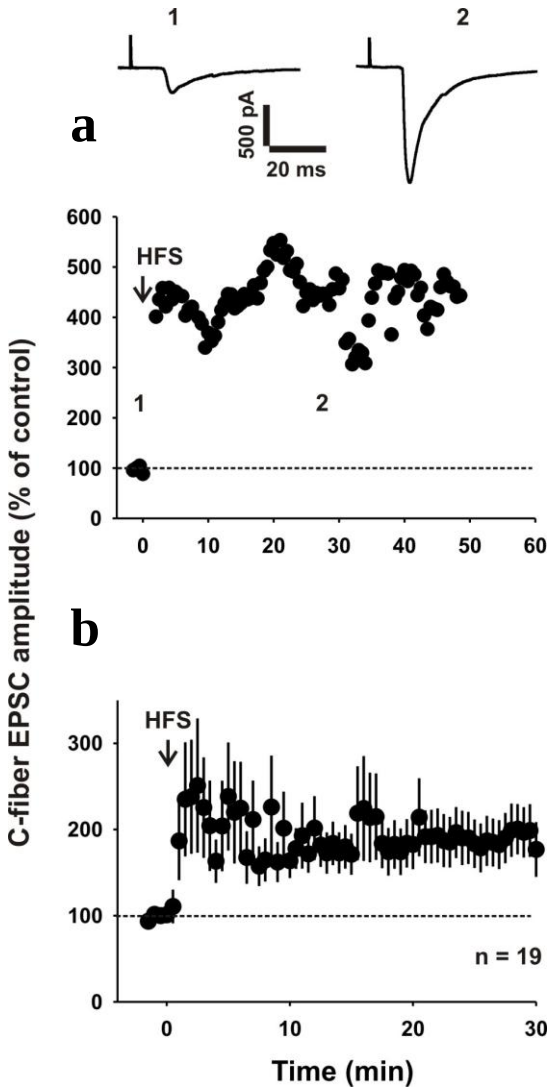


Figure 5: Induction of LTP following a conditioning stimulus. Here, LTP of C-fiber evoked EPSC amplitude (% of control) is plotted against time (min) and was induced following high-frequency stimulation (HFS) between primary afferents and spinal lamina I neurons. Evoked EPSC amplitudes increased up to 500% (**2**), compared to pre-observations (dotted line) (**1**) (**a**) and were significantly potentiated for at least 30 minutes (n = 19), ($p < 0.05$) (**b**) (Gruber-Schoffnegger, unpublished).

Further unpublished results of our group have shown that astrocytes become activated upon HFS (**Fig. 6**) (Gruber-Schoffnegger, unpublished).

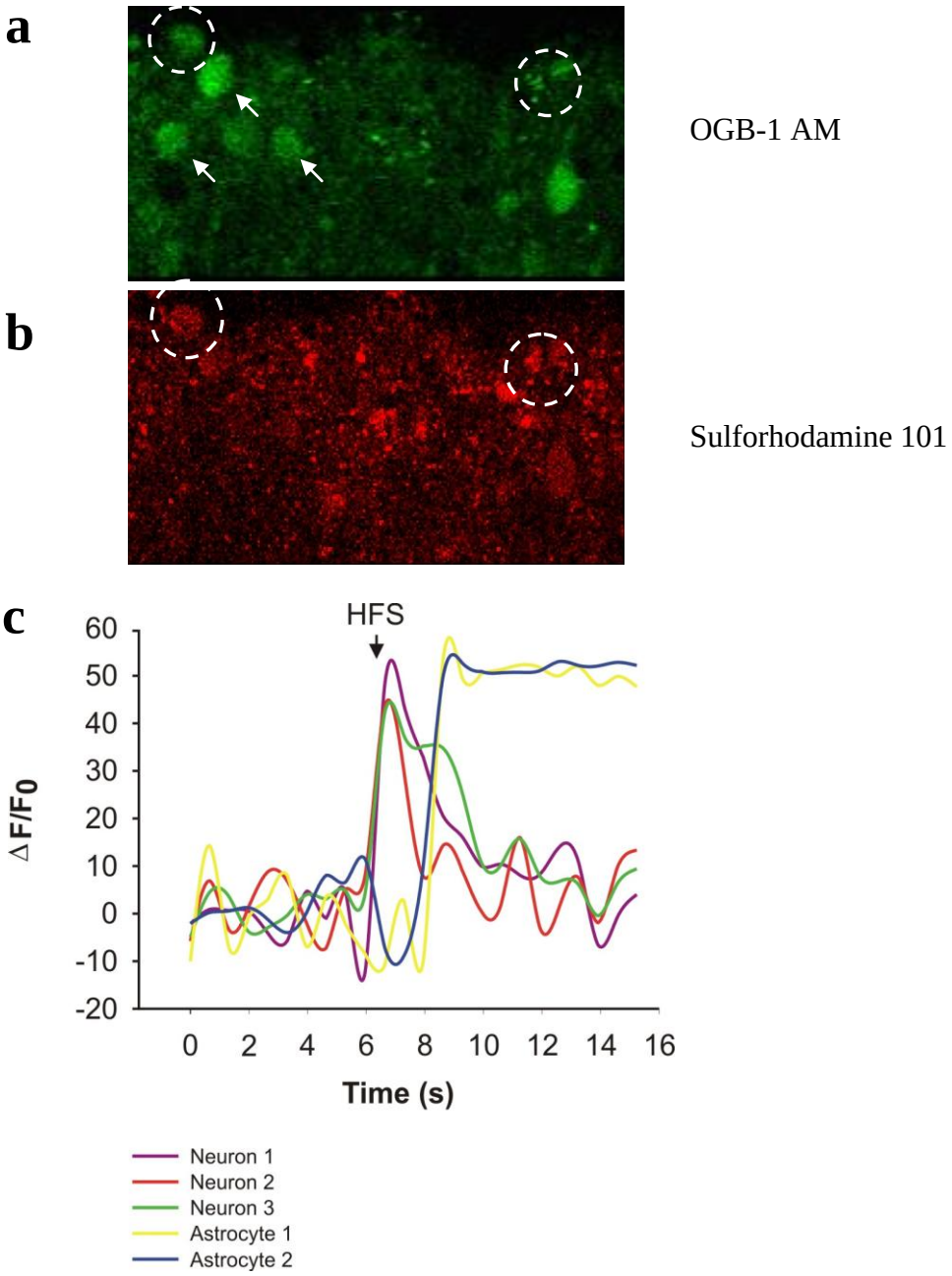


Figure 6: Astrocytes respond to HFS with an $[Ca^{2+}]_i$-rise. Ca^{2+}-imaging was performed in spinal cord slices labelled with the Ca^{2+}-sensitive dye oregon green BAPTA 1-AM (OGB-1 AM; green) (**a**) and the astrocyte specific marker sulfrhodamine 101 (red) (**b**). Marked neurons (arrows) and astrocytes (circles) are shown (**a**, **b**). Upon HFS, neurons reacted with a $[Ca^{2+}]_i$-rise and with a late, typical for astrocytes, delayed response, astrocytes showed $[Ca^{2+}]_i$-elevations (**c**) (Gruber-Schoffnegger, unpublished).

Further results of our group have shown that in the presence of minocycline, a specific microglial inhibitor (**Fig. 7a**) and fluorocitrate, an inhibitor of astrocytytic and microglial citrate cycle (**Fig. 7b**) LTP was no longer inducible anymore by HFS. The data even revealed a long-term depression (LTD) of recorded C-fiber evoked EPSC amplitudes in fluorocitrate treated slices (Gruber-Schoffnegger, unpublished).

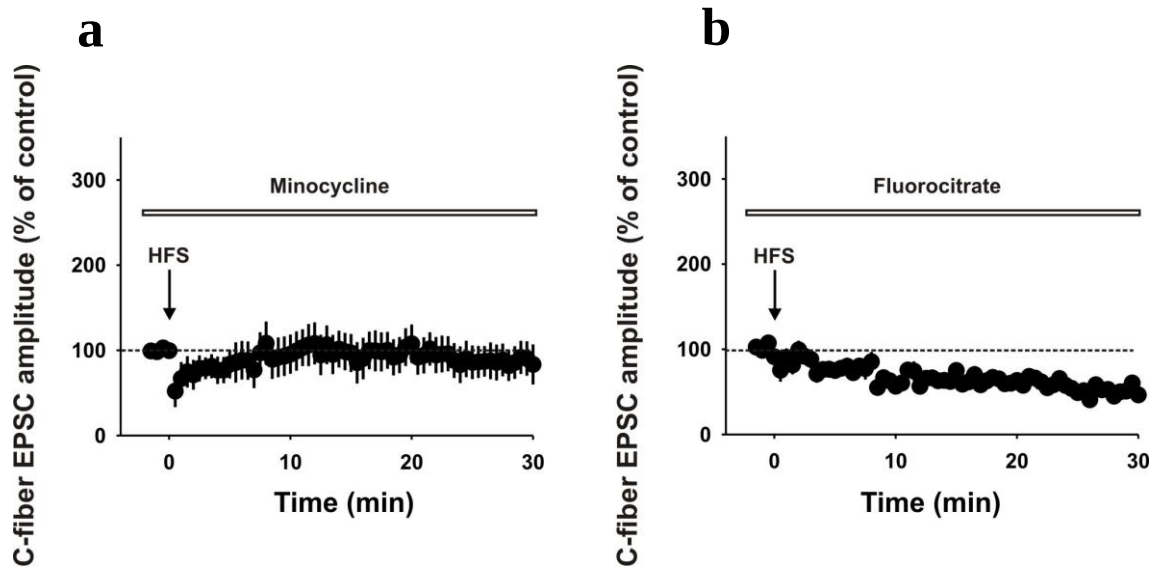


Figure 7: Glial inhibition prevents induction of LTP by HFS *in vitro*. Here, C-fiber evoked EPSC amplitude (% of control) in lamina I neurons is plotted against time (min). In presence of minocycline HFS had no effects on the strength of synaptic transmission. C-fiber evoked EPSC amplitude stayed at control value (dotted line) following HFS (n = 9). ($p > 0.05$) (**a**). In presence of fluorocitrate, HFS induced LTD of C-fiber evoked EPSC amplitude (n = 9), ($p < 0.05$) (**b**) (Gruber-Schoffnegger, unpublished).

It has also been shown that, glial blockade by fluorocitrate and minocycline prevented induction of LTP by HFS *in vivo* (**Fig. 8**) (Gruber-Schoffnegger, unpublished). Recent findings also confirm assumptions of glial cells' critical role in the induction of LTP (Henneberger et al., 2009).

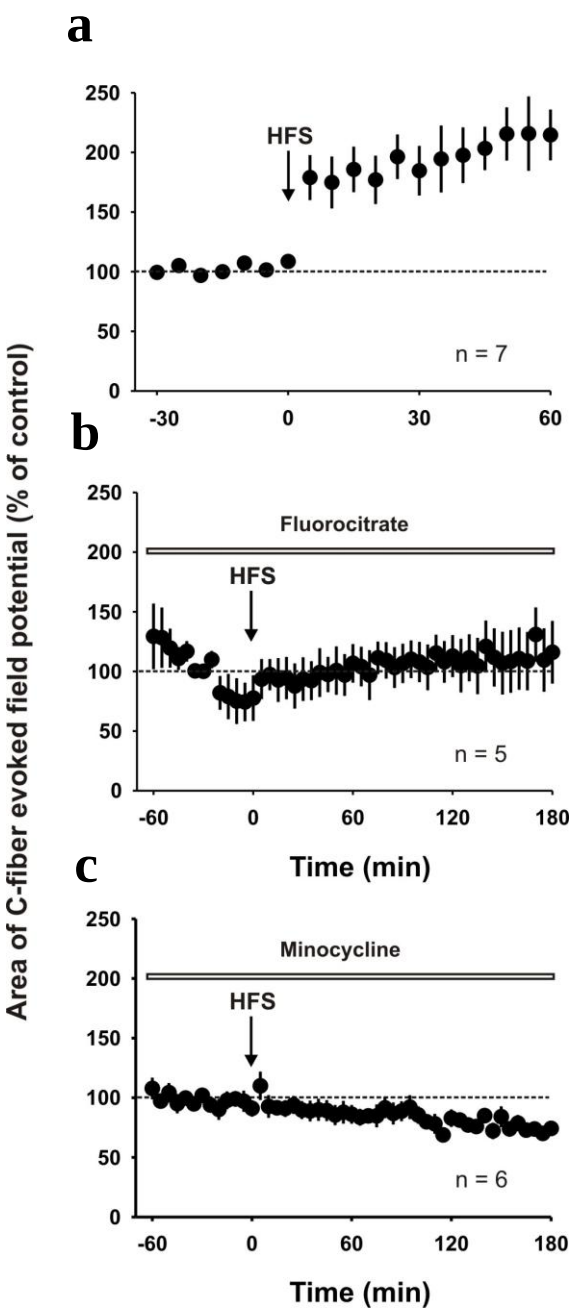


Figure 8: Glial inhibition prevents induction of LTP by HFS *in vivo*. Area of C-fiber evoked field potential (% of control) recorded in lamina I of the spinal cord is plotted against time (min). LTP of C-fiber evoked field potential was induced following HFS (n = 7), ($p < 0.05$) (**a**). Systemic administration of fluorocitrate (n = 5) (**b**) and minocycline (n = 6) (**c**) 60 minutes prior to HFS prevented induction of LTP significantly ($p < 0.05$). (Gruber-Schoffnegger, unpublished).

As reviewed above, IL-1ß has been shown to be released by activated glial cells (DeLeo et al., 2004; Ji and Strichartz, 2004; Watkins et al., 2001; Watkins and Maier, 2005). Furthermore IL-1ß has the potential to modulate the NMDA receptor (Zhang et al., 2008) which is of crucial importance for the induction of spinal LTP (Ikeda et al., 2006). Thus we tested the effects of IL-1ß on spinal lamina I neurons.

First, our group has analyzed how many lamina I neurons expressed the IL-1R and could therefore directly react to the application of the cytokine. Immunohistochemical data showed that 67 ± 5% of lamina I neurons express the IL-1R (**Fig. 9**) (Guber-Schoffnegger, unpublished).

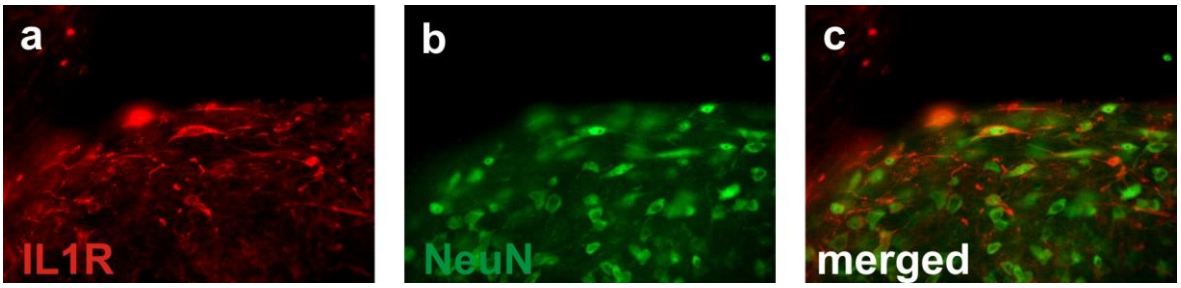


Figure 9: Expression of IL-1R in spinal dorsal Lamina I neurons in naïve rats. Immunohistochemical staining revealed expression of IL-1R in 67 ± 5% of stained neurons. IL-1R is shown in red (**a**), NeuN in green (**b**). Both pictures were merged to obtain overlapping expression (**c**) (Gruber-Schoffnegger, unpublished).

Since most of the lamina I neurons could potentially react to application of IL-1ß, electrophysiological recordings *in vitro* and *in vivo* were performed to study its effects on C-fiber evoked EPSC amplitudes (**Fig. 10**) (Guber-Schoffnegger, unpublished).

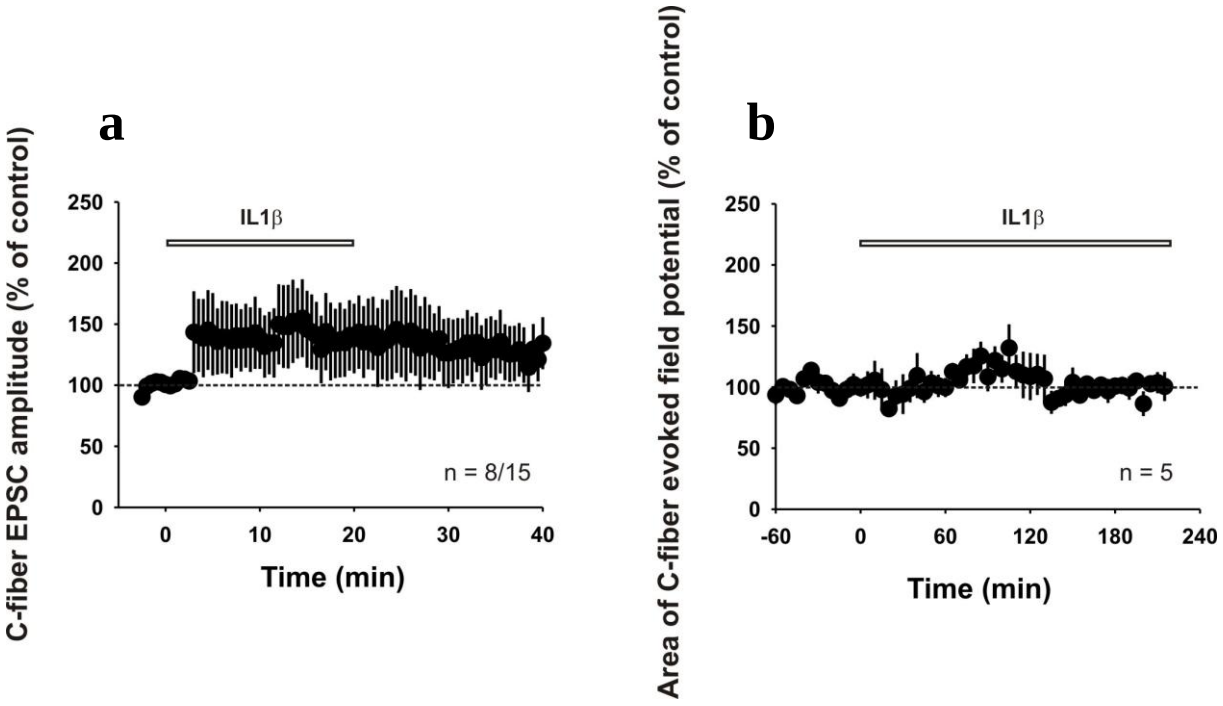


Figure 10: Effects of cytokine IL-1ß on the strength of synaptic transmission. IL-1ß increased the C-fiber evoked EPSC amplitude significantly compared to pre-observations (dotted line) *in vitro* (n = 8/15) ($p < 0.05$) (**a**). IL-1ß had no effects on C-fiber evoked field potentials *in vivo* (n = 5) ($p > 0.05$) (**b**). C-fibre evoked EPSC amplitude/field potential are plotted against time (min) (Gruber-Schoffnegger, unpublished).

Results of our group have shown that IL-1ß potentiated synaptic strength in spinal lamina I neurons *in vitro*. *In vivo* data revealed no effects of IL-1ß on magnitude of C-fiber evoked field potentials (Drdla, unpublished) (**Fig. 10**). We never tested if IL-1ß has differential effects on AMPAR- versus NMDAR-evoked EPSCs. Our group also tested whether IL-1ß thereby affected NMDAR and/or AMPAR mediated EPSCs between primary afferent fibers and spinal lamina I neurons. Results of our group revealed a considerable increase in C-fiber evoked NMDAR mediated EPSC amplitude after IL-1ß treatment (**Fig. 11b**) (Gruber-Schoffnegger, unpublished). These data suggest a crucial role of IL-1ß for the potentiation of C-fibre evoked NMDAR mediated EPSCs.

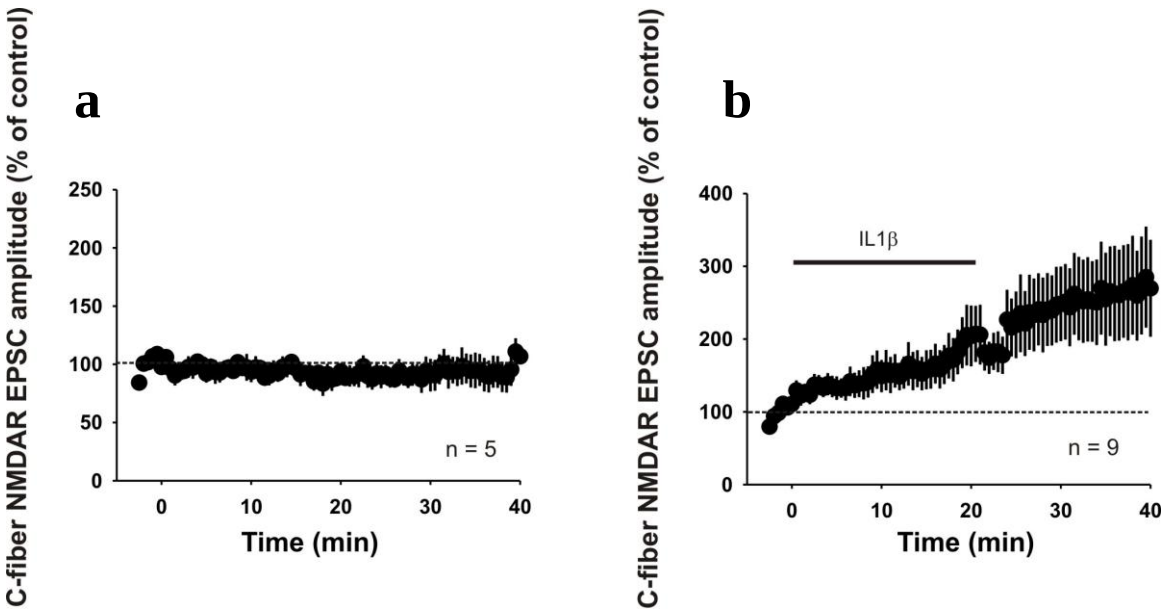


Figure 11: Effects of cytokine IL-1ß on NMDAR mediated currents. C-fiber evoked NMDAR mediated EPSC amplitude (% of control) is plotted against time (min) in control (n = 5) (**a**) and IL-1ß treated slices (n = 9) (**b).** IL-ß significantly potentiated C-fiber evoked NMDAR mediated EPSCs compared to pre-observations (dotted line) after application of IL-1ß ($p < 0.05$) (Gruber-Schoffnegger, unpublished).

The following data, including effects of IL-1ß on sEPSCs, AMPAR/NMDAR mediated currents, were collected within the framework of the diploma thesis to complete the research project.

2. Materials & Methods

2.1 Animals

All procedures were in accordance with European Communities Council directives (86/609/EEC) and were approved by the Austrian Federal Ministry for Education, Science and Culture.

2.2 Preparation of spinal cord slices

Spinal cord slices were obtained from young (18- to 23-day-old) Sprague-Dawley rats. Under deep ether anaesthesia rats were decapitated, the spinal cord was exposed by laminectomy and the lumbosacral segments with attached dorsal roots were excised. The dura mater and the ventral roots were removed. Transverse, 500 μm thick spinal cord slices with a long (8-10 mm) dorsal root were cut on a microslicer (DTK-1000, Dosaka EM, Kyoto, Japan). Slices were incubated in a solution that consisted of (mM): NaCl: 95, KCl 1.8, KH_2PO_4 1.2, $CaCl_2$ 0.5, $MgS0_4$ 7, $NaHCO_3$ 26, glucose 15, sucrose 50 and was oxygenated with 95% O_2, 5% CO_2, pH 7.4. A single slice was then transferred to the recording chamber where it was superfused with oxygenated recording solution. Recordings were performed at 31°C. Recording solution was identical to the incubation solution except for (mM): NaCl 127, $CaCl_2$ 2.4, $MgSO_4$ 1.3, and sucrose 0.

2.3 Recording and stimulation techniques

Neurons were visualized with an Olympus BX 50/51WI (Olympus Optical, Tokyo, Japan) microscope equipped with Dodt-infrared optics (Dodt and Zieglgänsberger, 1990) and a video camera system. Standard whole-cell patch-clamp recording techniques were used. Patch pipette were made from borosilicate glass and filled with intracellular solution consisting of (mM): potassium gluconate 120, KCl 20, $MgCl_2$ 2, Na_2ATP 2, Na-GTP 0.5, Hepes 20, EGTA 0.5, pH 7.28 with KOH and inserted into recording solution they had tip resistances of 2-5 MΩ.

Neurons in lamina I were selected for recording (**Fig. 12a**). The electrophysiological properties of the recorded neurons were investigated in voltage-clamp modes using an Axopatch 700B amplifier.

The dorsal root was stimulated with a suction electrode (**Fig. 12b**) with a constant current stimulator (World Precision Instruments, Stimulus Isolator, A360,

Sarasota, USA) and evoked excitatory postsynaptic currents (eEPSCs) were measured in the postsynaptic neuron.

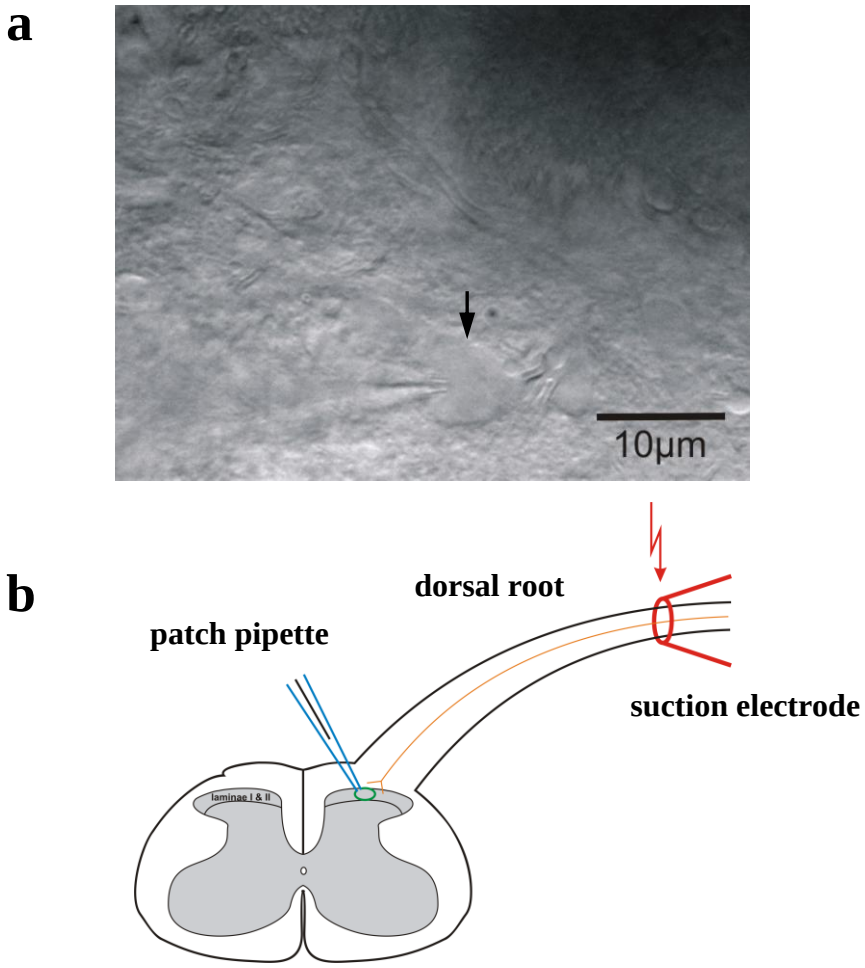


Figure 12: Transmission image of transverse slice with patch-clamped lamina I neuron (arrow) (**a**). Schematic of transverse slice with attached dorsal root stimulated via suction electrode (**b**).

2.4 Experimental protocol

The membrane potential was measured immediately after establishing the whole-cell configuration. Only cells with a resting membrane potential more negative than –50 mV were investigated further. Membrane resistance, membrane time constant, membrane capacitance, series resistance and leak current were measured by means of a hyperpolarizing voltage step from –70 mV to –80 mV.

Cells were usually excluded, if series resistance or holding current rose above 25 MΩ and above 50 pA respectively. The background noise threshold for sEPSCs recordings was up to 2.7 pA.

Afferent input was classified as C-fiber evoked, when calculated conduction velocity was below 2 m s^{-1}. Monosynaptic input was identified by the absence of failures in response to 10 consecutive stimuli given at 2 Hz and a jitter below 10% of response latencies. Conduction velocities of the responsible afferent fibers were calculated from the dorsal root length and the EPSC latency (**Fig. 13**). Synaptic delay of 1 ms was considered in calculation.

LTP was induced by high-frequency stimulation (HFS) (3 x 100 Hz, 10 s intervall) of the ipsilateral dorsal roots. To measure excitatory postsynaptic potentials (EPSCs) from neurons in lamina I, test pulses of 0.1 ms were given at a 30 s intervals. Effects of IL-1ß on evoked EPSC amplitudes were investigated. Therefore evoked EPSCs and evoked AMPAR mediated EPSCs were recorded at –70 mV, evoked NMDAR mediated EPSCs at –30 mV respectively. After three minutes baseline recording, IL-1ß was superfused for 20 minutes. Recordings then lasted for about 10 minutes for complete wash out of IL-1ß. In control experiments spinal cord slices were incubated with IL-1R antagonist for 10 minutes and again superfused with IL-1ß for 10 minutes. Spontaneous EPSCs (sEPSCs) were recorded to investigate the local global input at a holding potential of –75 mV. We decided to set time point of IL-1ß wash-in 10 ½ min after start of recording. At this time-point sEPSC rate/amplitude of recorded control cells became more stable. IL-1ß was washed out 21 min after start of recording.

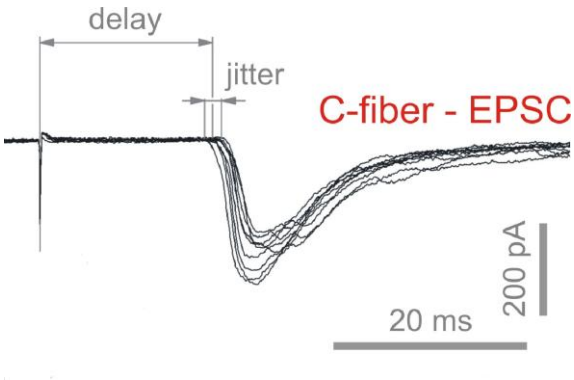


Figure 13: Example of C-fiber evoked monosynaptic responses in spinal lamina I neuron following electrical stimulation. Electrical stimulation induces C-fiber mediated EPSCs (pA) plotted against time (ms). Conduction velocity was calculated from the dorsal root length and sEPSC latency (delay). Different latencies of evoked EPSCs are called jitter.

2.5 Data analysis

The software pCLAMP 10.2 was used for data acquisitions and subsequent off-line analysis.
Additionally sEPSC frequencies and amplitudes were off-line analysed by use of MiniAnalysis software and visually checked afterwards.

2.6 Statistics

Data are presented as means ± SEM. Effects of IL-1ß on sEPSC frequency and amplitudes were analyzed using paired t-test/Student's t-test for intra/inter-group comparisons. Effects of IL-1ß on evoked AMPAR EPSCs were analyzed with a one-way ANOVA. Comparison of control and IL-1ß treated groups in AMPAR/NMDAR experiments were analyzed with a Student's t-test. A value of $P < 0.05$ was set as the level of statistical significance.

2.7 Application of drugs

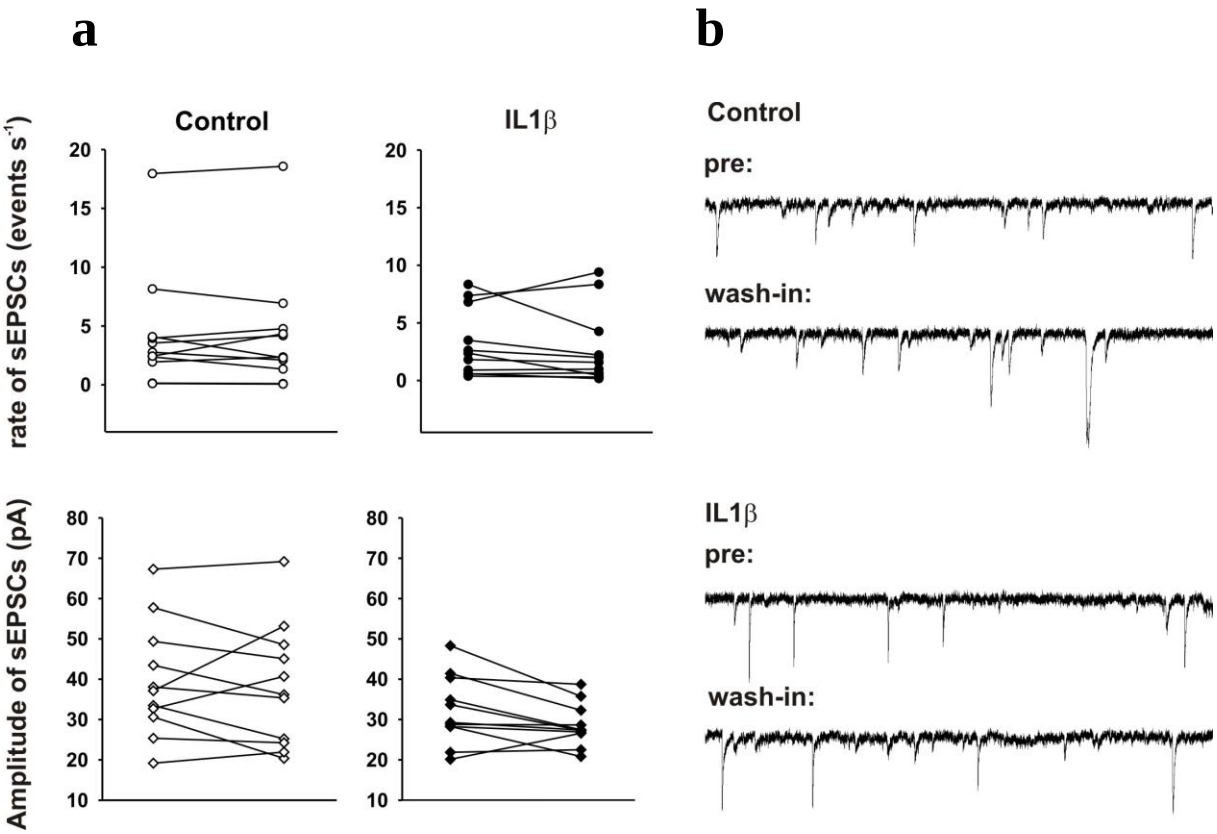
Spontaneous EPSCs were recorded in presence of bicuculline methiodide (bicuculline, 10 μM, Fluka) and strychnine (4 μM, Sigma), added to recording superfusion solution to block GABA A- and glycine- dependent receptors. Evoked AMPA-/ NMDA-EPSCs were also recorded in presence of bicuculline and strychnine.

C-fiber evoked AMPAR EPSCs were blocked with CNQX (10 μM, Ascent) and C-fiber evoked NMDAR EPSCs were blocked with D-AP5 (50 μM, Ascent). 10 μl IL-1ß (20 ng ml^{-1}, rat recombinant, R&D Systems) was added to 10 ml perfusion solution in spontaneous EPSC and C-fibre evoked EPSCs. In negative contol experiments IL-1R antagonist (40 μg ml^{-1}, human recombinant, AbD Serotec) was added.

3. Results

3.1 Effects of IL-1ß on sEPSC rate and amplitude

Recordings were obtained from 22 lamina I neurons. Eleven lamina I neurons were treated as controls, the other half was superfused with IL-1ß. There was a small but not significant decrease in rate (events s^{-1}) and amplitude (pA) of IL-1ß treated cells compared to controls ($p > 0.05$) (**Fig. 14**).



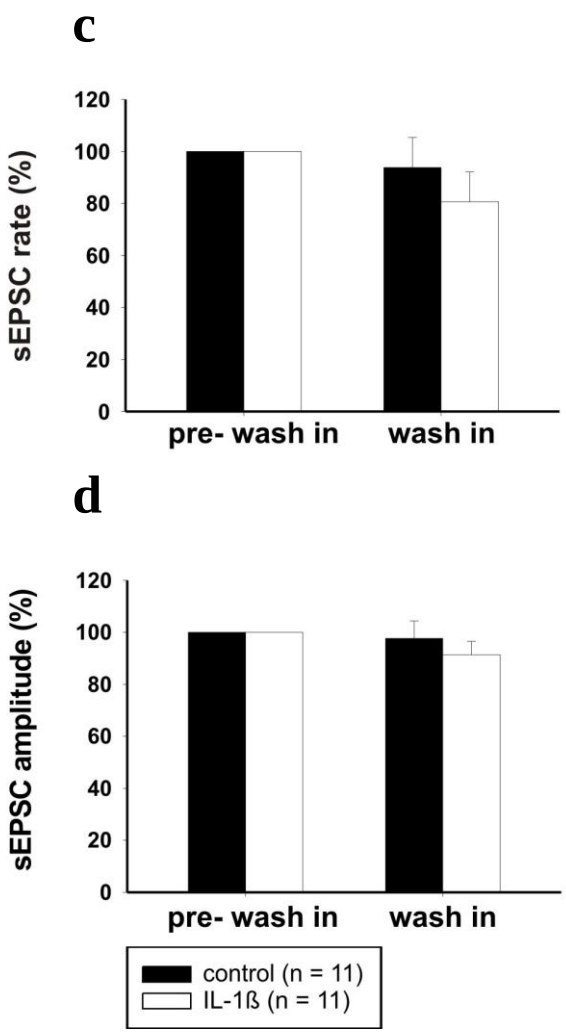


Figure 14: Recordings of sEPSCs in spinal lamina I neurons. Increase/decrease of sEPSC rates/amplitudes in control (n = 11) and IL-1ß treated cells (n = 11) is shown (**a**). Examples of sEPSC recorded traces before and after wash in of IL-1ß compared to control (**b**). Rate and amplitude (% of control) of sEPSCs in control (black charts) and IL-1ß treated slices were measured (white charts). sEPSC rate: Control: 93.8 ± 11.6% of pre-wash in phase; Added IL-1ß (20 ng ml^{-1}): 80.7 ± 11.5% of pre-wash in phase (**c**); sEPSC amplitude: Control: 97.6 ± 6.8% of pre-wash in phase; Added IL-1ß: 91.3 ± 5.2% of pre-wash in phase (**d**); Treatment of spinal cord slices with IL-1ß neither significantly affected sEPSC rate nor sEPSC amplitude ($P > 0.05$ compared to control); Means ± SEM of rates and amplitudes were calculated and pre-drug phase was normalized to wash in phase of IL-1ß.

3.2 Effects of IL- 1ß on AMPAR currents

Receptors important for primary afferent transmission are AMPA and NMDA receptors mainly. To check whether the major effect of IL-1ß is on AMPAR and /or the NMDAR component of the eEPSC, I measured AMPAR and NMDAR evoked EPSC amplitude separately. At first I obtained AMPAR mediated currents by application of bicuculline, strychnine and D-AP5 to selectively block NMDAR current. In eight cells, IL-1ß showed no wash in effect compared to control (n = 5) ($p > 0.05$) (**Fig. 15b**). Recordings of control cells which were not treated with IL-1ß did not show significant changes in synaptic transmission ($p > 0.05$). These findings suggest that IL-1ß has no direct effects on the strength of synaptic transmission mediated by AMPA receptors.

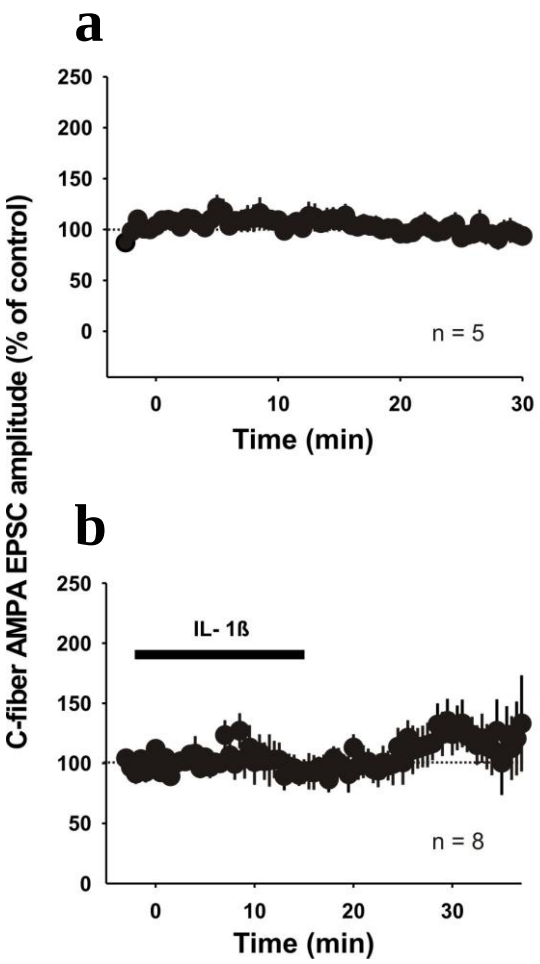


Figure 15: Effects of cytokine IL-1ß on evoked AMPAR mediated currents. C-fiber evoked AMPA EPSC amplitude (% of control) is plotted against time (min) in control (n = 5) (**A**) and

IL-1ß superfused cells (n = 8) (**B**). IL-1ß had no significant effects on C-fiber mediated AMPA EPSCs (P > 0.05 compared to control; Kruskal-Wallis One Way Analysis of Variance on Ranks); Means ± SEM of amplitudes were calculated and plotted against time (min); Amplitudes of individual C-fiber evoked EPSCs were normalized to predrug values.

3.3 Effects of IL-1ß on NMDAR currents

The second important receptors involved in synaptic transmission between primary afferent and post-synaptic terminals are NMDARs. To test whether IL-1ß has any effects on NMDA mediated response, C-fiber evoked NMDAR EPSC amplitudes were measured (**Fig. 11**) (Gruber-Schoffnegger, unpublished). Application of IL-1ß seemed to be sufficient for longer lasting potentiation of NMDAR mediated EPSC amplitudes. To prove involvement of IL-1ß in potentiation of evoked NMDAR responses, I recorded C-fiber evoked NMDAR EPSCs in presence of IL-1R antagonist (40 μg ml^{-1}). IL-1ß failed to potentiate C-fiber mediated NMDAR currents in all recorded cells (n = 6) pretreated with IL-1R antagonist compared to control (n = 6) (p > 0.05) (**Fig. 16b**). Recordings of control cells which were pre-treated with IL-1R antagonist did not show significant changes in synaptic transmission (p > 0.05) (**Fig. 16a**).

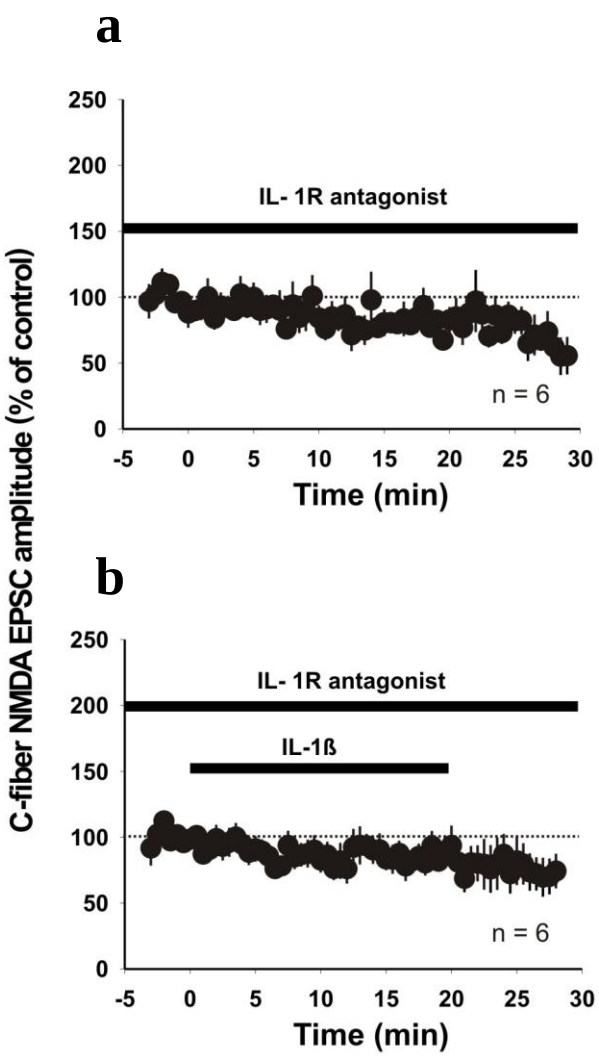


Figure 16: IL-1R antagonist inhibits IL-1ß mediated LTP of NMDAR mediated currents. C-fiber evoked NMDAR EPSC amplitude (% of control) in control (n = 6) (**a**) and IL-1ß superfused cells (n = 6) (**b**). Slices were pretreated with IL-1R antagonist. IL-ß had no significant effects on C-fiber mediated NMDAR EPSCs (P > 0.05 compared to IL-1R antagonist control); Means ± SEM of amplitudes were calculated and plotted against time (min); Amplitudes of individual C-fiber evoked EPSCs were normalized to predrug values.

4. Discussion

The present study supports hypothesis that IL-1ß, a proinflammatory cytokine importantly contributes to induction and maintenance of synaptic plasticity in the spinal dorsal horn. IL-1ß affected synaptic transmission, supporting our working hypothesis of glial dependent processes in synaptic plasticity.

At first, members of our group induced LTP following HFS in spinal lamina I neurons. LTP lasted until the end of the recording period for at least 30 minutes, in 19 lamina I neurons tested (**Fig. 5**) (Gruber-Schoffnegger, unpublished). Earlier studies investigated a postsynaptic, Ca^{2+}-dependent form of LTP induction in spinal lamina I neurons (Ikeda et al., 2003; Ikeda et al., 2006). Accumulating evidence indicates that glial cells contribute to the induction and maintenance of spinal LTP (Liu et al., 2007; Zhong et al., 2010). A Recent study showed, that sensory stimulation evokes astrocytic $[Ca^{2+}]_i$-rise within the barrel cortex of adult mice *in vivo* (Wang et al., 2006). Another study demonstrated an induced $[Ca^{2+}]_i$-rise in spinal dorsal horn astrocytes by the purinergic receptor agonist BzATP and reported Ca^{2+}-dependent forms of gliotransmission (Bardoni et al., 2010). Colleagues of our laboratory tested whether astrocytes showed a Ca^{2+}-rise in response to HFS, the first approach to prove involvement of glial cells in spinal LTP. After increase of $[Ca^{2+}]_i$ in neurons, also astrocytes reacted to stimulation of primary afferents with an increase in $[Ca^{2+}]_i$ (**Fig. 6**) (Gruber-Schoffnegger, unpublished). Convincing evidence exists that $[Ca^{2+}]_i$ variations can trigger release of Glutamate, ATP, D-Serine, cytokines like TNF-α and IL-1ß and prostaglandins (Santello and Volterra, 2009). In the next experiments my colleagues studied effects of glial inhibition, using minocycline or fluorocitrate, on the induction of LTP *in vitro* (**Fig. 7**) and *in vivo* (Fig.8) (Gruber-Schoffnegger, unpublished). Minocycline is a specific inhibitor of microglial activation and sufficiently prevented the induction of LTP *in vitro* and *in vivo* (**Fig.7a/8c**). Other results may also indicate the crucial role of microglia in induction of spinal LTP (Zhong et al., 2010). Question arises whether astrocytes

are also necessary for the induction of LTP in spinal dorsal horn neurons. Recent studies on astrocytes suggest a role downstream to microglia (Tanga et al., 2004) and late involvement of astrocytic activation in the maintenance of increased nociception (Zhuang et al., 2005; Zhuang et al., 2006). Interestingly, data of my colleagues indicated a long-term depression (LTD) of C-fibre evoked EPSC amplitudes following HFS in flurocitrate treated slices (**Fig. 7b**). The mechanism of decrease in EPSC amplitudes has not been identified yet. One study showed that field EPSPs were markedly depressed after several hours of superfusion with flurocitrate, due to lack of glutamate, a product of the citric acid cycle (Berg-Johnsen et al., 1993). It has also been shown that fluorocitrate inhibited microglial activation after peripheral nerve injury (Clark et al., 2007). Studies indicate that activated microglia and astrocytes release various proinflammatory factors such as IL-1ß (Watkins et al., 2001; Watkins et al., 2007). The proinflammatory cytokine IL-1ß is of special interest as recent findings have already shown that IL-1ß facilitates perception of noxious inputs, mediating thermal and mechanical hyperalgesia (Reeve et al., 2000; Sung et al., 2004). To address question how many lamina I neurons could possibly be affected by release of IL-1ß, immunohistochemical studies were performed. Data of my colleagues revealed that more than half of spinal lamina I neurons expressed the IL-1R (**Fig. 9**) (Gruber-Schoffnegger, unpublished). In another set of experiments we tested the reaction of spinal lamina I neurons to application of IL-1ß. I first measured rate and amplitude of spontaneous EPSCs (sEPSCs) after treatment with IL-1ß in lamina I neurons (**Fig. 14**). Results showed a small, but not significant decrease in sEPSC frequency and amplitude. In literature, a decrease in sEPSC frequency is stated as a decrease in release probability of presynaptic vesicles whereas a decrease in sEPSC amplitude is rather associated with postsynaptic modifications. Findings suggest that global input onto lamina I neurons is not affected by IL-1ß. In parallel a recent study demonstrated a slight increase of sEPSC frequency and amplitude after IL-1ß treatment in 50% of spinal lamina II neurons (Kawasaki et al., 2008b). Further, IL-1ß reduced

spontaneous inhibitory postsynaptic currents (sIPSCs) indicating that IL-1ß controls both excitatory and inhibitory synaptic transmission (Kawasaki et al., 2008b).

Recordings in lamina I neurons revealed that IL-1ß significantly potentiated synaptic strength of C-fiber evoked EPSCs *in vitro* (**Fig. 10a**) however failed to induce potentiation C-fiber evoked field potentials *in vivo* (**Fig. 10b**) (Gruber-Schoffnegger; Drdla, unpublished). To obtain further evidence in support of our results that IL-1ß could potentiate C-fibre evoked EPSCs in lamina I neurons, I tested effects of IL-1ß on AMPAR and NMDAR mediated currents. Application of IL-1ß did not significantly affect synaptic transmission of evoked AMPAR EPSCs compared to preobservations and control groups (**Fig. 15**). Withdrawal of IL-1ß showed also no effects on synaptic transmission. Interestingly, in contrast to AMPAR mediated currents, IL-1ß significantly potentiated C-fiber evoked NMDAR EPSCs (**Fig. 11**) (Gruber-Schoffnegger, unpublished). My colleagues still observed potentiation after wash out of IL-1ß, which suggests long lasting modifications of the NMDAR. These data are in parallel with other findings which reported potentiation of NMDA induced currents but not AMPA induced currents following application of IL-1ß (Kawasaki et al., 2008b). Results are also supported by the critical role of IL-1R signalling in modification of NMDAR subunit NR-1 (Zhang et al., 2008). In this study, a model of inflammatory hyperalgesia led to phosphorylation of the NR-1 subunit in superficial laminae of spinal dorsal horn. The authors applied an IL-1R antagonist which significantly inhibited phosphorylation of NR-1 and alleviated inflammatory hyperalgesia in rats (Zhang et al., 2008). Regarding these results I proved our hypothesis of cytokine induced LTP of C-fiber evoked NMDAR EPSCs by superfusion of IL-1R antagonist. In none of the tested neurons IL-1ß did potentiate NMDAR mediated currents (**Fig. 16**). IL-1R antagonist had also no effects on basal synaptic transmission in control. In conclusion, the present study demonstrates that glial cells act as a synaptic amplifier of NMDAR mediated currents in nociceptive pathways of spinal lamina I neurons.

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