Final report

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Organization and plasticity of neuronal microcircuits in the superficial spinal dorsal horn in chronic inflammatory and neuropathic pain

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The research carried out during the project period has been focused on two major aims: 1) Firstly, we intended to investigate the organization of neural microcircuits representing the first relay station of nociceptive sensory processing. 2) Secondly, in addition to studying the nociceptive mechanisms under normal conditions, we also intended to elucidate how information processing in nociceptive neural networks of the superficial spinal dorsal horn may change in chronic pain states.

To accomplish the major aims we have carried out several experiments. We studied the structural, functional and molecular properties and synaptic relations of neurons in the superficial spinal dorsal horn both in naive animals and in experimentally evoked chronic pain models. The studies resulted in a number of novel scientific discoveries, a short summary of which is given below.

1. Molecular organization of neural microcircuits in the superficial spinal dorsal horn

1.1. Numbers, densities, and co-localization of AMPA- and NMDA-type glutamate receptors (published)

Ionotropic glutamate receptors play important roles in spinal processing of nociceptive sensory signals and induction of central sensitization in chronic pain. We applied highly sensitive freeze-fracture replica labeling to laminae I–II of the spinal dorsal horn of rats and investigated the numbers, densities, and co-localization of AMPA- and NMDA-type glutamate receptors at individual postsynaptic membrane specializations with a high resolution. All glutamatergic postsynaptic membranes in laminae I–II expressed AMPA receptors, and most of them were also immunoreactive for the NR1 subunit of NMDA receptors. The numbers of gold particles for AMPA and NMDA receptors at individual postsynaptic membranes showed a linear correlation with the size of postsynaptic membrane specializations and varied in the range of 8 –214 and 5–232 with median values of 37 and 28, whereas their densities varied in the range of 325–3365/μm^2 and 102–2263/μm^2 with median

values of 1115/μm^2 and 777/μm^2, respectively. Virtually all glutamatergic postsynaptic membranes expressed GluR2, and most of them were also immunoreactive for GluR1. The numbers of gold particles for pan-AMPA, NR1, and GluR2 subunits showed a linear correlation with the size of postsynaptic surface areas. Concerning GluR1, there may be two populations of synapses with high and low GluR1 densities. In synapses larger than 0.1μm^2, GluR1 subunits were recovered in very low numbers.

1.2. Molecular organization of the endocannabinoid signaling apparatus

1.2.1. Neuronal and glial localization of the cannabinoid-1 receptor (published)

Although it is extensively documented that the cannabinoid-1 receptor (CB1-R) is strongly expressed in the superficial spinal dorsal horn, its cellular distribution is poorly defined, hampering our interpretation of the effect of cannabinoids on pain processing spinal neural circuits. Thus, we investigated the cellular distribution of CB1-Rs in laminae I and II of the rodent spinal dorsal horn with immunocytochemical methods. Axonal varicosities revealed a strong immunoreactivity for CB1-R, but no CB1-R expression was observed on dendrites and perikarya of neurons. Investigating the co-localization of CB1-R with markers of peptidergic and non-peptidergic primary afferents, and axon terminals of putative glutamatergic and GABAergic spinal neurons we found that nearly half of the peptidergic and more than 20% of the non-peptidergic nociceptive primary afferents, more than one third and approximately 20% of the axon terminals of putative glutamatergic and GABAergic spinal interneurons, respectively, were positively stained for CB1-R. In addition to axon terminals, almost half of the astrocytic and nearly 80% of microglial profiles were also immunolabeled for CB1-R. The findings suggest that the activity-dependent release of endogenous cannabinoids activates a complex signaling mechanism in pain processing spinal neural circuits into which both neurons and glial cells may contribute.

1.2.2. Differential distribution of DGLα and NAPE-PLD immunoreactivity (published)

In addition to cannabinoid-1 receptors, we investigated the distribution of diacylglycerol lipase-alpha (DGLα) and N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), enzymes synthesizing the endocannabinoid ligands, 2-arachidonoylglycerol (2-AG) and anandamide, respectively. Positive labeling was revealed only occasionally in axon terminals, but dendrites displayed strong immunoreactivity for both enzymes. However, the dendritic localization of DGLα and NAPE-PLD showed a remarkably different distribution. DGLα immunolabeling in dentrites was always revealed at membrane compartments in close vicinity to synapses. In contrast to this, dendritic NAPE-PLD labeling was never observed in

association with synaptic contacts. In addition to dendrites, a substantial proportion of astrocytic and microglial profiles were also immunolabeled for both DGLα and NAPE-PLD. Glial processes immunostained for DGLα were frequently found near to synapses in which the postsynaptic dendrite was immunoreactive for DGLα, whereas NAPE-PLD immunoreactivity on glial profiles at the vicinity of synapses was only occasionally observed. The results suggest that both neurons and glial cells can synthesize and release 2-AG and anandamide in the superficial spinal dorsal horn. 2-AG can primarily be released by postsynaptic dendrites and glial processes adjacent to synapses, whereas anandamide can predominantly be released from non-synaptic dendritic and glial compartments.

1.3. The role of RGS9-2 in opioid-mediated synaptic transmission (published)

The regulator of G protein signaling 9-2 (RGS9-2) is a constituent of G protein-coupled receptor (GPCR) macromolecular complexes with a major role in regulation of GPCR activity in the central nervous system. Previous in situ hybridization and Western blot studies revealed that RGS9-2 is expressed in the superficial dorsal horn of the spinal cord. In our study, we monitored tail withdrawal latencies to noxious thermal stimuli and performed in vitro whole-cell patch clamp electrophysiological recordings from neurons in lamina II of the spinal dorsal horn to examine the role of RGS9-2 in the dorsal horn of the spinal cord in nociceptive behaviors and opiate mediated modulation of synaptic transmission. Our findings obtained from RGS9 knockout mice indicate that the lack of RGS9-2 protein decreases sensitivity to thermal stimuli and to the analgesic actions of morphine in the tail immersion paradigm. This modulatory role of RGS9-2 on opiate-mediated responses was further supported by electrophysiological studies showing that hyperpolarization of neurons in lamina II of the spinal dorsal horn evoked by application of DAMGO ([d-Ala2, N-MePhe4, Gly-ol]-enkephalin, a mu opioid receptor agonist) was diminished in RGS9 knockout mice. The results indicate that RGS9-2 enhances the effect of morphine and may play a crucial role in opiate-mediated analgesic mechanisms at the level of the spinal cord.

1.4. Plasticity of HCN2 expression in inflammatory pain (published)

A great deal of experimental evidence has already been accumulated that hyperpolarization-activated and cyclic nucleotide-gated cation channels (HCN) expressed by peripheral nerve fibers contribute to the initiation of nerve activities leading to pain. Complementing these findings, we have demonstrated that HCN subunit 2 (HCN2) channel protein is also widely expressed by axon terminals of substance P (SP)-containing peptidergic nociceptive primary

afferents in laminae I-IIo of the spinal dorsal horn, and postulated that they may play a role in spinal pain processing. Now, we investigated how the expression of HCN2 ion channels in the spinal dorsal horn may change in inflammatory pain evoked by unilateral injection of complete Freund's adjuvant (CFA) into the hind paw of rats. We found that 3 days after CFA injection, when the nociceptive responsiveness of the inflamed hind paw had substantially increased, the numbers of HCN2-immunolabeled axon terminals were also significantly augmented in laminae I-IIo of the spinal dorsal horn ipsilateral to the site of CFA injection. The elevation of HCN2 immunoreactivity was paralleled by an increase in SP immunoreactivity. In addition, similarly to control animals, the co-localization between HCN2 and SP immunoreactivity was remarkably high, suggesting that central axon terminals of nociceptive primary afferents that increased their SP expression in response to CFA injection into the hind paw also increased their HCN2 expression. The results indicate that HCN2 ion channel mechanisms may play a role in SP-mediated spinal pain processing not only in naive animals but also in chronic inflammatory pain.

1.5. Effect of lamotrigine on synaptic transmission between nociceptive primary afferents and secondary sensory neurons (published)

It has been demonstrated that in the superficial spinal dorsal horn, Lamotrigine, which is known to block voltage-sensitive Na+ and N-type Ca2+ channels, depresses neural activities evoked by sustained activation of nociceptive primary afferent fibers. In this project, we studied how Lamotrigine exerts its inhibitory effect on spinal nociceptive information-processing mechanisms. We showed that Lamotrigine in an *in vitro* slice preparation effectively blocked synaptic transmission between primary afferents and secondary sensory neurons. Together with the robust increase in the failure rate and reduction in the amplitude of excitatory post-synaptic potentials (EPSPs) evoked by stimulation of nociceptive primary afferents, Lamotrigine caused a marked decrease in the number and amplitude of spontaneous EPSPs and a gradual shift of the resting membrane potential towards hyperpolarization. In addition, Lamotrigine treatment also changed the intrinsic firing pattern of superficial dorsal horn neurons. The results suggest that the effect of Lamotrigine on spinal nociceptive information-processing mechanisms is multiple: it depresses synaptic inputs from nociceptive primary afferents to secondary spinal sensory neurons and also weakens the intrinsic activities of nociceptive spinal neural circuits in the superficial spinal dorsal horn.

1.6. Differential expression patterns of KCC2 in spinal neurons (preliminary data)

GABA and glycine mediated hyperpolarizing inhibition is associated with a chloride-influx that depends on the inwardly directed chloride electrochemical gradient. In neurons, the extrusion of chloride from the cytosol primarily depends on the expression of an isoform of potassium-chloride co-transporters, KCC2. We investigated the cellular distribution of KCC2 in the superficial spinal dorsal horn of adult rats by using immunocytochemical methods both at the light and electron microscopic level. We demonstrated that perikarya and dendrites widely expressed KCC2, but axon terminals of nociceptive primary afferents and interneurons proved to be negative for KCC2 in the superficial spinal dorsal horn. Studying the somato-dendritic expression and distribution of KCC2 we found that it varied in a wide range from neuron to neuron. Neurons with high, medium and low level of KCC2 expression were equally recovered even in the group of neurons that expressed strong immunoreactivity for the beta3 subunit of $GABA_A$ receptor. Moreover, in single ultrathin sections we also observed dendritic segments that were negative for KCC2. Investigating KCC2 expression on NK1 immunoreactive neurons, that allowed us to study a large part of the somato-dendritic compartment of a selected subpopulation of neurons, we found that KCC2 presented a quite homogeneous distribution along the somato-dentritic membrane of individual neurons. The results suggest that $GABA_A$ receptor mediated synaptic mechanisms may evoke depolarization in axo-axonic synaptic contacts in the superficial spinal dorsal horn. In addition, the postsynaptic effect of $GABA_A$ receptor activation on the somato-dendritic membrane may also vary from neuron to neuron in laminae I-II of the spinal gray matter.

1.7. Expression and cellular localization of interleukin-1 receptor type 1 in Freund adjuvant-evoked inflammatory pain (preliminary data)

There is general agreement in the literature that interleukins play a role in spinal pain processing mechanisms. Due to the activation of nociceptive primary afferents interleukin-1β is released from glial cells which will act on neural interleukin-1 receptor type 1, resulting in the enhancement of neural activities. These mechanisms gain importance in chronic pain. Although the contribution of interleukins to the development of chronic pain is generally accepted, our present knowledge about the cellular expression of interleukin receptors in the spinal dorsal horn is insufficient and controversial. Thus, we investigated the expression and distribution of interleukin-1 receptor type 1 (IL1- R1) in the superficial spinal dorsal horn in naive adult male rats and in animals suffering in chronic inflammatory pain evoked by unilateral plantar injection of complete Freund adjuvant (CFA). Measuring the quantity of mRNAs with the TILDA method we found that CFA-evoked plantar inflammation evoked a

prominent elevation in the expression of IL1-R1 mRNA. In agreement with this, Western blot analysis showed a gradual increase in the quantity of IL1-R1 protein during the development of CFA-evoked inflammatory pain. Following this, we investigated the localization of IL1-R1 in the superficial spinal dorsal horn with immunocytochemical methods and found that in addition to neurons IL1-R1 is abundantly expressed by astrocytes and only sparsely by microglial cells in animals suffering form CFA-evoked inflammatory pain. Our data indicate that spinal astrocytes but not microglial cells can substantially contribute to interleukin-mediated signaling mechanisms which lead to the development of central sensitization of spinal pain processing neural circuits in CFA-evoked chronic inflammatory pain.

2. Cellular organization of neural microcircuits in the superficial spinal dorsal horn

2.1. Monosynaptic convergence of C- and Adelta-afferent fibers from different segmental dorsal roots on to single substantia gelatinosa neurons. (published)

Although it is known that each spinal cord segment receives thin-fiber inputs from several segmental dorsal roots, it remains unclear how these inputs converge at the cellular level. To study whether C- and Adelta-afferents from different roots can converge monosynaptically on to a single substantia gelatinosa (SG) neuron, we performed tight-seal recordings from SG neurons in the entire lumbar enlargement of the rat spinal cord with all six segmental (L1-L6) dorsal roots attached. It was found that one-third of SG neurons receive simultaneous monosynaptic inputs from two to four different segmental dorsal roots. For SG neurons from segment L4, the major monosynaptic input was from the L4-L6 roots, whereas for those located in segment L3 the input pattern was shifted to the L2-L5 roots. Based on these data, we proposed a new model of primary afferent organization where several C- or Adelta-fibers innervating one cutaneous region and ascending together in a common peripheral nerve may first diverge at the level of spinal nerves and enter the spinal cord through different segmental dorsal roots, but finally re-converge monosynaptically on to a single SG neuron.

2.2. Projection neurons with distinct axon-trajectories in the lateral spinal nucleus and lamina I (preliminary data)

In this experiment, we intended to trace the axons of projection neurons in the lateral part of lamina I and in the lateral spinal nucleus (LSN) by using patch-clamp recording and simultaneous intracellular biocytin labeling of single cells in the isolated spinal cord of young adult rats. Labeled neurons were reconstructed in 3D. We found, that despite a remarkable morphological variability, projection neurons in the lateral part of lamina I and in the LSN could be classified into four distinct categories on the basis of their axon-trajectories. The

majority of labeled neurons gave rise to an axon that crossed the midline in the anterior commissure and ascended in the contralateral anterolateral tract (ALT). A subset of these neurons gave rise to two long axon collaterals in the ipsilateral side, one of which ascended in the dorsal, while the other descended in the dorsolateral funiculus. A third set of neurons sent their main axon to the contralateral ALT through the posterior commissure. The main axon of neurons in the fourth group ran in the posterior commissure and after bypassing the central canal returned to the ipsilateral ALT through the anterior commissure. At the same time it gave short collaterals to the spinal gray matter on both sides of the spinal cord.

3. Exposure to Inhomogeneous Static Magnetic Field Ceases Mechanical Allodynia in Neuropathic Pain in Mice (published)

Magnetic therapy as a self-care intervention has led to the conduct of numerous human trials and animal experiments. Results concerning the analgesic efficacy of magnetic exposure, however, are inconsistent. By using a magnetic device generating an inhomogeneous static magnetic field (iSMF), here we studied how the whole-body exposure to iSMF may influence the mechanical withdrawal threshold (MWT) of the hind paw in different stages of neuropathic pain evoked by partial ligation of the sciatic nerve in mice. It was found that iSMF exposure did not prevent the decrease of MWT in the first postoperative week. A 2-week long iSMF treatment that was started just after the nerve ligation elevated MWT values to a modest extent. However, the effectiveness of a daily exposure to iSMF was much more prominent when it was applied between postoperative days 15 and 28. In this case, MWT was already noticeably increased after the first treatment and it practically reached the control values by the end of the 2-week long exposure period. The results suggest that exposure to iSMF cannot prevent the development of mechanical allodynia, but can inhibit processes that maintain the increased sensitivity to mechanical stimuli in neuropathic pain.

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