Pharmacological Mechanisms of Neck Muscle Nociception in a Translational Murine Model for Tension-Type Headache

Pharmacological Mechanisms of Neck Muscle Nociception in a Translational Murine Model for Tension-Type Headache

PhD Thesis by

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Table of Contents

The present thesis is based on three studies, which are referred to in the text by Roman numerals. The studies have been carried out in the period from 2007-2011 at Center for Sensory-Motor Interaction, Department of Health Science and Technology, Aalborg University and at the Biomedical Laboratory, Department of Pathology, Aalborg Hospital in collaboration with the Department of Neurosurgery, Ruhr University Bochum, Knappschaftskrankenhaus Bochum, Germany as well as Bayer Vital GmbH, Consumer Care Scientific Affairs, Leverkusen, Germany.

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Aalborg, Denmark, September 30th, 2011

Abbreviations

1400W	N-[[3-(Aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride;
A438079	3-[[5-(2,3-Dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride
ASA	Acetylsalicylic acid, Aspirin®
ATP	Adenosine 5´-triphosphate
α,β-meATP	α,β-methylene adenosine 5´-triphosphate
a.u.	Arbitrary units
c.f.	Confer
CNS	Central nervous system
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DMKG	German Migraine and Headache Society
ECG	Electrocardiogram
e.g.	Exempli gratia, for example
EFNS	European Federation of Neurological Sciences
EMG	Electromyogram
GABA	γ-aminobutyric acid
IASP	International Association for the Study of Pain
IHS	International headache Society
i.a.	Inter alia, amongst others
i.e.	Id est, that is
i.p.	Intraperitoneal
i.m.	Intramuscular
i.v.	Intravenously
I_0	Threshold intensity to elicit the jaw-opening reflex
I_{JOR}	Stimulus intensity to elicit reliably the jaw-opening reflex
JOR	Jaw-opening reflex
L-NMMA	NG-Monomethyl-L-arginine acetate
LTP	Long-term potentiation
NGF	Human β-nerve growth factor
NO	Nitric oxide

NOS		Nitric oxide synthase
	eNOS	Endothelial NOS
	iNOS	Inducible NOS
	nNOS	Neuronal NOS
NPLA		N^{ω}-propyl-L-arginine
n.s.		Not significant
NSAID		non-steroidal antiinflammatory drugs
P2X		Purinergic receptor, ionotropic
	P2X3	Purinergic receptor 3, homomeric
	P2X2/3	Purinergic receptor 2 and 3, heteromeric
	P2X4	Purinergic receptor 4
	P2X7	Purinergic receptor 7
PNS		Peripheral nervous system
RM ANOVA		Repeated measures analysis of variance
S.E.M.		Standard error mean
siRNA		Short interfering ribonucleic acid
TTH		Tension-type headache
	IETTH	Infrequent episodic TTH
	FETTH	Frequent episodic TTH
	CTTH	Chronic TTH
	PCTTH	Probable chronic TTH
VR		Vanilloid receptor
WDR		Wide dynamic range neurons

1. Introduction

The focus of this thesis lies on the basic research of pharmacological modulation of neck muscle nociceptive processing in translational murine model of tension-type headache (TTH), particularly in regard to potential pathophysiological TTH mechanisms and a putative impact on current and future treatment of TTH attacks.

1.1 Pain

The Statens Institut for Folkesundhed states that 19% of the Danish population suffer from chronic pain and are afflicted by chronic pain. Typically, chronic pain significantly lowers quality of life and causes great socioeconomic losses. The International Association for the Study of Pain (IASP, www.iasp-pain.org) defines pain as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."

Pain is more than a complex sensation, a subjective perception. There are many components accompanying or contributing to pain perception (Fig. 1). The so-called sensory-discriminative component is crucial for the identification of pain intensity, location and duration. The degree of unpleasantness is processed by the affective-emotional component. Depending on pain intensity and duration, vegetative (autonomous) body reactions can occur, such as cardiovascular (increase of heart rate and blood pressure) and glandular (sweating) responses. The motor component incorporates muscular responses such as increased tension or withdrawal reflexes. The evaluation of a painful sensation via comparison with former experiences is coordinated by the cognitive component that can lead to distinct pain expression patterns (psychomotor component) such as mimic and vocalization.

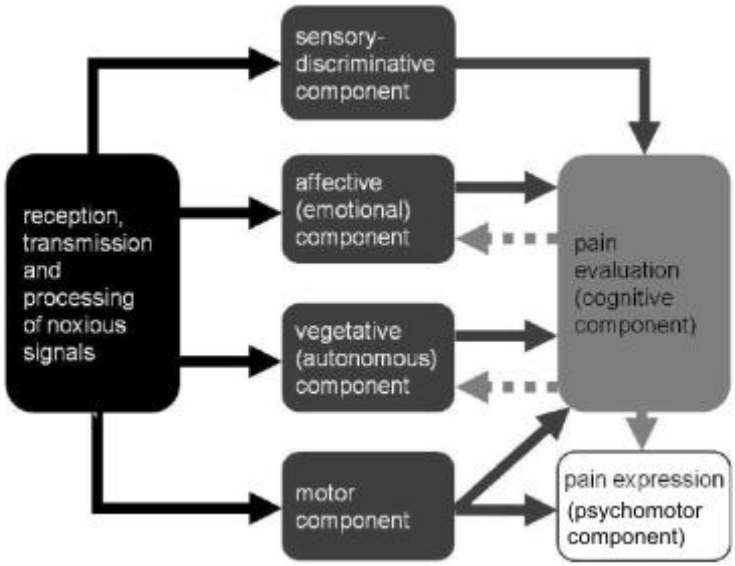

Figure 1: Schematic drawing of pain components activated by noxious signals. Sensory, affective, vegetative and motor components result in pain evaluation and expression. Vice versa, the cognitive component has also influence on the affective and vegetative component (dashed arrows).[1]

There are different pain states that can occur, i.e. most commonly acute and chronic pain. Typically, their definition bases upon the duration and frequency of perceived pain. The term acute pain is used in order to refer to recent onset, known origin and limited duration. Treatment of the cause typically relieves the pain. In contrast the term chronic pain describes persisting pain beyond normal healing time and longer duration, sometimes without obvious cause accompanied by changes in the central nervous system, often including severe social and psychological disturbances. The duration of chronic pain may vary on the type of pain. Within headache, chronification is typically referred to pain occurring on 15 or more days per month for more than three months (cf. 1.3.1).[2]

Typically, a stimulus perceived as painful either causes or can cause tissue damage with prolonged exposure time. Hence, pain processing serves as an essential warning and protective body function. In contrast to acute pain, with this body function properly working in its natural frame, chronic pain persists for at least six months mostly uncoupled of the initial painful incident. The pathophysiological mechanisms, i.e. physiological changes in pain processing outside the normal range, underlying the development of chronic pain are of cardinal clinical relevance for its treatment.

1.2 Nociception

Nociception describes the neuronal processing of afferent input from nociceptors usually associated with pain. Representing a physiological process, this includes different relay stations starting from the nociceptor, spinal cord, brainstem and the brain. Signal processing can be verified at each relay. The nociceptive transmission can be modulated by both endo- and exogenous factors at all those relay stations. Nociception can be described as an objective, quantifiable process independent from subjective experience. In contrast, it is impossible to objectively measure pain due to its nature as a subjective experience resulting from cognitive processing. The only way to quantify pain is relying on the subjects' description of his/her pain perception and to determine the quality of pain. However, activation of the nociceptive system does not always lead to pain. On the other hand, pain can be perceived in some cases without activation of the nociceptive system.

The following paragraphs of chapter 1.2 of this introduction focus on cutaneous, spinal and muscular nociception.

1.2.1 Peripheral nociceptive system

Nociceptive processing starts with excitation of nociceptors in the periphery. These free nerve endings (peripheral terminals) can respond to different stimulus modes, e.g. chemical, thermal or electrical stimuli, temperature or touch. This information is then forwarded along its axons to the spinal cord[3] and in the

trigeminal system via the brainstem.[4] The soma of these primary afferent sensory nerve fibers is localized in dorsal root ganglia. The primary sensory afferents terminate in the dorsal horn of the spinal cord via dorsal root ganglia or – in the trigeminal system – via trigeminal ganglia. Sensory nerve fibers can be categorized in three different categories according to anatomical and functional properties. These properties are mainly degree of myelination, conduction velocity and modulus coding. Aβ fibers mainly code for non-nociceptive (innocuous), tactile stimuli and have the highest degree of myelination and hence the fastest conduction velocity of sensory fibers (30 to 70 m/s). Aδ and C fibers are sensory fibers typically coding and transducing nociceptive information.[5] Aδ fibers have the lowest degree of myelination and are hence slower conducting (2 to 33 m/s). They typically mediate a sharp, well localized "first pain" sensation.[6] C fibers are the slowest conduction sensory fibers due to the lack of myelin isolation (0.4 to 1.8 m/s). Their activation is typically associated with a dull, burning "second pain" perception. Aδ and C fibers are sensory fibers can also code for and transduce innocuous information. Mediating nociceptive input from the periphery can be modulated via peptides and neurotransmitters resulting in increasing stages of nociceptive processing termed peripheral sensitization (cf. 1.2.3).[5]

1.2.2 Central nociceptive system

The first relay in the nociceptive transmission from the periphery to the brain is the spinal cord.[3] Its dorsal horn receives input from primary sensory afferents transmitting information from sensory receptors in skin, viscera, joints and muscle of trunk and limbs to the central nervous system (Fig. 2, left).

The primary sensory afferent neuron terminates in distinct laminae of the spinal dorsal horn to a secondary sensory afferent neuron and forms the first nociceptive synapse. The secondary sensory neuron is also referred to as projection neuron due to the direct transmission of the sensory input to higher brain centers. Aδ fibers terminate in lamina I (marginal layer) and V, C fibers in lamina II (substantia gelatinosa). Many lamina I neurons are nociceptive-specific and hence respond exclusively to noxious stimulation projecting to higher brain centers. The marginal layer also contains so-called wide dynamic range (WDR) neurons which receive both innocuous mechanical (Aβ fibers) and nociceptive input. The substantia gelatinosa contains almost exclusively both inhibitory and excitatory interneurons that can either be nociceptive-specific or respond additionally to innocuous stimuli. WDR neurons are the main type being present in lamina V that project to the brain stem and to the thalamus. Somatic and visceral nociceptive input converges in lamina V. These neurons can receive monosynaptic input from Aδ and Aβ fibers. Furthermore, they can receive C fiber input either directly (dendritic connection) or indirectly via C fiber synapsed interneurons. The main biochemical correlates of synaptic transmission from primary nociceptive to the secondary sensory neuron via interneurons are mediators such as excitatory glutamate and inhibitory GABA (γ-Aminobutyric acid).

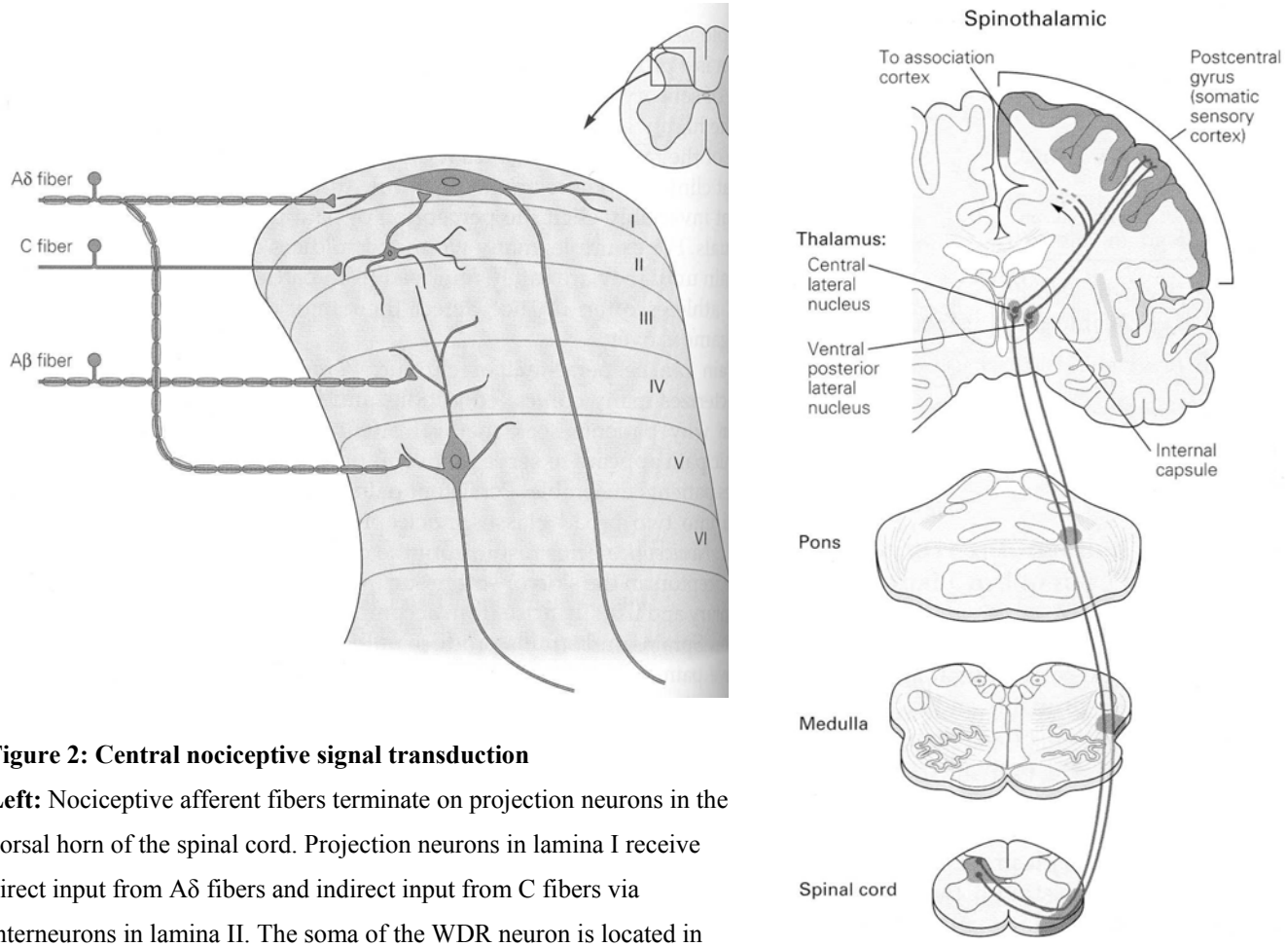

Figure 2: Central nociceptive signal transduction

Left: Nociceptive afferent fibers terminate on projection neurons in the dorsal horn of the spinal cord. Projection neurons in lamina I receive direct input from Aδ fibers and indirect input from C fibers via interneurons in lamina II. The soma of the WDR neuron is located in lamina V and has its dendrites in the laminae III to V. This WDR neuron receives convergent input from Aδ fibers in lamina V, from Aβ fibers in lamina IV and C fibers in lamina III.

Right: Spinothalamic ascending pathway that transmits nociceptive information from the spinal cord to higher centers. The spinothalamic tract is the most prominent ascending nociceptive pathway in the spinal cord. Nociceptive signaling from primary afferent neurons are relayed to projection neurons in the spin dorsal horn ascending directly to the thalamus and from there to higher cortical structures such as the somatosensory cortex.[7]

Excitatory (glutamatergic) interneurons increase response of these neurons whereas inhibitory (GABAergic) decrease this response.[8] This influences the output of the dorsal horn.[3]

With facial skin, lips, tooth pulp, oral and nasal cavities, mucosa of sinuses, cornea and meninges, peripheral nociceptive input is propagated within the trigeminal system.[4] Trigeminal afferents project via trigeminal ganglia to the brainstem and thalamus. The trigeminal ganglia synapse the mesencephalic nucleus, the principal sensory nucleus, the interstitial nucleus of the spinotrigeminal tract and the spinal trigeminal nucleus. Especially the nucleus interpolaris and caudalis within the spinal trigeminal nucleus have been associated with nociceptive processing in the trigeminal system. Besides further ascension of signal propagation, nociceptive input in spinal cord and brainstem can be integrated into certain reflex arcs.[4]

Typically, the purpose of such a spinal or brainstem reflex lies in the withdrawal of a limb (withdrawal reflex) or closing of an eye lid (blink reflex) protecting the corresponding body part. Due to the immense complexity and numerous cross-connections with the central nervous system, these reflex arcs can in turn be modulated. This is described more detailed in 1.4.1.

There are five ascending pathways for nociceptive transmission from the spinal dorsal horn to the brain. The spinothalamic, spinoreticular and spinomesencephalic tracts are considered to be the three major ascending nociceptive pathways in contrast to the cervicothalamic and spinohypothalamic tract (Fig. 2, right). Thus, the following description focuses on the major three nociceptive, ascending tracts.

The most prominent nociceptive pathway in the spinal cord is the spinothalamic tract. It comprises of axons of nociceptive specific and WDR neurons from laminae I and V-VII that project to the contralateral side of the spinal cord that ascend to the anterolateral white matter terminating in the thalamus followed by thalamocortical pathways. Processing of sensory and affective pain components occurs within two distinct thalamocortical pathways. Sensory information is transmitted from the lateral thalamus to the posterior insula, primary and secondary somatosensory cortex whereas affective pain components are processed via the axis medial thalamus to anterior cingulate cortex and anterior insula.[9] The prefrontal and the parietal cortices are involved in cognitive and attentional processes.[10,11] Integration of pain sensation, affect, fear and memory is thought to occur in a cortical-limbic pathway via projections via posterior parietal cortex from S1 and S2 and insula to amygdala and hippocampus. Autonomic fear and defensive responses are thought to be the result of activation of other ascending spinal pathways that directly access cortical structures such as amygdala, hippocampus, hypothalamus and periaqueductal grey.[12] Many neurons of the spinoreticular tract terminate in the reticular formation of medulla and pons projecting to the thalamus. The spinomesencephalic tract comprises of lamina I and V neurons projecting to mesencephalic reticular formation and the periaqueductal grey projecting in turn to the parabrachial nuclei via the spinoparabrachial tract. The spinomesencephalic tract is thought to contribute to the affective component of pain due to projections from the parabrachial nuclei to the amygdala.

1.2.3 Muscle pain – basics for muscle nociception and sensitization

Peripheral mechanisms of muscle nociception describe nociceptive signaling starting from innervated muscles to the spinal cord. Most nociceptors have a high activation threshold and do not respond to stimuli in the normal, everyday life situation. Nociceptors in the skeletal muscles are hence not excited by physiological movements or stretch.[13] Nonetheless, muscle nociceptors can be activated by chemical, thermal, mechanical or electrical stimuli. Well known endogenous algesic agents in the muscle are i.a. serotonin,

bradykinin (BKN), potassium ions, adenosine triphosphate (ATP) or hydrogen ions (H+). However, clinically relevant receptor molecules are purinergic P2X receptors (e.g. P2X3)[14,15] and the vanilloid receptor VR-1.16 Purinergic receptors bind ATP. This can lead to excitation of the primary nociceptive afferent. ATP is released both passively during cell trauma[17,18] and actively from skeletal muscles and neurons.[19-21] Furthermore, interstitial ATP concentrations in the skeletal muscle increase with muscle contraction and compression.[22-25] Intramuscular injections of ATP can cause muscle nociception and pain.[26,27] In contrast, the VR-1 receptor responds to heat and increases in concentrations of H+ ions. The sensitivity of VR-1 to protons is cardinal in conditions with lowered ph-values such as inflammation, ischemia or exhaustive muscle work. Sensitization of muscle nociceptive processing can occur on both the peripheral nociceptor (Fig. 3) and central spinal cord level.

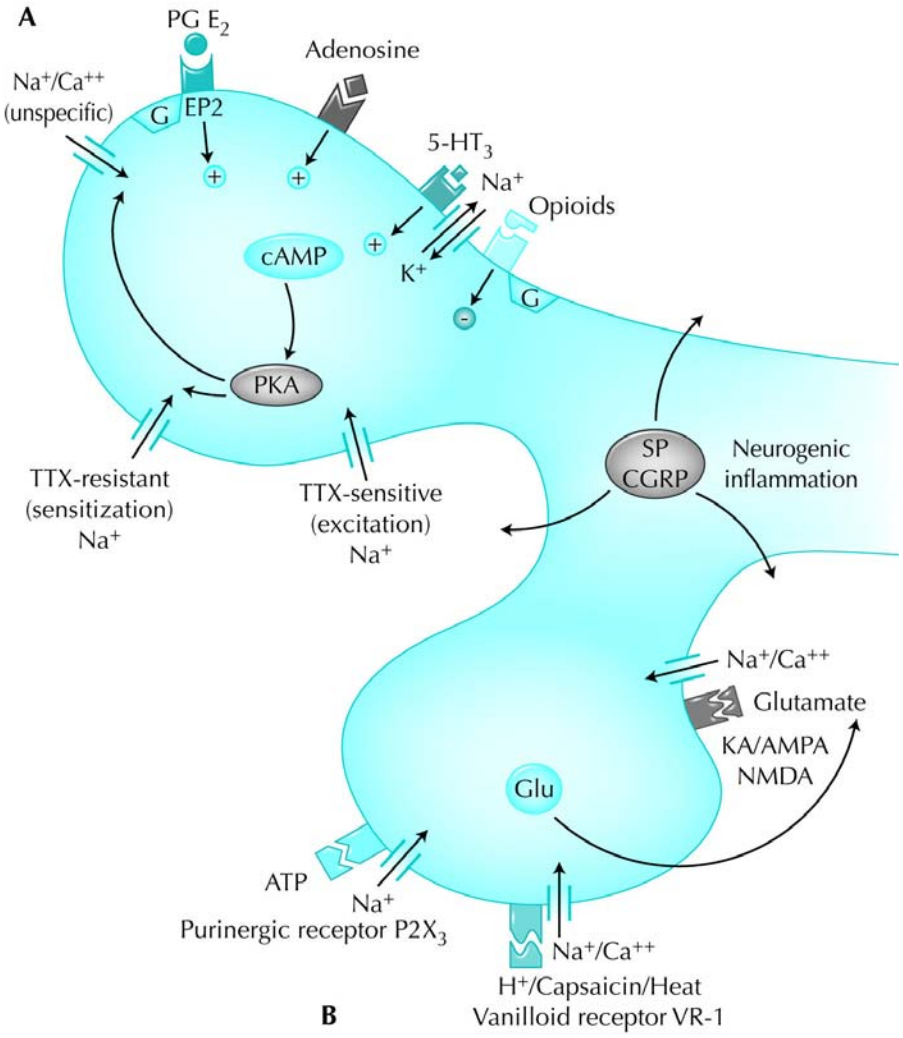

Figure 3: Schematic drawing of a nociceptive nerve ending showing membrane receptor molecules and intracellular events that increase the sensitivity of the ending.

Of clinical importance are the following processes. A, Sensitization is not an unspecific process because of the binding of the sensitizing substances (prostaglandin E2 [PG E2], serotonin [5-HT], adenosine) to specific membrane receptors, which induce intracellular cascades of events that increase the sensitivity of the Na+ channels by phosphorylation through activated protein kinase A (PKA). The larger ion currents that flow through the channel proteins of a sensitized ending render the ending more sensitive. B, Nociceptive endings are equipped with purinergic receptor molecules that bind adenosine triphosphate (ATP, eg, the P2X3 receptor) and vanilloid receptors (VR-1), which are sensitive to protons (H+) and heat. AMPA—alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; cAMP—cyclic adenosine monophosphate; CGRP—calcitonin gene-related peptide; G—G protein; Glu—glutamate; KA— kainic acid; SP—substance P; TTX—tetrodotoxin.[13]

In the periphery, mechanical sensitivity of nociceptors in pathologically altered tissue can be increased due to the release of various substances such as BKN. The sensitization is characterized by a decrease in the mechanical threshold, i.e. it takes lower intensities of mechanical stimuli to activate the nociceptor which explains local tenderness and increased sensitivity to movement of a pathologically altered muscle. Longer-lasting pathological alterations of muscle tissue are very likely not only associated with sensitization of muscle nociceptors but also with increased nociceptor innervation density of muscular tissue.[13]

A crucial role for the transition of acute to chronic muscle pain is central sensitization characterized by higher synaptic efficacy in spinal cord neurons resulting in their hyperexcitability. In other words, the response to nociceptive input is larger than before the sensitizing event. Neuroplastic changes describing central neuron hyperexcitability are typically independent from the initial peripheral input by which chronification is established. Changes in the spinal dorsal cord morphology consist of sprouting of afferent fibers within spinal terminals and broadening as well as creating new synaptic contacts.[13] Neurotransmitters involved in central sensitization are endogenous substance such as substance P, glutamate and nitric oxide (NO).

1.3 TTH and a translational mouse model on neck muscle nociception

1.3.1 TTH – classification & pathophysiology

Tension-type headache (TTH) is the most frequent primary headache with 1-year world-wide prevalence of 40 %.[28] Denmark has with 89% even twice the frequency.[29] According to the International Headache society (IHS, http://www.i-h-s.org), TTH attacks are characterized by episodes of headache lasting minutes to days. The pain is typically bilateral, pressing or tightening in quality and of mild to moderate intensity, and it does not worsen with routine physical activity. Nausea, photophobia or phonophobia may or may not be present depending on its category.

Headache independent from any other medical condition is referred to as a primary headache whereas headaches as a result of other, underlying medical conditions is referred to as a secondary headache. There are three major types of primary headaches with corresponding subtypes (IHS). This headache category consists of tensions-type headache (TTH), migraine and cluster headache. TTH subcategories are divided into infrequent episodic (IETTH), frequent episodic (FETTH), chronic (CTTH) and probable chronic (PCTTH) subtypes:

- Infrequent episodic TTH (IETTH)
  At least 10 episodes occurring on <1 day per month on average (<12 days per year)

- Frequent episodic TTH (FETTH)
  At least 10 episodes occurring on ≥1 but <15 days per month for at least 3 months (>12 and <180 days per year)
- Chronic TTH (CTTH):
  Headache occurring on ≥15 days per month on average for >3 months (≥180 days per year).
- Probable CTTH (PCTTH):
  Patients meeting one of these sets of criteria may also meet the criteria for one of the sub-forms of probable migraine. Though PCTTH is not attributed to another disorder, there is or has been medication overuse within the last two months with regular overuse for more than 3 months of one or more drugs that can be taken for acute and/or symptomatic treatment of headache.

Psychophysical studies in patients reveal involvement of neck muscle nociception in TTH pathophysiology.[30-34] The most significant abnormal finding in TTH patients is increased tenderness of pericranial muscles. Pericranial tenderness is positively correlated with both intensity and frequency of TTH.[35,36] In contrast to the vast clinical and socio-economic importance of TTH with estimated 3 billion DKK per year, its pathophysiology is still unclear. Peripheral pain mechanisms are most likely to play a role in ETTH whereas changes in central pain mechanisms are suspected to play a cardinal role in CTTH.[35] It was suspected that conversion of ETTH to CTTH could occur due to sensitization in the spinal dorsal horn or trigeminal nucleus induced by a prolonged nociceptive input. Nitric oxide (NO) may play a role in this sensitization process. NO is involved in the nociceptive transmission of the peripheral and central nervous system.[37-39] Increased NO levels may trigger central sensitization[40-42] whereas reduced NO levels is accompanied by reduced central sensitization.[43-45] Accordingly, the involvement of NO and its producing NOS (NO synthase) in TTH has been implicated.[46-48] Unspecific inhibition of NOS isoenzymes relieves TTH whereas increase in NO levels evokes delayed headache attacks similar to TTH. NO synthesis depends on oxidization of arginine to NO and citrulline catalyzed by three different NOS isoenzymes.[49-51] As there are three different NOS isoenzymes, it remains unclear which NOS is involved in TTH pathophysiology. Neuronal NOS (nNOS) is constitutively expressed in nervous system and skeletal muscles. Inducible iNOS can be expressed in a variety of cell types and has a pivotal role in the cytotoxic function of microphages. Endothelial eNOS was first found in vascular endothelial cells but can also be found in neuronal tissue and astrocytes.

Further important factors in TTH seem to be trigger factors, out of which mental and physical stress were reported to be the most frequent ones.[52-54] With still unclear pathophysiological mechanisms, the IHS

classification subcommittee encourages further research into the pathophysiological mechanisms and treatment of TTH.

1.3.2 Therapeutical approaches in the treatment of TTH

Basically, there are two options in TTH treatment: non-pharmacological management and pharmacotherapy.[55] In non-pharmacological treatment, psycho-behavioral treatments (EMG biofeedback, cognitive-behavioral therapy, relaxation training) as well as non-invasive physical therapy (i.a. posture improvement, massage, spinal manipulation), acupuncture and nerve block are applicable. EMG biofeedback techniques seem to be most reliable as its effects are sufficiently documented. The European Federation of Neurological Sciences (EFNS) recommends for acute treatment of TTH medication with cyclooxygenase (COX) inhibitors such as acetylsalicylic acid (ASA).[56] Combinations of COX inhibitors with caffeine should be regarded cautiously despite the increased efficacy of COX inhibitors due to additional caffeine. Caffeine withdrawal can in turn cause headache, especially in chronic daily headache associated with use of caffeine combination products.[57] The tricyclic antidepressant amitryptilin is a clinically relevant prophylactic drug in CTTH treatment.[58] So far, there is not sufficient reliable data on pharmacological therapeutic options such as muscle relaxants, serotonin or noradrenalin re-uptake inhibitors. Other treatment options are still in development. For example, the specific nNOS inhibitor NXN-188 dihydrochloride is tested in phase 2 multicenter study (NCT00959751, http://clinicaltrials.gov).

1.4 Animal model on TTH pathophysiology

1.4.1 A translational mouse model on neck muscle nociception

Up to now, there are only a few animal models on headache available[59] and, so far, only one with relevance to TTH.[33] This chapter deals with the background description of this one model which is suggested to be a translational model for the investigation of pathophysiological aspects of tension-type headache.

Based on the clinical finding of pericranial tenderness positively correlating with both TTH intensity and frequency, human experimental models for craniofacial muscle nociception were developed involving administration of diverse algogenic substances (hypertonic saline, human nerve growth factor and ATP) to different target muscles such as masseter, anterior temporalis, posterior temporalis, trapezius, splenius capitis and sternocleidomastoid.[27,60-63] The impact of neck muscle nociceptive input on brainstem nociceptive processing is described in one animal model. In anesthetized mice, administration of algesics (hypertonic saline, human nerve growth factor, ATP, ATP substrate analogon α,β-meATP) into semispinal neck muscles on brainstem nociception is documented.[26,64-67] Brainstem nociceptive processing is monitored via electromyographic (EMG) recording of the jaw-opening reflex (JOR) in the digastric muscle (Fig. 4).

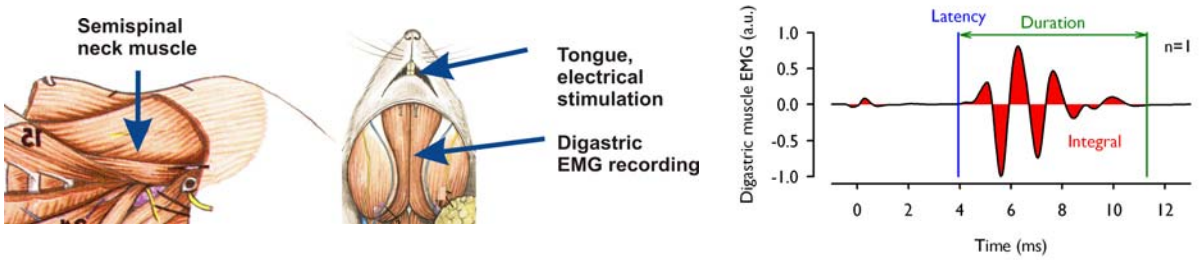

Figure 4: Murine model of neck muscle nociception
Left: semispinal neck muscle, site of local α,β-meATP application
Middle: electrical test stimulation of the tongue (upper bar), JOR EMG recording (lower bar)
Right: Single sweep recordings of the jaw opening reflex with integral (red), duration (green) and latency (grey).

The JOR can be elicited by electrical, thermal and mechanical stimulation of the craniofacial region.[68-73] Primary trigeminal afferents synapse on excitatory sensory neurons in the spinal trigeminal region which in turn project bilaterally to excitatory digastric motoneurons.[74,75]

Different craniofacial tissues such as neck muscles have convergent input to this reflex network. Due to this excitatory convergence, increased noxious input from the neck muscles via administered ATP leads to facilitation of the JOR. In this murine model, the JOR is elicited by electrical stimulation of tongue musculature (Fig. 4, middle and right). JOR integral, latency and duration are suitable parameters to evaluate brainstem nociception. Local intramuscular α,β-meATP administration into semispinal neck muscles (Fig. 4, left) induces sustained facilitation of brainstem nociception (Fig. 5). JOR facilitation was observed for at least four hours. Besides α,β-meATP, also hypertonic saline (5.8% NaCl) and the human β-nerve growth factor (NGF) are capable of inducing facilitation of brainstem nociceptive processing. NGF- induced facilitation in this model seems to

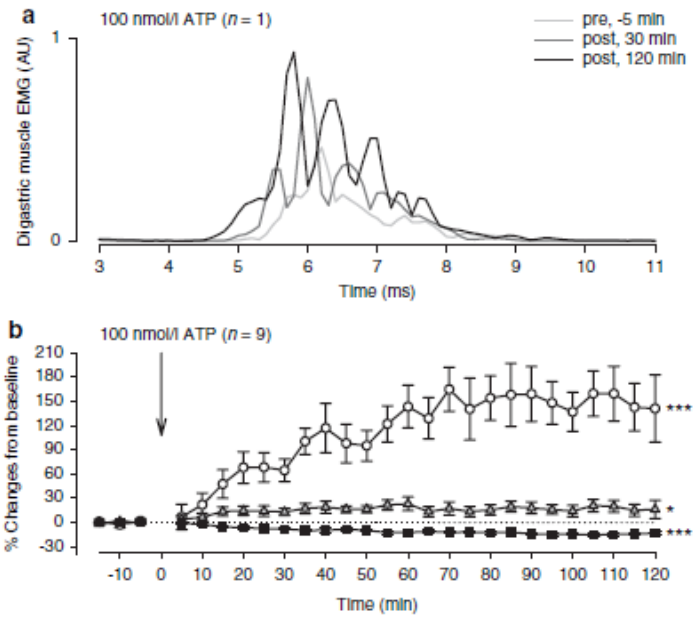

Figure 5: Reflex facilitation after intramuscular 100 nmol/l α,β-meATP
A: averages of 8 single sweep averages 5 min before (light grey), 30 min (dark grey) and 120 min after (black) α,β-meATP injection (100 nmol/l) into both semispinal neck muscles in one mouse. EMG activity is given in arbitrary units. B: Reflex integral (circles), duration (triangles) and latency (squares) from 15 min before to 120 min after α,β-meATP. Changes from baseline (from -15, -10 and -5 min) re expressed as arithmetic mean and standard error. Infusion start is indicated by an arrow. Friedman one way analysis of variance was performed. Asterisks represent the level of significance (*p<0,05; ***p<0,001).[26]

be mediated via C-fibers whereas α,β-meATP seems to mediate facilitation via excitation of Aδ fibers.[65] A comparison between TTH and the described animal model of neck muscle nociception is summarized in Table 1.

Table 1: Comparison of TTH and animal model properties.[76]

Property	TTH	Animal model
Pain correlative	Headache	Orofacial reflex facilitation
Myofascial correlative	Pericranial tenderness	Sensitization/noxious input from pericranial muscles
Location	Bilateral	Bilateral
Duration	30 min to 7 days	More than 4 hours

Altered nociception from pericranial muscles (tenderness vs. neck muscle nociception) as well as cranial effects (headache vs. reflex) are present in both cases. The described animal model seems to provide sufficient similarities as a surrogate model for the investigation of neck muscle nociceptive processing. Understanding its pathophysiological mechanisms in TTH is a prerequisite for its appropriate treatment.

1.4.2 The role of ATP and purinergic receptors in nociceptive processing and pain transmission

Focus of research in this model is the mechanism in facilitation of neck muscle nociception triggered by adenosine triphosphate (ATP) in mice (cf. 1.2.3). ATP seems to be an appropriate algesic stimulus in order to evoke noxious input from muscles according to three reasons:

- Increased interstitial ATP concentrations with muscle contraction.[22-24]
- ATP excites nociceptors on group III (Aδ fibers) and IV (C fibers) afferents in skeletal muscles.[77,78]
- Induction of pain and local tenderness via ATP injection into human trapezius muscle.[27]

More specifically, native ATP interacts with both ionotropic (P2X) and metabotropic (P2Y) receptors whereas its substrate analogon α,β-methylene ATP (α,β-meATP) interacts with P2X receptor with affinity to P2X3 homomers and P2X2/3 heteromers.[79,80] Both native ATP and α,β-meATP are able to activate and sensitize nociceptors.[81] α,β-meATP is a more appropriate algogenic substance than native ATP due to its slower degradation and its narrower receptor-binding profile.[82,83] Degradation studies have demonstrated dephosphorylation of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) with half-lives of about 15–20 min. In contrast, degradation of α,β-meATP to α,β-meADP occurs rather slowly, with approximately 80% remaining in tissue after 1 h. Both biochemical stability and narrow receptor profile are

the main reasons for selecting the P2X receptor agonist α,β-meATP in this translational murine model (cf, 1.3.3). P2X3 is almost exclusively expressed in sensory neurons.[84] Monomeric P2X3 and especially heteromeric P2X2/3 are known to be involved in nociceptive processing and, thus, are targets for future pain therapeutics.[85,86] Both P2X3 single P2X2/3 double knock-out mice showed reduced pain-related behavior.[87,88] Besides the neuronal P2X3 receptor, P2X7 receptors have recently been associated with nociception, too.[89-91] P2X7 seems to be involved in long-term potentiation (LTP) of spinal nociceptive processing and thus might mediate central neuroplastic changes.[92,93] LTP of synaptic transmission in the nociceptive system is thought to be involved in central sensitization, hyperalgesia, and chronic pain.[94] Thus, P2X mediated mechanisms in nociceptive processing are a promising target for future pain therapeutics.

1.5 Aim of the present thesis

The conceptual involvement of neck muscle nociception in TTH pathophysiology and hence, its importance for chronic pain therapy, is essential to obtain further extensive information about possible pathophysiological mechanism especially in regard to clinical relevance. Thus, the aim of the present thesis was a further, more detailed investigation of mechanisms in neck muscle nociception in this translational model with clinically relevant and potentially future drugs. In order to evaluate these mechanisms, the effect of systemically administered drugs on α,β-meATP-induced JOR-facilitation was examined (Study I, II, III).

1) Study I

   Due to the reported general role of NOS isoenzymes in both animal model[95] and TTH,[48] involvement of nNOS and iNOS in α,β-meATP-induced facilitation of neck muscle nociceptive processing was hypothesized. The particular focus lied on the role of nNOS in induction and maintenance of purinergic facilitation.

2) Study II

   ASA is of clinical relevance in treatment of acute TTH attacks.[56] Thus, the effect of ASA was evaluated on the induction and maintenance of α,β-meATP-induced facilitation of neck muscle nociceptive processing and its reversal and prevention was hypothesized, respectively. The particular focus lied in the subsequent administration ASA mimicking acute treatment of TTH in this translational animal model.

3) Study III

   The involvement of P2X7 receptor was examined regarding its putative role in the maintenance of

α,β-meATP-induced facilitation of neck muscle nociceptive processing. It was hypothesized that P2X7 antagonism reverses the α,β-meATP effect.

Clarifying possible mechanisms that contribute both to induction and maintenance of purinergic facilitation of brainstem nociception in mice could illustrate a further step in elucidating TTH pathophysiology as well as current and future treatment options.

Parts of these studies were presented at international conferences.[96,97]

2 Methods

Mechanisms of neck muscle nociception in mice were investigated by the use of pharmacological and electrophysiological means. Applied methods were essential to provide more insight into putative pathophysiological mechanisms in TTH and to point to possible new treatment approaches for TTH patients. This chapter provides a presentation of the applied methods.

2.1 Animal preparation

2.1.1 Animals

In this thesis, electrophysiological experiments were performed in adult male C57BL/6 mice (n =107, approximately 12 weeks old; Taconic, www.taconic.com). All procedures received institutional approval from the local ethics committee. The principles of laboratory animal care and use of laboratory animals (European Council Directive of November 24, 1986(86/609/EEC)) were followed. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Detailed descriptions of anesthesia, surgery and electrophysiological recording are published in detail.[98]

2.1.2 Preparation and microsurgery

Mice were anesthetized by an initial intraperitoneal (i.p.) injection of a 0.5% pentobarbital sodium salt solution (Amgros I/S, www.amgros.dk) with a dose of 70 mg/kg. Depth of anesthesia was checked by ensuring that noxious pinch stimulation (blunt forceps) of hindpaw, forepaw, and ear did not evoke any sensorimotor reflexes. When the animal was sufficiently deeply anesthetized, the skin of the throat and neck were carefully shaved and lidocaine hydrochloride gel (Xylocaine® 2%, AstraZeneca A/S, www.astrazeneca.com) was applied to the skin of the throat to induce local anesthesia. Dexpanthenol eye ointment (Bepanthen®, Roche, www.roche.com) was applied to cornea and conjunctiva of both eyes to protect them from drying. The right external jugular vein was catheterized for continuous administration of a 2% methohexital sodium salt solution (Brevimytal®, Hikma, www.hikma.de) with a dose of 60 mg/kg per hour corresponding to a flow rate of about 0.07 ml/h for a 23 g mouse. A pair of Teflon-coated stainless steel wires (140 µm diameter) was inserted into the right anterior digastric muscle to record electromyographic activity (EMG) and the jaw-opening reflex via a differential amplifier. After tracheotomy, animals were placed in a stereotactic frame and were artificially respired with a stroke volume of about 150 µl and about

200 strokes per min (MiniVent Model 845, Harvard Apparatus, www.harvardapparatus.com). Body core temperature was maintained at 37.3°C with a heating blanket and a fine rectal thermal probe (FMI GmbH, www.fmigmbh.de). One platinum needle electrode each (300 µm diameter) was subcutaneously inserted into right forepaw and left hind paw to record the electrocardiogram (ECG) via a differential amplifier. Two stainless steel needle electrodes (150 µm diameter) were longitudinally inserted into the tongue musculature (parallel, 2 mm distance) in order to apply electrical stimuli and to evoke the jaw-opening reflex. The oral cavity was filled up with white vaseline (Riemser Arzneimittel AG, www.riemser.de) to protect oral mucous membrane from drying. Neck skin was locally anesthetized via Xylocaine®. Semispinal neck muscles on both sides were carefully exposed. One injection cannula each (0.4 mm diameter) was inserted into the muscle belly of both semispinal neck muscles. Each cannula was connected via thin and short tubing to a liquid switch (CMA/110, CMA Microdialysis AB, www.microdialysis.se). Glass microsyringes (1 ml) were connected to the liquid switch by thin tubing and were fixed in a microdialysis pump (CMA 102, www.microdialysis.se). Use of this liquid switch enabled repeated injection at the same i.m. site without changing cannula and without interruption of flow during the in vivo experiment. This procedure allowed bilateral induction of noxious input from neck muscles in order to mimic bilateral neck muscle pain in tension-type headache patients. Saline (control) or α,β-meATP (Sigma-Aldrich Chemie Gmbh, www.sigmaaldrich.com) was i.m. administered with a volume of 20 µl per semispinal neck muscle during a time period of one minute. After preparation, the anesthetized animal was rested for at least one hour. During this time period level of anesthesia and heart rate were routinely checked and documented, and depth of anesthesia was maintained.

2.2 Electrophysiology

All electrical signals (EMG, ECG) were recorded by bioamplifiers. EMG signals were filtered with 500 to 5000 HZ, ECG signals with 500 to 1000 HZ. EMG signals led into a data collection system (CED Micro1401, CED, Cambridge Electronic Design Limited, www.ced.co.uk) and a personal computer using Signal® software program (CED, Cambridge Electronic Design Limited, www.ced.co.uk).
The jaw-opening reflex (JOR) was elicited by electrical stimulation of afferent nerve fibers in the tongue musculature via two needle electrodes with rectangular electrical pulses of 500 µs duration and a stimulation frequency of 0.1 Hz (Fig. 1.3.3.1, middle, right). Reflex responses were recorded in the anterior digastric muscle by electromyography (EMG). Reflex responses elicited by a single stimulus were quantified by measuring its onset latency, duration and integral (Fig. 1.3.3.1, right). The duration covers the time window between onset latency and end of the reflex response in the digastric muscle EMG. In this time window, the

reflex integral (area under the curve) was calculated. The electrical threshold of the jaw-opening reflex was determined by applying one series of increasing and decreasing stimulus intensities from 0 to 2 mA in steps of 100 µA. The lowest stimulus intensity that just evoked a reflex response was defined as the reflex threshold. Test stimulus intensity was adjusted to approximately 150% of the reflex threshold. The jaw-opening reflex was evoked in series of eight stimuli. These series were repeated every 5 min.

2.3 Pharmacology and drug administration

In general, drugs were i.p. injected in different dosages before or after i.m. α,β-meATP infusion. α,β-meATP and drugs were dissolved in physiological saline solution. I.p. injection of saline solution served as a control. Since intramuscular saline infusion was demonstrated to have no effect on the jaw-opening reflex in this murine model,[65,67] intramuscular saline infusion as control for ATP administration was not performed again in order to reduce the amount of animals used.

Study I

Administered substances were the nNOS inhibitor NPLA (N^{ω}-propyl-L-arginine; Tocris Bioscience, www.tocris.com), iNOS inhibitor 1400W (N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride; Tocris Bioscience, www.tocris.com), isotonic saline (0.9% sodium chloride) and α,β-meATP (α,β-methylene adenosine 5´-triphosphate, Sigma-Aldrich, www.sigmaaldrich.com). Two different experiments were conducted in 39 mice.

In the first experiment, NPLA or vehicle were administered i.p. after stable baseline recording prior to i.m. α,β-meATP infusion. Vehicle was isotonic saline (100 µl). Due to preceding pilot experiments, three different dosages of NPLA (100 µl) were applied (0.5, 1 and 2 mg/kg). Six consecutive reflex series were recorded before α,β-meATP infusion (1 µM) into semispinal neck muscles. The jaw-opening reflex was then monitored for 90 min.

In the second experiment, α,β-meATP was i.m. infused into both semispinal neck muscles after three stable baseline recordings. 90 min subsequent to α,β-meATP administration, an i.p. injection of 2 mg/kg NPLA, 2 mg/kg 1400W (100 µl) or saline, was performed. Reflex monitoring continued for at least 60 min.

Study II

Administered substances were aspirin® i.v. (ASA, d,l-lysinacetylsalicylat, Bayer Vital, www.bayer.com), sodium chloride solution and α,β-meATP. α,β-meATP was resolved in isotonic saline solution. ASA was resolved in aqua ad iniectibilia. As control served i.p. injection of a saline solution isoosmolar to 15 mg/ml ASA (corresponding to 60 mg/kg). Isotonic saline was diluted with aqua ad iniectibilia to a mean osmolarity

of 190 mosmol/l. Two different experiments were conducted in 42 mice.

In the first experiment, the effect of subsequently administered ASA on α,β-meATP-induced reflex facilitation was investigated. After stable baseline recording, α,β-meATP (100 nM) was infused i.m. For the following 60 min, purinergic reflex facilitation was observed. Then, either saline or ASA was administered i.p. and the reflex was then monitored for further 90 min. Three different dosages of ASA (15, 30 and 60 mg/kg) were applied (100 µl) according to preceding pilot experiments and recommended maximal daily ASA dosage in humans (60 mg/kg).

In the second experiment, the effect of precedingly administered ASA or saline on α,β-meATP-induced reflex facilitation was investigated. After stable baseline recordings, the maximal ASA dosage (60 mg/kg) was i.p. injected and the reflex monitored for the following 60 min. α,β-meATP was i.m. infused. The reflex was monitored for at least further 60 min. In a further series, saline was i.p. administered after stable baseline recordings and the reflex was recorded for the following 30 min. Then, α,β-meATP was i.m. infused and the reflex was recorded for at least 60 min.

Study III

Administered substances were A438079 (3-[[5-(2,3-Dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride, Tocris Bioscience, www.tocris.com), sodium chloride solution and α,β-meATP. α,β-meATP and A438079 were resolved in isotonic saline solution.

The experiments were conducted in 20 mice. The effect of subsequently i.m. or i.p. administered A438079 on α,β-meATP-induced reflex facilitation was investigated. After stable baseline recording, 100 nM α,β-meATP was infused i.m. Purinergic reflex facilitation was observed for at least the following 90 min. Then, either saline or A438079 were administered either i.m. (100 µM) or i.p. (150, 300 µmol/kg). The reflex was then monitored for at least further 60 min.

3 Discussion of study I-III

In the utilized model, observed purinergic facilitation as well as its modulation via precedingly or subsequently administered drugs or saline is most probably independent of mechanical induction such as volume effects. Both i.m. and i.p. injected isotonic saline[65,67,99] do change neither the basal nor the facilitated jaw-opening reflex. This contradicts any local volume effects due to injection and instead suggests pharmacological actions of drugs.

The presented data in this thesis provides further insights in mechanisms of neck muscle nociception in the utilized translational murine model of TTH. The NOS isoenzymes nNOS and iNOS play a divergent role in the induction and maintenance in purinergic facilitation. Furthermore, the induction and maintenance of α,β-meATP -induced facilitation can be both prevented and reversed by ASA in clinically relevant dosages, respectively. Moreover, the P2X7 receptor plays a functional role in the maintenance of purinergic facilitation.

3.1 Study I

Systemic administration of unspecific NOS inhibitor L-NMMA dose-dependently prevented and reversed sustained purinergic facilitation of brainstem nociception.[95] This study investigated the involvement of nNOS both in the induction and maintenance of α,β-meATP-induced facilitation of neck muscle nociceptive processing in anesthetized mice. Neither preceding saline nor NPLA affected the basal JOR. Inhibition of nNOS prevented the induction of purinergic facilitation but did not modulate its maintenance. Hence, involvement of iNOS on established reflex facilitation was additionally evaluated. Inhibition of iNOS reversed the facilitated reflex. Study I demonstrates the divergent role of NOS isoenzymes in α,β-meATP-induced facilitation of neck muscle nociception. Whereas nNOS is crucial for its induction, iNOS is involved in its maintenance.

NOS isoenzymes and their synthesized NO is involved in nociceptive and pain processing based on human[46,47,100,101] and experimental animal models.[40,45,102-105] A number of studies in animal experimental pain studies on the pharmacological inhibition of nNOS and on nNOS deficient mice document this isoenzymes involvement in central nociception.[43,106-111] Nonetheless, NOS contribution to nociceptive processing is not restricted to the central nervous system but can also be observed in the periphery.[102,112] Constitutive expression of nNOS in skeletal muscles[113,114] implicates putative peripheral nNOS physiological mechanisms as presented in study I. Taken together, the prevented purinergic facilitation via preceding nNOS inhibition can principally take place in different tissues such as neck muscle, peripheral and central nervous system.

Nociceptive events from muscle and peripheral nervous system could explain why the NPLA effect was restricted to the prevention of the α,β-meATP effect.

The involvement of iNOS in different animal pain models was demonstrated for both peripheral and central nociceptive mechanisms, too.[112,115-117] Inhibition of iNOS via intrathecally administered 1400W attenuated carrageenan- and formalin-induced hyperalgesia in rats.[115,116] Protein expression levels in the subnucleus caudalis of all three isoenzymes were up-regulated after capsaicin injection into the rat masseter muscle.[117] Pretreatment with NOS inhibitors attenuated capsaicin-induced masseter hypersensitivity. Additionally, iNOS and nNOS overexpression in peripheral and central nervous system was demonstrated in a murine model of mononeuropathy induced by sciatic nerve chronic constriction injury.[112] With nNOS organizing only the induction of purinergic facilitation of brainstem nociception in the utilized model, central nervous system action of iNOS seems logical. Divergent roles of nNOS and iNOS in peripheral and central animal pain models suggest orchestration of NOS isoenzymes at different sites and with different impact.[107,112,117,118] Hypothetically, depotentiation via iNOS inhibition could occur in the central nervous system but at which level remains speculative.

The development of chronic TTH might be due central sensitization induced by prolonged noxious input from pericranial myofascial tissues.[119,120] NO is implicated in pathophysiological mechanisms of central sensitization.[121] In consequence, the impact of unspecific NOS inhibition on chronic TTH patients was investigated.[46,47] Administration of the unspecific NOS inhibitor L-NMMA lead to reduced muscle hardness and headache intensity. Accordingly, TTH patients might benefit from specific NOS isoenzyme inhibitors in clinical practice without impairing NO signaling in systems not related to TTH pathophysiological mechanisms. Selective NOS inhibitors for clinical studies might be available in the near future. A phase 2 multicenter study in migraneurs with aura on the impact, efficacy and side effects of the nNOS inhibitor NXN-188 dihydrochloride was recently finished (NCT00959751, http://clinicaltrials.gov). This demonstrates new opportunities for the investigation of NOS isoenzyme involvement in pathophysiological mechanisms of neck muscle nociception.

3.2 Study II

ASA is a clinically relevant drug recommended by the EFNS[56] and DMKG[122] for self-medication of TTH attacks. The focus of this study lied on the impact of subsequently applied ASA in anesthetized mice mimicking the acute treatment of acute TTH attacks. Additionally to subsequent injection, ASA was nonetheless also administered preceding to intramuscular α,β-meATP infusion in order to evaluate the impact

of ASA on both induction and maintenance of purinergic facilitation in two distinct set-ups. Purinergic facilitation of neck muscle nociception was prevented and reversed with preceding and subsequent ASA, respectively. With clinically significant dosages recommended for treatment of acute TTH attacks, ASA interfered with both induction and reversal of the α,β-meATP effect in anesthetized mice.

The analgesic properties of ASA for the treatment of TTH are well documented.[123-126] Typically, dosages of 1000 mg ranging from 650 mg to 1200 mg are orally administered. In case of self-medication with ASA, the German Migraine and Headache Society (DMKG, http://www.dmkg.de) recommends a single oral dosage of 1000 mg per day. Similarly, EFNS recommendations include oral ASA dosage up to 1000 mg per day for self-medication (http://www.efns.org). In animal experiments, antinociceptive and analgetic dosages of ASA range from 10 to 400 mg/kg depending on the model used.[127-133] ASA ED50 values for humans and animals - and thus ASA efficacy - may not be directly comparable. Hence, one study established correlations between human and murine ED50 values enabling dosage estimation from mice to humans.[134] Accordingly, in the presented study applied dosages of 15, 30 and 60 mg/kg in mice would correspond to single oral dosages of 660, 780 and 1030 mg in humans. Thus, applied ASA dosages in this study cover a clinically relevant range.

Both peripheral and central ASA modes of actions are well known. The antinociceptive and analgetic property of ASA in the periphery is usually associated with antiinflammatory action. However, data from non-inflammatory pain studies in human on brainstem[135] and cortical nociceptive processing[136,137] and in animals on trigeminal[127] and brainstem nociceptive processing[130,138] suggests an additional central mode of action for ASA, possibly mediated via COX inhibition.[130,139] This central, antinociceptive ASA effect alone could be accountable for observed ASA effects in study II.

Nevertheless, the central antinociceptive ASA action is possibly not restricted or even partially independent from COX inhibiting mechanisms. In the utilized animal model of purinergic facilitation of neck muscle nociceptive processing, the COX-unspecific inhibitor indomethacin failed to exert antinociception on the α,β-meATP effect.[140] Divergent action of ASA and indomethacin was also reported in other animal pain models.[132,141,142] Moreover, a ASA might exert its antinociceptive action via interaction with NOS isoenzymes.[131,143,144] As discussed in study I, NO and NOS isoenzymes play an important role not only in nociceptive processing but is also implicated in pathophysiological mechanisms of central sensitization[121] that could be relevant in TTH pathophysiology. Another reported ASA mode of action its dosage dependent effect on mitochondrial oxidative phosphorylation.[145] Up to now, there is no evidence for the latter route to be implicated in ASA-mediated antinociceptive mechanisms. A central nervous system ASA antinociceptive mode of action via COX and/or NOS may be accountable for presented observations in study II.

The facilitation of brainstem nociceptive processing in the current murine model lasts for at least four hours[67] resembling fulfilled IHS diagnostic criteria for both episodic and chronic TTH attack duration between 30 min < 7 days. Nociceptive input from neck muscles is thought of as the main trigger for episodic TTH. Prolonged exposition might induce central sensitization that would lead to chronification. The single, non-repetitive administration of α,β-meATP possibly implies resemblance to a rather acute than chronic translational murine model. Nevertheless, hypothesized peripheral driven induction and centrally established purinergic facilitation (cf. study I and II) could incorporate a transitional step from peripheral to central sensitization. Accordingly, a recently applied reflex model in TTH patients demonstrated a similar divergence.[146] Predominantly in chronic TTH patients, the so-called trigemino-cervical reflex showed abnormal size or latency but was not observed in episodic TTH patients pointing to established central sensitization with chronification.

Study II demonstrates ASA effects on facilitated brainstem nociceptive processing in this translational murine model that supports the treatment of acute attacks of tension-type headache with ASA in analogy to the recommendation of the DMKG and EFNS.

3.3 Study III

P2X7 receptor pharmacology is gaining increasing interest with regard to its therapeutic potential in chronic pain states.[147] In this study, it was hypothesized that α,β-meATP-induced facilitation of brainstem nociception involves purinergic signaling via P2X7 receptor in anesthetized mice. Study III focused on the administration of the P2X7 receptor antagonist A438079 with established JOR facilitation. This particular set-up allowed mimicry for treatment of acute TTH attacks. Purinergic facilitation was reversed only with systemical but not with intramuscular administration of P2X7 antagonist A438079 in a dose-dependent manner.

There is evidence for P2X7 participation and modulation of nociceptive transmission and pain processing. The role of P2X7 in increased pain processing is demonstrated in animals via antagonistic P2X7 action.[148,149] Additionally, inhibition of P2X7 prevents induction of LTP in both in-vivo and in-vitro and alleviates mechanical allodynia in the rat.[92] Accordingly, siRNA-mediated P2X7 down-regulation prevents LTP induction and inhibits mechanical allodynia. P2X7 gene disruption results in abolished hypersensitivity in inflammatory and neuropathic pain models despite preserved normal nociceptive processing in mice.[89] Taken together, participation of P2X7 in divergent elevated states of nociceptive transmission and pain processing seems evident.

It remains not entirely clear, in which tissue P2X7 antagonisms impacts purinergic facilitation in the present study. P2X7 is localized in glial cells such as microglia,[150] macrophages and monocytes[79,151] and neuronal tissue.[152] With systemic A438079 administration, brain to plasma ratio is 2:1 suggesting a pronounced central nervous system A438079 distribution.90 P2X7 is presynaptically present in rat cortical nerve terminals[153] and localized in excitatory terminals in the rat hippocampus.[154] P2X7 receptor mRNA is expressed in neurons, oligodendrocytes and microglia in the rat brain.[155] However, there are implications that glial P2X7 might contribute to purinergic facilitation in the present study. Antagonism of glial metabolism inhibits α,β-meATP-induced LTP of neuronal excitation in the rat superficial spinal dorsal horn.[93] Furthermore, ATP-release from nerve terminals and dorsal root ganglia neurons enables neuron-glia signaling.[150,156,157] Similarly, a P2 receptor-mediated bi-directional communication is described in dissociated murine sensory trigeminal ganglia, probably utilizing ATP for communication.[158] Still, a direct link between peripherally α,β-meATP-driven facilitation and a microglial involvement via P2X7 signalling is yet missing but this synergistic cross-talk can be considered. Hence, it remains unclear whether P2X7 antagonistic effects in the present study are localized peripherally or rather centrally.

Study III defined a further component in the α,β-meATP-induced facilitation of neck muscle nociception implying a cardinal role for P2X7 receptor signaling in maintaining sensitization. These results may point to involvement of P2X7 mediated signaling in TTH pathophysiology and may suggest potential future targets for pharmacological treatment of TTH.

4 Conclusion and perspectives

With this data, new insight is provided into the pharmacological modulation of purinergic facilitation of neck muscle nociception in anesthetized mice. This translational model is regarded as suitable for the investigation of putative pathophysiological TTH mechanisms. The following conclusion is sorted according to clinical relevance of performed studies.

Accordingly, the routine clinical practice in the treatment of acute TTH attacks via ASA is confirmed with study II. This outcome emphasizes the validity of the utilized animal model for the investigation of possible TTH pathophysiological mechanisms not restricted to murine neck muscle nociception. ASA is highly recommended only for the acute treatment of ETTH attacks.[56,122] In turn, this could imply that the current murine model reflects rather ETTH than CTTH mechanisms, and hence, could resemble a rather peripheral than central mechanism of sensitization. This would correspond to the current concept of TTH pathophysiology. Nonetheless, it remains to be clarified whether the α,β-meATP effect in this model is founded on prolonged excitation of purinergic sensory afferents in the neck muscles or it involves central sensitization. Furthermore, the actual mode of ASA action in this model needs to be clarified as the ASA effect seems to involve other mechanisms than the COX system. In this regard, it is self-evident to use other COX inhibitors that are clinically relevant in TTH treatment, such as Ibuprofen® and Paracetamol®.

Based on the reported involvement of NO and NOS isoenzymes in TTH, the outcome of study I points to new targets for future pharmacological treatment options, i.e. drugs specifically inhibiting NOS isoenzymes. Possibly, iNOS inhibitors could gain clinical relevance in the acute treatment of TTH attacks whereas nNOS inhibitors could be used for TTH prophylaxis. Up to date, there are no specific NOS inhibitors commercially available that are approved for clinical purposes. On the other hand, administration of NO donors could demonstrate pro-nociceptive responses in the current model confirming human experimental data of TTH pathophysiology. Combined with specific inhibition of NOS isoenzymes (i.p. vs. i.m.), a more detailed exploration of NO and NOS mechanistic involvement in purinergic facilitation seems necessary. Finally, localization of the tissue involved is inevitable in which inhibition of nNOS and iNOS exerts preventive and reversing effects on purinergic facilitation. This would additionally clarify the involvement of peripheral and central structures in this murine model.

P2X receptors gain increasing interest as targets for future therapeuticals in chronic pain states. Up to date, there are comparably few reports on the involvement of P2X7 receptor in nociceptive processing. Nonetheless, a wider field of interest opens when taking P2X7 distribution in neuronal and glial cells into account, with particular regard to neuron-glia actions in nociception and nociceptive sensitization.

Nonetheless, clarification is needed for the localization of observed P2X7 antagonistic effects in this model, especially regarding the involved tissue in peripheral and central structures. Furthermore, it needs to be elucidated that P2X7 signaling plays a role in TTH pathophysiology. Only then, future clinical application of P2X7 inhibitors in the treatment of acute TTH attacks is justified.

Moreover, the chronological sequence of purinergic facilitation of neck muscle nociception and the interaction of known components is of special interest. It remains to be demonstrated, at which instances NOS isoenzymes, P2X7 receptor and ASA-related mechanisms are recruited in α,β-meATP-mediated effects. This would enable enhanced understanding of the physiological mechanisms in α,β-meATP-induced facilitation of neck muscle nociception in mice and with putative implication in TTH pathophysiology.

Of further interest is the applicability of the murine model with induction of purinergic facilitation from other muscles such as temporalis, splenius capitis and trapezius muscles. Due to the reported involvement of different pericranial and craniofacial muscles in TTH pathophysiology, it is logical to switch to abovementioned muscles to investigate the impact of i.m. α,β-meATP infusion on neck muscle nociceptive processing.

It has not been demonstrated yet, whether both purinergic facilitation and TTH pathophysiology involves up-regulation of nociception-specific receptors such as the P2X3 receptor. As reported in several animal experimental models, translational inhibition of nociceptive receptors via administration of short interfering RNA (siRNA) results in diminished transduction of nociceptive signals and a decreased capacity to induce increased levels of nociceptive processing. Hence, it is necessary to investigate in which tissue prevention and reversal of purinergic facilitation can be induced with translational down-regulation via e.g. P2X3 sequence-specific siRNA. Possibly, chronification of TTH parallels with up-regulation of these nociceptive units and, hence, would point to new aspects in TTH pathophysiology and treatment options.

Finally, a re-translation from mice to humans is inevitable. Though, there are not many human experimental reflex models that are regarded as suitable for the investigation of TTH pathophysiology. Recently, the trigemino-cervical reflex (TCR) gained attention due to reported altered reflexes in TTH patients compared with healthy volunteers. As the TCR can be recorded from different neck muscles such as the semispinalis capitis, this model offers the possibility of switching from mice to humans.

5 Danish summary

Spændingshovedpine (SPH) er den hyppigste primære hovedpine med 1-års verdensomspændende prævalens på 40%. Danmark har med 89% selv to gange frekvensen. Den mest betydningsfulde abnorme fund i SPH patienter er øget ømhed af pericranial muskler, som er positivt korreleret med både intensitet og hyppigheden af hovedpine. Trods den store kliniske og socio-økonomiske betydning af SPH, er dens patofysiologi stadig stort set ukendt. Det nuværende koncept foreslår inddragelse af perifere smerter mekanismer i udviklingen af SPH og ændringer i det centrale smerter mekanismer, der fører til sine chronification. Baseret på kliniske konstateringen af pericranial ømhed positivt korrelerede med både hovedpine intensitet og hyppighed, en mus model på øget nakke muskel følsomhed blev udviklet med henblik på at undersøge formodede patofysiologiske mekanismer af SPH, der er baseret på intramuskulær (i.m.) infusion af α,β methylen adenosin 5'-trifosfat (α,β-meATP). På grund af sammenkoblede neurale netværk, i.m. α,β-meATP administration forårsager en øget hjernestammen refleks, den såkaldte kæbe-åbning refleks (KÅR), som kan bruges til at overvåge virkningerne af nakke muskler nociceptive input. Denne model betragtes som egnet til undersøgelse af formodede patofysiologiske mekanismer i SPH.

Denne afhandling indeholder detaljerede elektrofysiologiske og farmakologiske undersøgelser af α,β-meATP-induceret lettelse af nakke muskel nociception. Formålet var at identificere mekaniske komponenter i denne model gør det muligt for fremskrivning til SPH med særligt hensyn til fremtiden eller i øjeblikket relevante terapeutiske mål i behandlingen af SPH angreb. Afhandlingen består af tre undersøgelser:

Studie I:

Undersøge virkningen af specifikke nitrogenoxid syntase (NOS)-hæmmere på α,β-meATP effekt.
Uspecifik hæmning af NOS isoenzymer reducerer hovedpine smerte intensitet. Alligevel vides det ikke, hvilken af de tre isoenzymer (neuronal nNOS, inducerbare iNOS, endotelial eNOS) er involveret. I overensstemmelse hermed, uspecifikke NOS hæmning forebygger og tilbagefører α,β-meATP medieret KÅR lettelse uden nogen viden om, hvilke NOS isoenzymer er mekanisk involveret. Den største fokus i denne undersøgelse løjet i formodet engagement nNOS. Desuden blev hæmning af iNOS undersøgt.

Studie II

Kontrol af kliniske relevans af acetylsalicylsyre (Aspirin ®, ASS) i denne model.
ASS er anbefalet af European Federation of Neurological Sciences (EFNS, www.EFNS.org) som et effektivt lægemiddel i self-behandling af SPH angreb. Omfanget af denne undersøgelse var at efterprøve de kliniske betydning af ASS i SPH behandling i denne translationelle mus model af nakke muskel nociception.

Administration af ASA med lettere KÅR var af særlig betydning, da det angiveligt efterlignede behandling af en akut SPH angreb.

Studie III

Udforskning af inddragelse af P2X7 receptorer i α, β-meATP-medieret KÅR lettelse.
Purinergic receptorer (P2X) er involveret i nociceptive signalering. Nuværende udtalelsen foreslås disse receptor typer til at have et betydeligt potentiale i fremtidige behandling af smertetilstande. Ajourførte, viden om receptor subtype P2X7 og dens rolle i nociceptive forarbejdning er relativt sparsom. Alligevel er der overbevisende dokumentation for, at P2X7 deltager i fysiologiske processer involveret med forhøjede tilstande af nociceptive forarbejdning. Således denne undersøgelse hypotese tilbageførsel af KÅR lettelse via P2X7 hæmning.

Alle tre undersøgelser er baseret på elektrisk stimulation af tungen for at fremkalde og registrere KÅR fra digastric muskler i bedøvede mus. Infusion af α, β-meATP i semispinal nakke musklerne udløst KÅR lettelse. De farmakologiske effekter af forskellige NOS-hæmmere, ASS og P2X7 antagonist på KÅR lettelse blev vurderet før og/eller efterfølgende til α,β-meATP infusion. Alle lægemidler blev injiceret systemisk. Den P2X7 antagonist blev givet yderligere ind semispinale nakke musklerne. Både systemisk og intramuskulær injektion af isotonisk saltvand påvirker ikke KÅR kredsløb. Derfor blev saltvand brugt som kontrol i alle tre undersøgelser.

Den første undersøgelse viste divergerende inddragelse af nNOS og iNOS isoenzymer. Ud fra følgende betragtninger nNOS hæmning resulterede i forebyggelse af KÅR lettelse, kunne dens tilbageførsel kun opnås via iNOS hæmning. Dette indebærer inddragelse af forskellige NOS isoenzymer på forskellige stadier af øget nakke muskel nociception, som medfører de samme terapeutiske strategier i behandling af akut SPH angreb.

Den anden undersøgelse dokumenteret både vending og forebyggelse af α, β-meATP-medieret KÅR lettelse via ASA. Da denne model betragtes som egnet til undersøgelse af hovedpine patofysiologiske mekanismer, bekræfter denne data IHS anbefalinger for behandling af akut SPH angreb med ASS.

I den tredje undersøgelse, fører P2X7 hæmning tilbageførsel af α,β-meATP virkning. Dette indebærer en afgørende inddragelse af P2X7 receptor i purinergic signalering i den aktuelle model. Disse resultater tyder derfor P2X7 antagonist som potentielle fremtidige mål i behandlingen af SPH.

Afhandlingens resultat er et vigtigt skridt for at belyse komponenter involveret i α,β-meATP-medieret øget følsomhed i nakke muskler hos mus. Derfor, det giver ny mekanistisk indsigt og potentielle fremtidige behandlingsmuligheder for SPH, de hyppigste primære hovedpine.

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