Interaction of Transient Receptor Potential Vanilloid 1 (TRPV1) with G-protein coupled receptors and TRP ion channels

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Abbreviation

A	alanine
AC	adenylylcyclase(s)
ASIC	acid sensing ion channel
AgCl	silver chloride
AKAP	A kinase anchoring protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANKTM1 / p120	old names for TRPA1
ANOVA	analysis if variance
ATP	adenosine triphosphate
BSA	bovine serum albumin
CA	cinnamaldehyde
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
capsa	capsaicin
CB	cannabinoid receptor
Cdk5	cyclin-dependent kinase 5
cDNA	copy desoxyribonucleic acid
CFA	Complete Freund´s Adjuvant
CGRP	calcitonin gene-related peptide
CIB	Calcium Imaging Buffer
CREB	cAMP response element binding protein
CTRL	control
Da	dalton
DAG	diacylglycerol
DAMGO	D-Ala^2, N-$MePhe^4$, Gly^5-ol-enkephalin
DMSO	dimethyl sulphoxide
DNA	desoxyribonucleic acid
DOPA	dihydroxyphenylalanine
DOR	δ-opioid receptor
DRG	dorsal root ganglion
DTT	dithiothreitol
E. coli	Escherichia coli

ECS	extracellular buffer
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal regulated kinase
F340/F380	ratio of fluorescence at 340 nm to that at 380 nM
f	femto (10^{-15})
FBS	fetal bovine serum
g	gram (s)
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
HEK 293	human embryonic kidney cells 293
HEPES	4-2hydroxyethyl-1-piperazineethanesulfonic acid
I	current
IB4	isolectin B4
IBMX	isobutylmethylxanthin
ICS	intracellular buffer
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
k	kilo
K_D	dissociation constant
KCl	potassium chloride
LB	Luria-Bertani
LC	Locus coeruleus
LTP	long-term potentiation
M	molar
MAPK	mitogen activated protein kinase
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolare
MOR	μ-opioid receptor
mRNA	messenger RNA
MuO	mustard oil
mV	millivolt

n	number
nA	nanoampere
NA	nucleus accumbens
NGF	nerve growth factor
nM	nanomolar
nm	nanometer
n.s.	not significant
NLX	nalaxone
NMDA	N-methyl-D-asparate
OIH	opioid induced hyperalgesia
OWIH	opioid withdrawal induced hyperalgesia
pA	picoampere
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
PUFA	polyunsaturated fatty acid
PVD	polyvinylidene fluoride
RTX	resiniferatoxin
s	second
S	serine
SP	substance P
TG	trigeminal ganglion
TM	transmembrane domain
TNFα	tumor necrosis factor alpha
TRIS	tris (hydroxymethyl) amino-methane
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1
V	volt
YFP	yellow fluorescent protein

1. Introduction

Injury and inflammation of peripheral tissue stimulates electrical activity of sensory dorsal root ganglion (DRG) neurons (“nociceptors”). These impulses can be modulated by excitatory and inhibitory ion channels and receptors, and are eventually transmitted to the central nervous system where they are translated into the perception of “pain”. Among the most prominent nociceptor membrane proteins are excitatory transient receptor potential (TRP) channels and inhibitory opioid receptors. The interplay between these membrane proteins and their signalling pathways shall be elucidated here.

The aims of this doctoral thesis are, first, to investigate the involvement of the excitatory ion channel TRPV1 (Transient Receptor Potential Vanilloid 1) during withdrawal from inhibitory (analgesic) drugs (opioids) and second, the influence of a related ion channel TRPA1 (Transient Receptor Potential Ankyrin 1) on TRPV1 activity. Besides inflammatory mediators, both channels are activated by pungent components such as capsaicin (TRPV1) and mustard oil (TRPA1), and play a critical role in pain sensation and in the development of enhanced sensitivity to painful stimuli (“hyperalgesia”) typically associated with tissue injury. Both channels are co-expressed in nociceptors.

Opioids produce analgesia (pain inhibition) by activation of G_i-protein-coupled opioid receptors and subsequent dampening of neuronal excitability. However, after prolonged opioid treatment and abrupt withdrawal, paradoxical hyperalgesia can arise. Although the precise molecular mechanism is not yet fully understood, this is generally thought to result from neuroplastic changes in the peripheral and central nervous systems that lead to sensitization of pronociceptive pathways. In the following, the role of TRPV1 in opioid withdrawal-induced hyperalgesia will be investigated in the peripheral nervous system. Behavioural studies indicated that, in addition to TRPV1, the TRPA1 channel also plays a key role in pain transduction, especially during pathological conditions triggered by tissue damage and inflammation. TRPV1-mediated responses in neurons have a characteristic voltage dependency that is influenced by extracellular Ca2+ and by the type and concentration of TRPV1-specific agonists. Because of the prominent role of both TRP channels in inflammatory pain, we decided to investigate the functional relevance of interactions between TRPA1 and TRPV1, and whether TRPV1-mediated responses can be modulated by TRPA1.

1.1. Pain

Pain is generally defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Loeser and Treede 2008). Major pain syndromes are classified into nociceptive, inflammatory and neuropathic pain (Patapoutian et al. 2009). Temporal classification distinguishes between acute and chronic pain. Nociceptive pain includes somatic and visceral pain and is generated by noxious stimuli that act on peripheral nociceptors. Nociceptive pain, which occurs clinically in the settings of acute trauma, is protective and functions to prevent further tissue damage. Inflammatory pain develops with damaged or inflamed tissue. Chemical mediators produced and released from the primary sensory terminal and from non-neuronal cells (e.g., fibroblasts, mast cells, neutrophils and platelets) directly stimulate and/or sensitize nociceptors to chemical and mechanical stimuli. The latter phenomenon leads to behaviourally observable hyperalgesia and is classified into primary (sensitization that occurs directly at the site of tissue injury) and secondary hyperalgesia (sensitization that occurs in surrounding undamaged tissues). Hyperalgesia results from sensitization of ion channels in the membrane of nociceptors and an alteration in nociceptor excitability (Julius and Basbaum 2001). Molecular mechanisms regarding the sensitization of the capsaicin receptor TRPV1 will be introduced in more detail in chapter 1.2. A special case of hyperalgesia is allodynia, where normally innocuous stimuli induce pain sensation. Besides peripheral sensitization of nociceptors, central changes are induced, which may even result in an activation of normally non-nociceptive neurons by noxious stimuli (Patapoutian et al. 2009).

Neuropathic pain results from injury / dysfunction of the peripheral, autonomic or central nervous system (Backonja 2003). Neuropathic pain is associated with abnormal sensations like spontaneous pain and pain hypersensitivity, which occur both centrally and peripherally.

One mechanism implicated in the development of opioid withdrawal-induced hyperalgesia shares similarities with mechanisms thought to underly the development of neuropathic pain. Activation of μ-opioid receptors in the dorsal horn of the spinal cord can lead to hyperalgesia via stimulation of the excitatory amino acid neurotransmitters system. While the stimulation of a μ-opioid receptor initially hyperpolarizes central neurons by activating inwardly rectifying potassium channels, ongoing stimulation of the μ-receptor can result in upregulation of intracellular messengers (e.g. cAMP, phosphokinase C), activation of the N-methyl-D aspartate receptor system, and result in enhanced neuronal excitability (Mao et al.

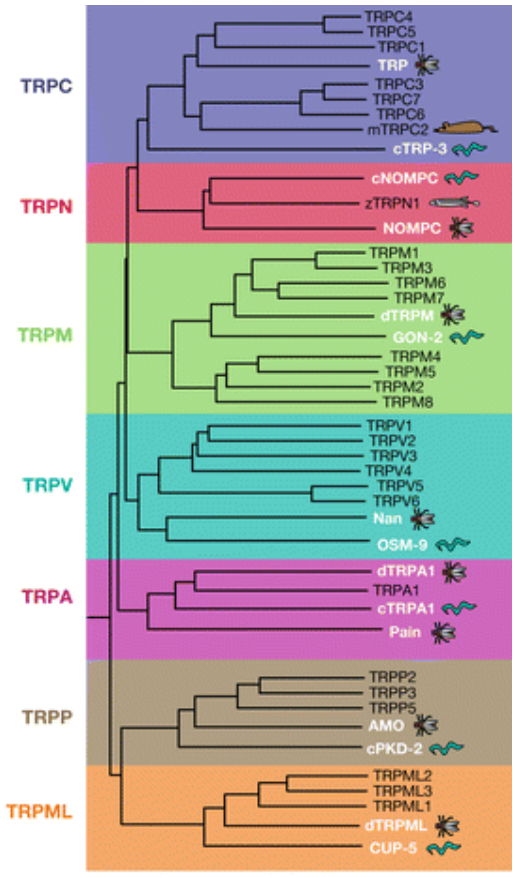
1994, 1995). In the present work we demonstrate the existence of another molecular mechanism for opioid withdrawal-induced hyperalgesia in peripheral sensory neurons.

1.2. Transient receptor potential ion channel family

One of the major classes of membrane proteins detecting noxious stimuli is the Transient Receptor Potential (TRP) ion channel family (Clapham 2003; Dhaka et al. 2006; Julius and Basbaum 2001). In the following chapter two members of the TRP channel family, TRPV1 and TRPA1, will be introduced.

All members of the TRP ion channel family share the common features of six transmembrane domains with diverse extents of sequence homology and permeability to cations. They play critical roles in response to all major classes of external stimuli, including light, sound, chemicals, temperature and touch. Some are also able to detect alterations of osmolarity. Furthermore, they can be considered as multiple signal integrators, due to their ability to modify responses of one signal by another (Venkatachalam and Montell 2007).

All currently known members of the TRP channel superfamily and their evolutionary relationships are presented in Figure. 1.1.



Venkatachalam K, Montell C. 2007.
Annu. Rev. Biochem. 76:387–417

Figure 1.1. Phylogenic tree showing the relatedness of TRP proteins. The dendrogram of vertebrate TRPs includes mostly human TRPs, except for mouse TRPC2 (cartoon of a mouse) and zebrafish TRPN1 (cartoon of a zebrafish). White text and cartoons highlight the TRP proteins from worms and flies. One C. elegans and one Drosophila member of each subfamily are included (modified from Venkatachalam (Venkatachalam and Montell 2007).

A common feature of all TRP channels is the formation of homo- or heterotetramers of four single subunits (Clapham 2003). Each subunit has six transmembrane (TM) domains with intracellular amino (N-) - and carboxy (C-) - terminal domains and a pore loop between domain five and six. Except for the TRPM subfamily, TRP channels contain multiple N-terminal ankyrin repeats, a 33- residue sequence motif which is known to mediate protein-protein interactions (Mosavi et al. 2004).

1.2.1. TRPV1

The first discovered temperature-sensitive ion channel was TRPV1, formerly known as VR1. TRPV1, initially detected in small to medium sized neurons in sensory ganglia, is also found in many other regions in the central nervous system (Mezey et al. 2000; Roberts et al. 2004) and in some non-neuronal tissues such as epidermal keratinocytes of human skin (Southall et al. 2003), gastric epithelial cells (Kato et al. 2003), and in epithelial cells of the urothelium and smooth muscle (Birder et al. 2001). Besides the rat TRPV1 cloned in 1997 by Caterina *et al*. (rVR1, GenBank AY445519), partially homologous human (AJ277028; homology 86 %), guinea pig (85%), rabbit (86%), chicken (65%) and pig (84%) TRPV1 sequences were later identified (Correll et al. 2004; Gavva et al. 2004; Hayes et al. 2000; Jordt and Julius 2002; Ohta et al. 2005; Phelps et al. 2005; Savidge et al. 2002).

TRPV1 is activated by numerous stimuli such as noxious heat (>43°C), capsaicin (pungent compound of hot chilli pepper) (Caterina et al. 2000; Caterina et al. 1997; Davis et al. 2000) and many other chemicals, including endocannabinoids (anandamide) (Zygmunt et al. 1999), camphor (Xu et al. 2005), and the pungent compounds present in black pepper (piperine) (McNamara et al. 2005), garlic (allicin) (Macpherson et al. 2005), ginger (gingerol) (Dedov et al. 2002) and clove oil (eugenol) (Yang et al. 2003). TRPV1-mediated cation influx initiated by the application of noxious chemicals or temperatures is further enhanced by low pH (Caterina et al. 1997). An acidic pH $\leq$ 5.9, which is characteristic for injured tissue, induces a shift in thermal threshold activation from > 43°C to 20°C (Montell 2005). In addition, TRPV1 is activated by venoms from cnidarians and spiders (Cuypers et al. 2006; Siemens et al. 2006).

Activation of TRPV1 results in an influx of mainly Ca^{2+} but also other cations like Na^{+}, K^{+} and Mg^{2+} can enter the cell through this channel. Its permeability for calcium ions is ten times higher than for sodium ions. The influx of cations provokes membrane depolarisation and subsequent release of inflammatory neuropeptides, most notably substance P (SP) and calcitonin gene related peptide (CGRP), which play a fundamental role in the development of neurogenic inflammation and generation of electrical impulses (Tominaga 2007).

In agreement with other TRP ion channels, TRPV1 has six TM domains and a short, pore-forming hydrophobic stretch between the fifth and sixth TM domains (Fig. 1.2.) (Caterina et al. 1997). Its N-terminus contains three ankyrin-repeat domains and its C-terminus has been proposed to serve as a determinant of subunit tetramerisation and to contribute to important aspects of channel function (Garcia-Sanz et al. 2007). The ankyrin repeat consists of a ~ 33-residue motif and binds many cytosolic proteins (Sedgwick and Smerdon 1999). Calmodulin, a calcium-binding protein, binds to the first ankyrin repeat domain of TRPV1 (Rosenbaum et al. 2004). TRPV1 is proposed to have a tetrameric structure with homotetramers as a predominant form (Kedei et al. 2001), although a heterooligomerisation with TRPV3, which is coexpressed with TRPV1 in DRG neurons, was observed in heterologous expression systems using co-immunoprecipitation (see chapter 3.2.6.).

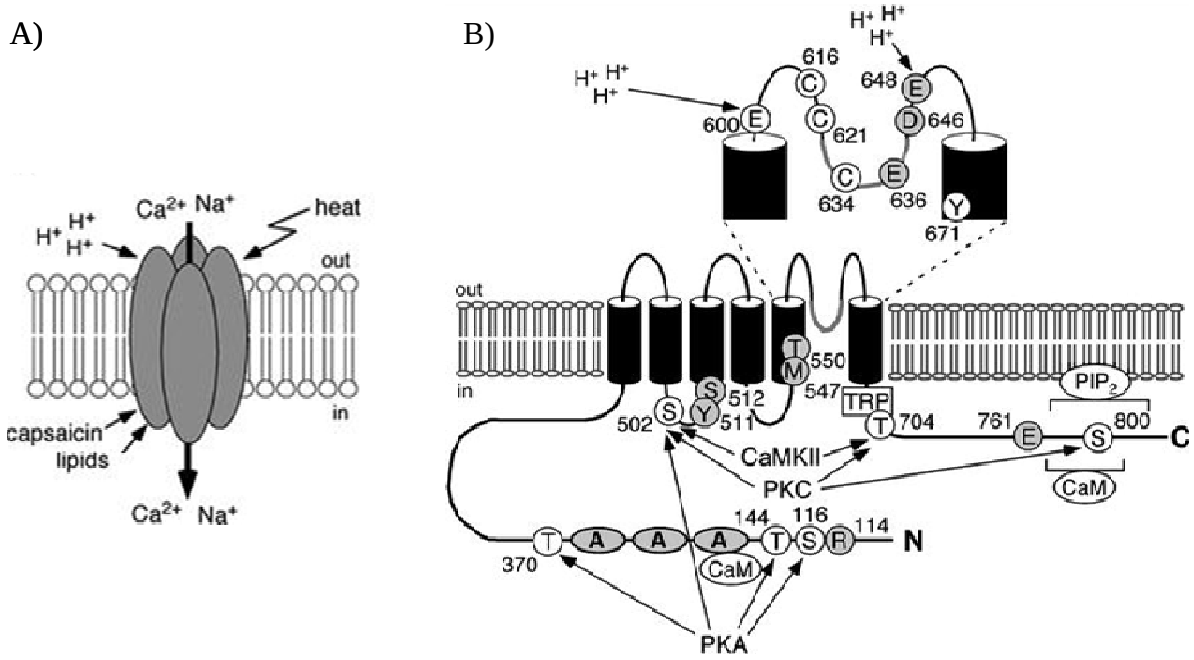


Fig. 1.2. A) Proposed tetrameric structure of TRPV1 in the plasma membrane. B) Regions and amino acids involved in TRPV1 function. Residues reported to be involved in vanilloid binding are presented in grey. "TRP" in a box indicates a TRP domain (tetramerization and transduction domain that stabilizes channel subunits and couples stimuli sensing to channel gating). Phosphatidylinositol 4,5-

bisphosphate (PIP_2) binds to the indicated domain in the C-terminus. CaM binds to both C- and N-termini. "A" indicates an ankyrin repeat. PKA, PKC or CaM kinase II can phosphorylate serine (S) or threonine (T) residues indicated by arrows. Protons act on the two glutamine acids (E) in the extracellular loop indicated by arrows. Modified from Tominaga (Tominaga and Tominaga 2005).

Capsaicin and heat evoking TRPV1 activity is potentiated in the presence of extracellular protons, in part by lowering the threshold for channel activation. Extracellular protons can also be viewed as agonists themselves because acidification down to pH < 6.0 leads to the opening of the channel at ambient temperature, suggesting an action of protons primarily by increasing the probability of channel opening (Baumann and Martenson 2000; Tominaga et al. 1998). Mutation studies showed that the glutamine residue at position 600 (Glu 600, located in a putative extracellular domain) and the glutamine residue at position 648 (Glu 648) are important for proton binding. Whereas Glu 600 functions as a regulator site for proton potentiation, Glu 648 is involved in direct proton-induced TRPV1 activation (Jordt et al. 2000). Moreover, protons permeate the non-selective channel pore in the presence of acidic extracellular milieu, leading in substantial intracellular acidification (Hellwig et al. 2004).

1.2.2. Sensitization of TRPV1

Injury increases pain sensation by elevating the sensitivity of nociceptors to thermal and mechanical stimuli. This phenomenon results partly from the production and release of chemical mediators from sensory nerve terminals and from non-neuronal cells in the tissue. TRPV1 apparently plays a fundamental role in the sensitization of nociceptors and in the development of thermal hyperalgesia since TRPV1-deficient mice lack both phenomena (Caterina et al. 2000; Davis et al. 2000). This is due to sensitization of TRPV1 by inflammatory mediators and upregulation of TRPV1 protein expression. The number of TRPV1 positive cells in small to medium sized DRG neurons increases after induction of paw inflammation by Complete Freund´s Adjuvant (CFA) and/or treatment with inflammatory mediators like nerve growth factor (NGF) (Amaya et al. 2004; Breese et al. 2005; Ji et al. 2002). Inflammatory mediators can also alter neuronal excitability directly by interacting with TRPV1 (e.g. protons, adenosine triphosphate, lipids) and indirectly by binding to metabotropic receptors (e. g. NGF and bradykinin) (Woolf and Salter 2000).

One of the main responses to injury is tissue acidosis. The degree of associated pain is well correlated with the magnitude of acidification (Reeh and Steen 1996). Protons activate the TRPV1 channel when the extracellular pH drops below 6, and enhance responses to capsaicin

and heat (pH 6-8), resulting in an increase of nociceptor excitability even at normal body temperature (Jordt et al. 2000; Welch et al. 2000).

In addition, several bioactive peptides are produced and released from non-neuronal cells or derived from plasma proteins at the site of injury. Bradykinin, a nonapeptide, induces immediate membrane depolarization as well as sensitization to other noxious stimuli when applied to nociceptors (Burgess et al. 1989). Bradykinin binds to G-protein-coupled receptors (bradykinin receptor 2; BK_2) to stimulate phospholipase C (PLC)-catalyzed hydrolysis of phospho-inositol phosphate 2 (PIP_2) into inositol-phosphate 3 (IP_3) and diacylglycerol (DAG). IP_3 and DAG activate protein kinase C (PKC), which directly phosphorylates TRPV1 preferentially at the serine residues 502 and 800 (Ser502 and Ser800) (Bhave et al. 2003; Numazaki et al. 2002). Both residues are also involved in re-phosphorylation of TRPV1 after calcium-dependent desensitization (Mandadi et al. 2004). Other inflammatory mediators like adenosine triphosphate (ATP), prostaglandins, trypsin or tryptase also activate G_q-coupled receptors followed by a downstream activation of PKC (Dai et al. 2004; Moriyama et al. 2005; Moriyama et al. 2003; Sugiura et al. 2002; Tominaga et al. 2001). Phosphorylation of TRPV1 by PKC results in a potentiation of capsaicin- or proton-evoked responses and in a reduction of the thermal threshold for TRPV1 activation at body temperature. Activation of proteinase-activated (PAR2)-receptors also leads to a PKC-mediated sensitization of TRPV1 (Dai et al. 2004).

Prostaglandins may also modulate capsaicin- or heat-sensitivity of TRPV1 by activating the protein kinase A (PKA)-pathway. Serine at position 116 (Ser 116) and threonine at position 370 (Thr 370) are reportedly phosphorylated by PKA. Phosphorylation of Ser 116 inhibits dephosphorylation of TRPV1 caused by capsaicin. Phosphorylation of Thr 370, Thr 144 and Ser 502 are thought to be involved in heat-evoked TRPV1 responses (Bhave et al. 2002; Mohapatra and Nau 2003; Rathee et al. 2002).

Besides PKC and PKA, TRPV1 is phosphorylated and sensitized by the Ca^{2+}/CaM-dependent kinase II (CaMKII), the tyrosine kinase Src, and the cyclin-dependent kinase 5 (CdK5) (Bhave et al. 2002; Jung et al. 2004; Lee et al. 2005; Mohapatra and Nau 2003; Numazaki et al. 2002; Olah et al. 2002; Pareek et al. 2007; Premkumar and Ahern 2000).

The TRPV1 channel is also activated by the membrane-derived lipids anandamide, oleoylethanolamide (OEA) and some lipoxygenase products (Ahern 2003; Hwang et al. 2000; Zygmunt et al. 1999). Another important lipid is PIP_2, which possibly interacts in an inhibitory way with amino acids 777-820. PLC-induced PIP_2 hydrolysis into DAG and IP_3 results in TRPV1 activation (Chuang et al. 2001). However, other studies have shown an

activation of TRPV1 by PIP_2 in excised patches (Stein et al. 2006), leading to a controversial discussion (Rohacs et al. 2008).

PKA activation is not solely a downstream effect of inflammatory mediators, but can also result from an increase of intracellular cAMP, caused by a compensatory upregulation of adenylylcyclase (AC) activity during withdrawal of chronically applied opioids (Levine and Taiwo 1989; Sharma et al. 1975). In this context, the first part of the thesis investigates the sensitization of TRPV1 during opioid withdrawal. Opioid mediated effects during acute application and after withdrawal of chronic application and their underlying molecular mechanisms will be introduced in more detail in chapter 1.3.

1.2.3. TRPA1

TRPA1, formerly known as ANKTM1 or p120 was first isolated in 1999 by Jaquemar *et al*. in a screen for transformation-sensitive proteins in cultured fibroblasts (Jaquemar et al. 1999). TRPA1 is homologous in sequence to other proteins belonging to the TRP channel family and was identified as a novel thermo TRP in 2003 (Story et al. 2003). Mouse TRPA1 has fourteen predicted N-terminal ankyrin domains followed by six TM domains (see Figure 1.4.) and is probably the sole mammalian member of a distant subfamily of TRP channels. After extensive analysis concerning the expression and function of TRPA1, it was proposed to be a candidate receptor for noxious cold temperature (Caspani and Heppenstall 2009).

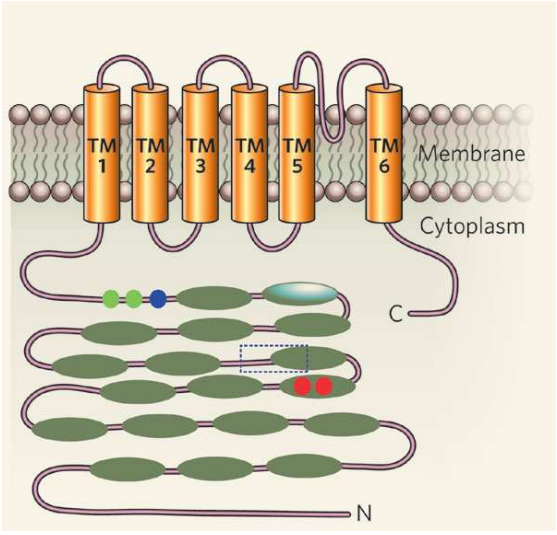


Fig. 1.4. Structure of one TRPA1 subunit, modified from Caterina (Caterina 2007).

TRPA1 is expressed in non-myelinated C-or lightly myelinated Aδ-fibres of DRG neurons that sense temperature and/or noxious stimuli. Similar to TRPV1 it is expressed in CGRP- and SP-positive neurons (peptidergic nociceptors), but rarely co-localized with the cool and

menthol activated channel TRPM8 (Peier et al. 2002). TRPA1 is also found in non-peptidergic nociceptors, which can be labelled with isolectin B4 (IB4) (Bautista et al. 2006; Kobayashi et al. 2005; Linte et al. 2007; Story et al. 2003). Ninety-seven percent of TRPA1 positive neurons also express TRPV1 and 30 % of TRPV1-positive neurons express TRPA1. Moreover, TRPA1 is expressed in murine sympathetic neurons from the superior cervical ganglion (Smith et al. 2004), in nodose ganglia (Nagata et al. 2005) and in hair cells of the inner ear and the vestibular system (Corey et al. 2004). Recently, the channel was found in human motor neurons of the spinal cord, in ventral roots and basal keratinocytes (Anand et al. 2008), in neurons throughout the bladder together with TRPV1 (Du et al. 2008; Streng et al. 2008), in rat geniculate ganglia (Katsura et al. 2006), in vagal nerve afferents innervating the airways (Nassenstein et al. 2008) and in the spinal cord (Andrade et al. 2008). Non-neuronal TRPA1 protein expression was demonstrated in human joint cells (synoviocytes) (Kochukov et al. 2006), in the intestine, heart, lung, skeletal muscles and pancreas (Stokes et al. 2006).

Evidence for TRPA1 as a cold sensor came from electrophysiological experiments and microfluorimetry of TRPA1 transfected Chinese hamster ovary (CHO) cells, where temperatures below 17°C or the presence of icilin (cooling agent) significantly increase TRPA1 activity (Story et al. 2003).
This was discussed controversially by other groups, showing a failure of TRPA1 activation by cold (Jordt et al. 2004) and no correlation between mustard oil responses and cool sensitivity in somatosensory neurons (Babes et al. 2004; Jordt et al. 2004; Bautista et al. 2006). Even the generation of TRPA1 null mice by two independent laboratories did not resolve this controversy (Bautista et al. 2006; Kwan et al. 2006). Our laboratory showed that cold-induced activation of TRPA1 in expression systems is an indirect effect, caused by cold-induced calcium release from intracellular stores and subsequent calcium-dependent activation of the channel (Zurborg 2007). Karashima *et al*. demonstrated a calcium-independent and calcium store-independent activation of heterologously expressed TRPA1 by cold. Moreover, they identified a subset of cold-sensitive trigeminal ganglion (TG) neurons relying on TRPA1 for their cold response and gave behavioural evidence that TRPA1 is required for the normal nociceptive response to noxious cold (Karashima et al. 2009).

TRPA1 can be chemically activated by many exogenous and endogenous substances, environmental irritants and pungent compounds. These include constituents of wasabi, horseradish, mustard oil (isothiocyanates), garlic (allicin), cinnamon oil (cinnamaldehyde),

marijuana (tetrahydrocannabinol), ginger and clove oil (eugenol). Such pungent compounds are all electrophils that activate TRPA1 through covalent modification of reactive amino acids such as cysteins (Hinman et al. 2006; Macpherson et al. 2007a). Other TRPA1 activators include components of tear gas, cigarette smoke and industrial pollutants (acrolein), formaldehyde (the most commonly used substance to assay chemical nociception in rodents), acetaldehyde (an intermediate substrate of ethanol metabolism) and 4-hydroxynonenal (4-HNE) (Bandell et al. 2004; Bang et al. 2007; Bautista et al. 2006; Jordt et al. 2004; Macpherson et al. 2005; Macpherson et al. 2007b; McNamara et al. 2007; Trevisani et al. 2007). The endogenous 4-HNE and 15-deoxy prostaglandin J2 (PGJ2) both produced through lipid peroxidation or spontaneous dehydration, may be responsible for the pathological effects of oxidative stress (Andersson et al. 2008; Cruz-Orengo et al. 2008; Materazzi et al. 2008; Trevisani et al. 2007). Reactive oxygen and nitrogen species (nitrooleic acid, hydrogen peroxide and hydrogen sulphide) as well as bradykinin have also been demonstrated to activate TRPA1 (Basbaum et al. 2009; Sawada et al. 2008; Takahashi et al. 2008; Yoshida et al. 2006; Bandell et al. 2004).TRPA1 null mice showed substantially decreased responses to bradykinin at the cellular and behavioural level (Bautista et al. 2006; Kwan et al. 2006).

TRPA1 agonists are usually structurally unrelated. Some of the substances activate TRPA1 via the classical ‘lock-and-key’ principle. However, this model has been challenged by two recent publications (Cebi and Koert 2007). They showed that the activation of the channel by some agonists such as allylisothiocyanate results in covalent modifications of TRPA1 (Hinman et al. 2006; Macpherson et al. 2007a). The group of David Julius demonstrated that structurally distinct electrophiles (e.g. isothiocyanates, N-methyl-maleidmide) are strong activators of human TRPA1. The nucleophilic counterparts of these electrophiles are cysteine residues. Site-directed mutagenesis studies showed that three cysteines and a lysine residue within the N-terminus (Cys619, Cys639, Cys663, and Lys708) are critical for the activation of TRPA1 (Hinman et al. 2006). These amino acids are directly modified by the electrophilic agonists and the activation does not depend on structure but on reactivity. Another group also identified three cysteines in the N-terminus of mouse TRPA1 (Cys415, Cys422, Cys622) as targets for electrophilic agonists such as isothiocyanates, cinnamaldehyde types, and iodoacetamides (Macpherson et al. 2007a). Many electrophilic reactions are irreversible and the mechanisms of channel inactivation are not clearly solved. However, mutational analysis revealed that structurally unrelated TRPA1 agonists, such as 2-aminophenyl borane (2-APB) and δ-9-tetrahydrocannabinol (THC), are able to activate the channel by an independent biochemical pathway (Hinman et al. 2006). Intracellular free polyphosphates may also play a

crucial role by keeping TRPA1 in the needed conformation for channel gating by pungent chemicals such as AITC and allicin (Kim and Cavanaugh 2007).

Besides the activation by pungent compounds and, possibly, cold temperatures, the multiple ankyrin repeats of TRPA1 may form a gating spring capable of transducing mechanical force and thereby facilitating channel opening (Corey et al. 2004; Howard and Bechstedt 2004). Hill *et al*. detected mammalian TRPA1 activation by membrane crenations in heterologous expression systems (Hill and Schaefer 2007) and a worm ortholog of TRPA was sensitive to mechanical pressure applied via a suction pipette (Kindt et al. 2007). However, TRPA1 deficient mice display only weak deficits in mechanosensory behaviour and results remain inconsistent (Bautista et al. 2006; Kwan et al. 2006; Petrus et al. 2007).

Approximately 30-50 % TRPV1 expressing small to medium sized sensory neurons co-express TRPA1 and almost all TRPA1 positive neurons also express TRPV1. Moreover, currents induced by mustard oil- and WIN55,212 (TRPA1 agonists) were almost exclusively detected in TRPV1 positive cells, suggesting an interaction between TRPV1 and TRPA1. Such interactions may lead to potentiated nociceptor excitability and pain sensation. Therefore, I investigated the modulation of TRPV1 activity through TRPA1 stimulation.

1.3. Opioids

Opioids are the most powerful drugs for treatment of severe pain (Zollner and Stein 2007). The alkaloid morphine was isolated by Friedrich Wilhelm A. Sertürner two hundred years ago from the opium poppy plant *Papaver somniferum* (Papaveraceae). Opium use was first documented 4000 b.C. by the Sumerians. The latex (raw opium) was extracted by incision of the opium poppy capsule. Besides morphine, other analgesic alkaloids like codeine, noscapine, papaverine and thebaine are ingredients of the opium poppy latex (Friderichs and Strassburger 2002). Opioids mediate their analgesic action through G-protein coupled opioid receptors. The opioid receptors are divided into three main groups, the μ-, δ- and κ-opioid receptors, based on results of early binding studies and bioassays (Lord et al. 1977; Martin et al. 1976; Pert and Snyder 1973; Stein and Zollner 2009). The existence and classification of the three opioid receptor types was confirmed by cloning. The δ-opioid receptor was cloned simultaneously by two independent research groups in 1992 (Evans et al. 1992; Kieffer et al. 1992). Later on, the μ- and κ-opioid receptors were cloned (Meng et al. 1993; Wang et al. 1993).

According to their ability to activate opioid receptors, opioids are classified as agonists, partial agonists, and antagonists. The reversible binding of an agonist causes a conformational change of the receptor followed by an activation of G-proteins and intracellular signalling pathways. They eventually provoke a measurable biological response (e.g. analgesia) (Hulme et al. 1999). Synthetic fentanyl is one of the most potent opioid agonists with a high affinity to μ-opioid receptors (MOR). Another synthetic highly potent MOR agonist is the peptide D-Ala2-N-MePhe4-Gly5-ol-enkephalin (DAMGO), which is widely used in pharmacological research. Buprenorphine acts as a partial MOR agonist but as an antagonist at κ-opioid receptors (KOR). Other agonist-antagonists have different affinities at the three receptor types (Huang et al. 2001). Antagonists do not induce a biological response after reversible binding to receptors. The most common opioid receptor antagonists are naloxone and naltrexone, both binding to three opioid receptors but with a preference to MOR.

Besides the numerous exogenous agonists, endogenous opioid receptor agonists have also been identified. The first endogenous opioid peptides were Met-and Leu- enkephalin (Hughes et al. 1975), followed by β-endorphin and dynorphin, which were discovered in the late seventies (Goldstein et al. 1979; Li and Chung 1976; Li et al. 1976). β-Endorphin consists of 31 amino acids and is processed from the precursor protein proopiomelanocortin (POMC). It is an agonist at MOR and DOR. Endomorphin 1 (Tyr-Pro-Trp-Phe-NH2) and 2 (Tyr-Pro-Phe-Phe-NH2), discovered in the late nineties, are selective agonists of MOR (Zadina et al. 1997). Prodynorphin and proenkephalin are precursors of dynorphin (KOR agonist) and Met-and Leu-enkephalin (DOR agonists), respectively. Endogenous opioid peptides are predominantly expressed in the brain, in the dorsal horn of the spinal cord and in immune cells (Endres-Becker 2007).

1.3.1. μ-opioid receptor

μ-opioid receptors (MOR) are expressed in the central (cortex, thalamus, hypothalamus, limbic system, brainstem) and peripheral nervous system, by neuroendocrine (pituitary, adrenals), immune, and ectodermal cells (Duncan 1999; Stein and Zollner 2009). In the periphery, they are synthesized in cell bodies of DRG neurons and intra-axonally transported via microtubules to central and peripheral terminals (Stein et al. 2003). Moreover, they occur in high concentrations in the gastrointestinal tract and in the urinary bladder, where they mediate a reduction in intestinal motility and micturition.

Opioid receptors are G-protein coupled receptors (GPCR) which are classified into four groups concerning their interacting G-proteins: G_s ("stimulatory"), $G_{i/o}$ ("inhibitory"/"other"), $G_{12/13}$ and $G_{q/11}$. Heterotrimeric G-proteins consist of an α-subunit (G_α), which binds GDP/GTP, a β-subunit and a γ-subunit, which form a non-dissociable complex ($G_{\beta\gamma}$). The activation of an opioid receptor by an agonist induces conformational changes, allowing intracellular coupling of mainly $G_{i/o}$ proteins to the C-terminus (Stein and Zollner 2009). Thereby the receptor functions as a guanine nucleotide exchange factor (GEF) that exchanges guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G_α-subunit. This is followed by the dissociation of the $G_{\alpha,}$ (binding GTP) from the $G_{\beta\gamma}$ dimer and the receptor. G_α and $G_{\beta\gamma}$ can then activate different signalling cascades and effector proteins. The $G_{\beta\gamma}$-subunit directly interacts with voltage dependent ion channels (Clapham and Neer 1997). $G_{\beta\gamma}$ presynaptically suppresses the activity of N- ("neuron"), P/Q- ("purkinje cell") and R-type ("remaining") calcium channels, resulting in an inhibition of the generation and transmission of electrical stimuli in nociceptive peripheral and/or central nervous system (CNS) neurons (Akins and McCleskey 1993; Borgland et al. 2001; Irnaten et al. 2003; Schroeder and McCleskey 1993).

Moreover, $G_{\beta\gamma}$ inhibits purinergic P_2X receptors and tetrodotoxin (TTX) resistant sodium channels, which are mainly expressed on nociceptors and important in nociception. Postsynaptically, $G_{\beta\gamma}$-subunits activate voltage-dependent and G-protein-gated inwardly rectifying (GIRK) potassium channels in the CNS, causing postsynaptic hyperpolarisation (North et al. 1987; Torrecilla et al. 2002) and thereby preventing generation and/or propagation of action potentials (Zollner and Stein 2007). The extracellular signal-regulated kinase (ERK)- and mitogen-activated protein kinase (MAPK)-system is also activated by $G_{\beta\gamma}$ (Belcheva et al. 1998; Li and Chang 1996). In addition, the release of proinflammatory and pronociceptive neuropeptides (e.g. substance P; SP) from central and peripheral terminals of sensory neurons is inhibited (Kondo et al. 2005; Yaksh 1988).

Through $G_{\alpha i}$ opioids cause a reduction in adenylylcyclase (AC) activity. ACs are lyase enzymes that catalyze the conversion of adenosine-5'-triphosphate (ATP) to 3', 5'-cyclic adenosine-monophosphate (cAMP) (Law et al. 2000). The second messenger cAMP regulates other proteins like cAMP-dependent protein kinase A (PKA) or cyclic-nucleotide gated ion channels. PKA consists of two regulatory subunits (R) that bind to two catalytic subunits (C). Each regulatory subunit possesses two binding pockets for cAMP-molecules. If cAMP-molecules bind to the regulatory subunit, the R-C complex dissociates and the catalytic subunit is released, now able to transfer phospho-groups to the amino acids serine and

threonine (serine/threonine-kinase). The guiding of PKA to the target protein is accomplished by A-kinase anchoring proteins (AKAP) (Dell'Acqua and Scott 1997). Phosphorylation of proteins is an important control mechanism in signal transduction and in the regulation of enzyme- or transcription factor-activity. PKA is involved in a wide range of processes such as transcription, metabolism, cell cycle progression and apoptosis (Gjertsen and Doskeland 1995; Hubbard and Cohen 1993; Huggenvik et al. 1991; Matten et al. 1994; Smith et al. 1993). One target of phosphorylation by PKA in the nucleus is the cAMP Response Element Binding Protein (CREB), which increases transcription in its phosphorylated state. In the current thesis I investigated whether TRPV1, whose phosphorylation by PKA can lead to channel sensitization/resensitization can occur during opioid withdrawal. Previous studies from our laboratory have shown that opioids inhibit the activity of TRPV1 in a naloxone- and pertussis toxin (PTX)-sensitive manner via the cAMP/PKA pathway (Endres-Becker 2007). Coexpression of TRPV1 and MOR in small to medium diameter-sized neurons was shown, and the application of morphine or DAMGO significantly decreased capsaicin-induced TRPV1 currents in whole cell patch clamp experiments. These effects were reversed by naloxone, PTX, forskolin (FSK) and the stable cAMP-analogon 8-Br-cAMP. Washout experiments revealed that additional capsaicin applications progressively increased TRPV1 activity after removal of morphine (Endres-Becker et al. 2007).

1.3.2. Opioid withdrawal-induced hyperalgesia

Withdrawal of opioids can result in hyperalgesia (Drdla et al. 2009), which has been well documented both in animal studies (Mao et al. 1995; Nestler and Aghajanian 1997) and clinical reports (Angst et al. 2003; Compton et al. 2003; Doverty et al. 2001). Despite intensive work, the neurobiological mechanisms of opioid withdrawal induced hyperalgesia (OWIH) are not fully clarified. Early *in vitro* studies in cell lines showed that continuous ("chronic") morphine administration induces a compensatory increase in AC activity and intracellular cAMP concentrations (Brandt et al. 1976; Sharma et al. 1975). Increased cAMP leads to activation of cAMP-dependent PKA (Avidor-Reiss et al. 1997; Bie et al. 2005). In addition, the expression of the catalytic subunit of PKA is upregulated during opioid withdrawal (Lane-Ladd et al. 1997). PKA in turn phosphorylates and thereby sensitizes receptor proteins. *In vitro* studies identified cAMP-mediated increased synaptic transmission and augmented hyperpolarization-activated currents in central neurons (Williams et al. 2001). Firing rates of locus coeruleus (LC) neurons were reduced by acutely applied opioids, whereas normal levels were reached during prolonged opioid administration. Withdrawal of

opioid agonists or application of opioid antagonists resulted in higher firing rates due to cAMP-upregulation (Kogan et al. 1992). In addition, Drdla *et al.* showed a "long-term potentiation" (LTP) of synaptic strength in spinal cord pain pathways after abrupt withdrawal of opioids. Under physiological conditions LTP is a mechanism for learning and memory in the brain (Drdla et al. 2009).

Opioid withdrawal can also induce an upregulation of transcription factors, particularly CREB (cAMP response element binding protein) and ΔFosB (Nestler 2004), which are responsible for enhanced expression of neuropeptides, neurotransmitter synthesizing enzymes, neurotransmitter receptors, signalling proteins, and other transcription factors leading to increased neuronal excitability (Lonze and Ginty 2002; Mayr and Montminy 2001). Continuously applied opioids and their withdrawal also influence MAPK-/ERK signal transduction (Asensio et al. 2006; Ferrer-Alcon et al. 2004). While ERK1-/ERK2-activity is reduced during chronic application of opioids (Muller and Unterwald 2004), it is dramatically increased during opioid withdrawal (Schulz and Hollt 1998). On the spinal level opioid withdrawal causes an activation of calcium-dependent PKC, inducing phosphorylation and sensitization of spinal NMDA receptors (Mao et al. 1994). Furthermore, dynorphins, which activate pronociceptive signalling cascades, are released (Vanderah et al. 2000). In the periphery adrenergic and adenosine receptors are phosphorylated and sensitized by kinases (Aley et al. 1995; Aley and Levine 1997c).

Numerous animal and clinical studies have described hyperalgesia to mechanical and thermal stimuli during opioid withdrawal (Angst and Clark 2006). These stimuli were applied peripherally and are known to activate TRPV1 which plays a fundamental role in the development of inflammatory hyperalgesia. Therefore, I hypothesized that TRPV1 participates in the development of hyperalgesia during opioid withdrawal.

2. Objectives

My overall hypothesis is that TRPV1 can be sensitized by interactions both with inhibitory opioid receptors and with excitatory TRP channels.

Until now, opioid withdrawal-induced hypersensitivity, often associated with thermal hyperalgesia, was mostly explained by enhanced neuronal excitability via activation of NMDA receptors at the central/spinal level. Since TRPV1 plays a fundamental role in thermal hyperalgesia, the current work investigates the role of peripheral sensory neurons and TRPV1 in opioid withdrawal-induced hyperalgesia.

Hypothesis 1: TRPV1 sensitization underlies opioid withdrawal-induced hyperalgesia. This sensitization is mediated via PKA and phosphorylation at specific TRPV1 phosphorylation sites.

TRPV1 and TRPA1 are co-expressed in sensory neurons, they can be activated by similar chemical compounds and are involved in increased pain sensitivity during inflammation. In the second part of the thesis I investigated signalling pathways and direct protein-protein interactions between TRPV1 and TRPA1.

Hypothesis 2: TRPV1 is sensitized by interaction with TRPA1 via PKA signalling pathways.

3. Animals, material and methods

3.1. Materials

3.1.1. Cell lines and bacteria

Escherichia coli (E.coli) DH5α	Invitrogen, Karlsruhe, Deutschland
HEK 293 (human embryonic kidney cells)	German collection of microorganisms and cell cultures (DSMZ), Braunschweig, Deutschland
HEK 293 Tet - On	Kind gift of Prof. Paul Heppenstall

3.1.2. Animals and animal housing

Male Wistar rats (140-200 g) were individually housed, maintained in a 12 h light/dark cycle with a temperature controlled environment, and given food ad libitum. The animal protocol was approved by the state animal care and use committee and the guidelines on ethical standards for investigations of experimental pain in animals were followed (Zimmermann 1983).

3.1.3. Chemicals

Rotiphorese 40 (Acryl amide)	Carl Roth, Karlsruhe, GER
Ammonium persulfate (APS)	Sigma-Aldrich, Steinheim, GER
BAPTA-AM	Sigma-Aldrich, Steinheim, GER
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, GER
Bromphenolblue	Sigma-Aldrich, Steinheim, GER
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Steinheim, GER
Capsaicin	Sigma-Aldrich, Steinheim, GER
Complete-Mini	Roche Diagnostics, Mannheim, GER
Deoxycholat (Doc)	Sigma-Aldrich, Steinheim, GER
Dithiothreitol (DTT)	Roche Diagnostics, Mannheim, GER
Doxycycline	Sigma-Aldrich, Steinheim, GER
Ethanol	Mallinckrodt Baker, Deventer, NL
Ethylendiamine-tetraacetat (EDTA)	Sigma-Aldrich, Steinheim, GER

Ethylenglycol-bis-(2-aminoethylethyl)-tetraacetic acid (EGTA)	Sigma-Aldrich, Steinheim, GER
Forskolin	Sigma-Aldrich, Steinheim, GER
Fura-2/AM	Invitrogen, Karlsruhe, GER
G 418 Disulfat	Sigma-Aldrich, Steinheim, GER
Glucose	Sigma-Aldrich, Steinheim, GER
Glycerine	Sigma-Aldrich, Steinheim, GER
Glycine	Sigma-Aldrich, Steinheim, GER
α-1-Glycoprotein	Sigma-Aldrich, Steinheim, GER
H-89	Sigma-Aldrich, Steinheim, GER
HEPES	Sigma-Aldrich, Steinheim, GER
Hydrochloric acid (HCL)	Sigma-Aldrich, Steinheim, GER
3-Isobutyl-1-methylxanthin (IBMX)	Sigma-Aldrich, Steinheim, GER
Isopropanol	Sigma-Aldrich, Steinheim, GER
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Steinheim, GER
β-2-Mercaptoethanol	Sigma-Aldrich, Steinheim, GER
Methanol	Mallinckrodt Baker, Deventer, NL
Milk	Carl Roth, Karlsruhe, GER
Phosphatase inhibitor cocktail	Sigma-Aldrich, Steinheim, GER
Phospho-Stop	Roche Diagnostics, Mannheim, GER
Pluronic F-127	Invitrogen, Karlsruhe, GER
Polyethylenimmine (PEI)	Sigma-Aldrich, Steinheim, GER
Poly-L-lysine	Sigma Aldrich, Steinheim, GER
Polyoxyethylenesorbitan monolaurat (Tween® 20)	Sigma Aldrich, Steinheim, GER
Potassium chloride (KCl)	Sigma-Aldrich, Steinheim, GER
Potassium hydroxide (KOH)	Sigma-Aldrich, Steinheim, GER
Protein G-agarose	Roche Diagnostics, Mannheim, GER
Resiniferatoxin (RTX)	Sigma-Aldrich, Steinheim, GER
[3H]-Resiniferatoxin ([3H]-RTX)	Perkin Elmer LAS, Rodgau-Jugesheim, GER
Saccharose	Carl Roth, Karlsruhe, GER
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, GER

Sodium deoxycholat monohydrate	Sigma-Aldrich, Steinheim, GER
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Steinheim, GER
Sodium hydroxide (NaOH)	Sigma-Aldrich, Steinheim, GER
Sodium orthovanadate (Na3VO4)	Sigma-Aldrich, Steinheim, GER
SQ 22,536	Sigma-Aldrich, Steinheim, GER
TEMED	Carl Roth, Karlsruhe, GER
Tris-(hydroxymethyl)-amino methane (TRIS)	Roche Diagnostics, Mannheim, GER
Triton X 100	Sigma-Aldrich, Steinheim, GER
Trizma® Pre-set crystals	Roche Diagnostics, Mannheim, GER

3.1.4. Media, buffer

Dimethyl sulphoxide (DMSO)	Merck, Darmstadt, GER
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Steinheim, GER
Foetal bovine serum (FBS)	Biochrom, Berlin, GER
Foetal bovine serum Tet-On (FBS Tet-On)	Clontech Laboratories, Mountain View, USA
L-glutamine	GIBCO Invitrogen, Paisley, GB
Horse serum	Biochrom AG, Berlin, GER
LB agar	Invitrogen, Karlsruhe, GER
LB medium	Invitrogen, Karlsruhe, GER
Minimal essential medium (MEM) alpha medium	GIBCO Invitrogen, Paisley, GB
MEM Earle's medium	Biochrom, Berlin, GER
Penicillin (10000U)/Streptomycin (10000µg/ml)	Biochrom, Berlin, GER
Phosphate buffered saline (PBS); 0,1 M	Biochrom, Berlin, GER
Trypsin (0,05 %) / EDTA (0,02 %) in PBS	Biochrom, Berlin, GER

Calcium Imaging Buffer (CIB)	2 mM $CaCl_{2;}$ 10 mM Glucose; 20 mM HEPES; 5 mM KCl; 140 mM NaCl; adjusted at pH 7,4 with NaOH

Extracellular solution (ECS)	2 mM $CaCl_2$; 10 mM Glucose; 10 mM HEPES; 5 mM KCl; 2 mM $MgCl_2$; 140 mM NaCl; adjusted at pH 7,4 with NaOH
ECS calcium free	10 mM Glucose; 10 mM HEPES; 5 mM KCl; 2 mM $MgCl_2$; 140 mM NaCl; adjusted at pH 7,4 with NaOH
Fura-2/AM solution	50 µg Fura-2/AM diluted in 10 µl Pluronic solution and 50 µl DMSO
Intra cellular solution (ICS)	5 mM EGTA; 10 mM HEPES; 140 mM KCl; 2 mM $MgCl_2$; adjusted at pH 7,4 with KOH
Pluronic solution	20 % Pluronic F-127 in DMSO
2 X SDS sample buffer	126 mM TRIS-HCl; 4 % SDS; 20 % Glycerin; 0,02 % Bromphenolblau; 10 % β-2-Mercaptoethanol
SDS running buffer	192 mM Glycin; 25 mM TRIS; 0,1% SDS, pH 8,3 - 8,8
Lysis buffer (Ripa)	1 mM EDTA; 150 mM NaCl; 0,5 % Natrium-Deoxycholat; 0,1% SDS; 50 mM TRIS; 0,5% Triton X 100; 1:1000 DTT, 1:10 Phospho-Stop; 1:7 Complete-Mini
TBS	7,7 mM TRIS-HCl; 150 mM NaCl, pH 7,6
TBS-Tween	TBS Puffer, 0,01% Tween 20
Blotting buffer	25 mM TRIS; 192 mM Glycin; 20% Methanol
Binding buffer	Trizma® Pre-set crystals; BSA 0,25 mg/ml

3.1.5. Reaction systems

Bio-Rad protein assay	Bio-Rad Laboratories, München, GER
cAMP Biotrak Enzymimmunoassay (EIA) system	GE Healthcare, Buckinghamshire, GB

Enhanced chemiluminescent and chemifluorescent labelling reagents (ECL)	GE Healthcare, Buckinghamshire, GB
Developing and fixing solution for Western Blot	Adefo Chemie, Dützenbach, GER
Fugene® 6	Roche Diagnostics, Mannheim, GER
Qiafilter™Plasmid Maxi Kit	Qiagen, Hilden, GER
Precision Plus Protein Standard	Bio-Rad Laboratories, Munich, GER

3.1.6. Expendable materials

Borosilicate glass capillaries	Hilgenberg, Malsfeld, GER
Cell culture flasks (75 cm^2 growth area)	BD Bioscience, Palo Alto, USA
Cell culture plates (9,2 cm^2 growth area)	TPP®, Trasadingen, Schweiz
Cell culture plates (60,1 cm^2 growth area)	TPP®, Trasadingen, Schweiz
Cell scraper	TPP®, Trasadingen, Schweiz
GF/B glasfiber filter	Whatman, Brentford, GB
QIA Shredder	Qiagen, Hilden, GER
Silver wire (0,25 mm)	World Precision Instruments, Saratosa, USA

3.1.7. Technical equipment

Microscope Eclipse TE 2000-S	Nikon, Japan
Objective S Fluor 40-fach 1.3 oil	Nikon, Japan
SensiCam	PCO, Kehlheim, GER
Microscope Axiovert 25	Carl Zeiss, Göttingen, GER
Microscope Axiovert 200	Carl Zeiss, Göttingen, GER
Objective A Plan 10-fach 7,025 Ph1	Carl Zeiss, Göttingen, GER
Biofuge fresco	Heraeus, Kleinostheim, GER
Multifuge K4	Heraeus, Kleinostheim, GER
Centrifuge Avanti TM J-25	Beckmann, Munich, GER
CO_2 cell incubator	Heraeus, Kleinostheim, GER
Electrophoresis chamber Power Pac 1000	Bio-Rad Laboratories, Munich, D
Fuji X-Ray Film Processor RG II	Fuji Photo Film, Düsseldorf, GER
1414 Liquid Scintillation Counter	Perkin Elmer Wallac, Freiburg, GER
Amplifier EPC-10	HEKA, Lambrecht, GER
HS 18 Laminar Airflow	Heraeus, Kleinostheim, GER

Micromanipulator 5171	Eppendorf, Hamburg, GER
Micropipette puller P-97	Sutter Instrument, Novato, USA
Photometer Gene Quant II	Pharmacia Biotech, Piscataway, USA
Pump SCI Q 323	Watson Marlow, Rommerskirchen, GER
Polychrome	Till Photonics, Gräfeling, GER
SPECTRAmax® spectrophometer	Molecular Devices, Sunnyvale, USA
Thermo block	Eppendorf, Hamburg, GER
UV 1601 spectrophometer	Shimadzu, Duisburg, GER
Varifuge 3.OR	Heraeus, Kleinostheim, GER
Homogenizer Ultra-Turrax T8	IKA®-Werke, Staufen, GER

3.1.8. Antibodies

TRPV1 (VR1) N-terminus (rabbit)	Neuromics, Edina, USA
GFP (mouse monoclonal)	Abcam, Cambridge, UK
A cyclase V/VI (rabbit polyclonal)	Santa Cruz, Edina, USA
Phosphoserine (rabbit, polyclonal)	Abcam, Cambridge, UK
Phosphothreonine (rabbit, polyclonal)	Abcam, Cambridge, UK
TRPA1 (ANKTM1) (goat, polyclonal)	Santa Cruz, Edina, USA
ADCY 3 (rabbit, polyclonal)	Abcam, Cambridge, UK
TRPA1-Mix (rabbit, polyclonal)	Kindly provided by Prof. Heppenstall
Peroxidase-conjugated Affini Pure Rabbit Anti-mouse IgG	Jackson Immuno Research Lab., INC, Suffolk, UK
Peroxidase-conjugated Affini Pure Goat Anti-rabbit IgG	Jackson Immuno Research Lab., INC, Suffolk, UK
Rabbit Anti-goat IgG HRP	Santa Cruz, Edina, USA

3.2. Methods

3.2.1. Experimental procedures with animals

Cultures of dorsal root ganglion (DRG) neurons

Rats were killed by isoflurane anaesthesia and DRG neurons were removed. Tissues were placed immediately on ice in 1 ml cold sterile Modified Eagle Medium (MEM) complemented with 1% penicillin and streptomycin. DRG were digested with rat collagenase

type 2 (3mg/ml) in MEM for 50 min at 37° C. Subsequently, 1 mg/ml trypsin type 1 was added for 10 min at 37° C. After digestion DRG were carefully dissociated by mechanical agitation (pipetted up and down 20 times) and filtered carefully through a 40 µm filter to remove impurities. Eighty mg of BSA in 4 ml MEM were added and the solution was centrifuged at 500 g for 5 min at 4 °C. The cell pellet was resuspended in 5 ml MEM/penicillin/streptomycin by hand shaking and centrifuged again at 300 g for 5 min at 4 °C. The pellet was mechanically resuspended in 3 ml medium and 300 µl of the mixture were transferred to polylysine coated culture dishes. After 1 h incubation at 37 °C, 1.7 ml MEM complemented with 10 % horse serum and 1% penicillin/streptomycin were added. Electrophysiological experiments were performed 24 to 48 h after the culture.

Behavioural experiments

Thermal hyperalgesia during opioid withdrawal was analyzed using the Hargreaves test. Behavioural experiments were performed by Oliver Fischer (Dep. of Anaesthesiology and Intensive Care Medicine, Charité, Campus Benjamin Franklin). The time necessary for the animal to remove its hindpaw after thermal stimulation was measured to determine the pain sensitivity (paw withdrawal latency [PWL]) (Hargreaves *et al.* 1988). The animal was placed in a transparent plastic chamber with a glass floor (Analgesia Meter; model 336; IITC Life Science, Woodland Hills, USA). Thermal stimuli were applied to the hindpaw by a heat-emitting lamp, which was directed at the bottom of the chamber floor. The experiment was stopped at the latest after 35 sec to avoid tissue damage (cut-off). PWL was measured twice per paw in an interval of 30 sec. The mean of both values was used for statistical analysis.
Prior to the experiment, fentanyl was injected into the right hindpaw (0.05 – 0.9 µg in 20 µl H_2O and 0.7 µg in 20 µl H_2O, respectively) three times every 10 minutes. PWL was investigated 5 and 60 min after the last fentanyl injection. In separate experiments naloxone, H89 (100, 500 and 1000 ng) and capsazepine (0.1, 1, 10, 100 and 1000 ng) were injected simultaneously with fentanyl (0.7 µg).

Additionally, protecting and flinching behaviour of rats after intraplantar (i.pl.) capsaicin injection (30 µg in 10 µl ethanol) during opioid application or withdrawal was ascertained for 20 min.

3.2.2. Cell biological techniques

Culture of HEK 293 and HEK 293 Tet-On cells

Experiments were performed in HEK 293 cells because they do not constitutively express TRPV1, TRPA1 or MOR (Endres-Becker 2007) (control experiments, data not shown). To investigate the interaction of TRPV1 and MOR, wild type HEK 293 cells were used. In the second part the HEK 293 Tet-On® Advanced cell line was chosen because it stably expresses a reverse tetracycline-controlled transactivator protein. An important advantage of Tet-On systems is the ability of high expression levels after addition of doxycycline to the culture medium. This is important, because TRPA1 seems to be toxic when constitutively expressed in HEK293 cells and because TRPA1 is downregulated in a time dependent manner (Story *et al.* 2003). HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37° C and 5 % CO_2 in a cell incubator. They were splitted 1:3 - 1:10 every second to third day depending on the confluence. The adherent cells were rinsed in DMEM from the culture flask ground, transferred to a 50 ml cell culture tube and centrifuged for 10 min at 400 g and room temperature. Afterwards the supernatant was removed and the cell pellet was resuspended in fresh media. Culture of HEK 293 Tet-On Advanced cells followed the same procedure as described above, except for using MEM alpha medium supplemended with 10 % Tet system approved FBS, 1 % penicillin/streptomycin, 4 mM L-glutamine and 200 µg/ ml geneticin (G 418).

Transient transfection

Transfection aims at the incorporation of foreign DNA into eukaryotic cells (Lottspeich 2006) and is classified as transient or stable. Stably transfected cells permanently express, in a constitutive or inducible manner, an exogenous DNA that has been introduced into the genome of the host cells. In transient transfection the exogenous cDNA is present as a plasmid within the cell and persists up to several days. Transfection of cDNA needs a carrier system. A common method uses the transport of nucleic acid through the membrane via lipofection. Liposomes are vesicles that can easily merge with the cell membrane since they both consist of a phospholipid bilayer. Negatively charged DNA molecules bind to positively charged liposomes and form DNA-lipid-complexes that can easily penetrate the cell membrane by endocytosis.

Usually the cDNA of interest is inserted into a vector system which can carry a gene resistant against antibiotics that acts as a marker for further selection in case of stable transfection. Vectors used in this study were pcDNA 3.1 (Invitrogen) and pTRE 2 (Clontech). The pcDNA 3.1 is a 5.4 kb vector designed for high level stable and transient expression in mammalian cells. It contains a human cytomegalovirus (CMV) immediate-early promoter for high-level constitutive expression and multiple cloning sites (MCS) in the forward and reverse orientations to facilitate cloning of ampicillin and neomycin resistance genes. The plasmid pTRE 2 has a Tet-responsive promoter. It carries an MCS immediately downstream of the Tet-responsive promoter (P_{hCMV-1}) and contains an ampicillin resistance gene. The Tet-system provides efficient, precise and reversible control over time and level of gene expression in eukaryotic cells. It comprises two complementary circuits, described as the Tet-Off and the Tet-On system. In each system a recombinant tetracycline controlled transcription factor (tTA or rtTA) interacts with a responsive promotor (P_{tet}) to drive expression of the gene of interest. Thus, expression is regulated by tetracycline or its derivates activating DNA binding of tTA and rtTA transcription factors. In Tet-On systems rtTA requires a tetracycline ligand (e.g doxycycline) for DNA binding and transcription (Gossen and Bujard 1992). The cDNA of TRPV1 was labelled with yellow fluorescent protein (YFP) to enable selection of TRPV1-expressing HEK 293 and HEK Tet-On by exposure to blue light.
All utilized vectors, the embedded plasmids and their origin and source are listed below:

Plasmid	Vector	Source	Species
wt MOR	pcDNA 3.1	Christian Zöllner, Charité Berlin, Anaesthesiol.	Rat
wt TRPV1-YFP	pcDNA 3.1	Michael Schaefer, Charité Berlin, Pharmacology	Rat
S116A TRPV1-YFP	pcDNA 3.1	Christian Zöllner, Charité Berlin, Anaesthesiol.	Rat
S502A TRPV1-YFP	pcDNA 3.1	Christian Zöllner, Charité Berlin, Anaesthesiol.	Rat
S774A TRPV1-YFP	pcDNA 3.1	Christian Zöllner, Charité Berlin, Anaesthesiol.	Rat
T144A TRPV1-YFP	pcDNA 3.1	Christian Zöllner, Charité Berlin, Anaesthesiol.	Rat
wt TRPA1	pTRE 2	Paul Heppenstall, Charité Berlin, Anaesthesiol.	Human
si RNA CTRL plasmide		Christian Zöllner, Charité Berlin, Anaesthesiol.	
si RNA AC 3		Christian Zöllner, Charité Berlin, Anaesthesiol.	
si RNA AC 5		Christian Zöllner, Charité Berlin, Anaesthesiol.	

HEK 293 or HEK Tet-On cells were cultured in special culture plates corresponding to the experimental method (see Table 1-6). After 1-2 d, at a cell confluence of approximately 80%,

cells were transiently co-transfected with either plasmids of wildtype/mutant TRPV1-YFP and MOR (for the first part of the study) or wildtype/mutant TRPV1-YFP and wildtype TRPA1 (for the second part of the study). Transfection was performed using Fugene® 6 (Roche Diagnostics) according to the manufacturer's instructions. For electrophysiology transfected cells were separated using trypsin 1 d prior to the experiment. For this purpose the adherent cells were washed twice with 1 ml of phosphate buffer saline (PBS) and incubated for 3 min at 37 °C with 500 µl trypsin. Dissociation was stopped by adding 1 ml medium. Afterwards cells were centrifuged with 500 g at room temperature for 5 min, resuspended in 1 ml fresh medium and lightly plated on poly-l-lysine coated glass coverslips. In HEK Tet-On cells gene expression was induced after addition of 1µg/ml doxycycline. All experiments were performed 24 to 48 h after transfection. TRPV1-YFP-expressing HEK 293 and HEK Tet-On were selected by exposure to blue light.

Table 1) Transfection scheme of the first part of the study for calcium imaging and electrophysiological experiments.

Plasmids (µg)	Culture plate	Fugene® (µl)	Cell line	Transfection media (µl)
0,5 wt TRPV1/ 2 MOR	34 mm Ø with coverslips	3	HEK 293	97
0,5 S116A TRPV1/ 2 MOR				
0,5 S502A TRPV1/ 2 MOR				
0,5 S774A TRPV1/ 2 MOR				
0,5 T144A TRPV1/ 2 MOR				
0,5 wt TRPV1/ 2 MOR/ 2 si AC3				
0,5 wt TRPV1/ 2 MOR/ 2 si AC5				
0,5 wt TRPV1/ 2 MOR/ 2 CTRL-si AC				

Table 2) Transfection scheme of the second part of the study for calcium imaging and electrophysiological experiments.

Plasmids (µg)	Culture plate	Fugene® (µl)	Cell line	Transfection media (µl)
0,5 wt TRPV1/ 0,5 wt TRPA1	34 mm Ø	3	HEK Tet-On	97

0,5 S116A TRPV1/ 0,5 wt TRPA1	with coverslips			
0,5 S502A TRPV1/ 0,5 wt TRPA1				
0,5 S774A TRPV1/ 0,5 wt TRPA1				
0,5 T144A TRPV1/ 0,5 wt TRPA1				
0,5 wt TRPV1/ 0,5 Ptre2 (mock)				
0,5 pcDNA3.1/0,5 wt TRPA1 (mock)				

Table 3) Transfection scheme of the second part of the study for EIA.

Plasmids (µg)	Culture plate	Fugene® (µl)	Cell line	Transfection media (µl)
0,025 wt TRPV1/ 0,025 wt TRPA1	96 well	0,5	HEK Tet-On	4,5
0,025 wt TRPV1/ 0,025 Ptre2 (mock)				

Table 4) Transfection scheme of the first part of the study for radioligand binding studies and (co-) immunoprecipitations.

Plasmids (µg)	Culture plate	Fugene® (µl)	Cell line	Transfection media (µl)
3 wt TRPV1/ 12 MOR	87 mm Ø	18	HEK 293	582

Table 5) Transfection scheme of the second part of the study for radioligand binding studies and (co-) immunoprecipitations.

Plasmids (µg)	Culture plate	Fugene® (µl)	Cell line	Transfection media (µl)
3 wt TRPV1/ 3 wt TRPA1	87 mm Ø	18	HEK Tet-On	582
3 wt TRPV1/ 3 Ptre2 (mock)				

Transformation and amplification of plasmid - DNA

To obtain sufficient quantities for transfection plasmid DNA was amplified in Escherichia coli cells (DH5α) and purified using the Qiafilter^{TM}Plasmid Maxi Kit. Transformation of plasmid DNA was realized by heat shock. Briefly, 1 µl of plasmid DNA was added to 20 µl

DH5α suspension and incubated for 20 min on ice. The cells were heat-shocked for 30 sec at 42 °C and then cooled on ice for 2 min. Afterwards 300 µl SOC medium was subjoined and the mixture was centrifuged at 300 rpm and 37 °C for 1 h. Finally, transformed bacteria were plated on Luria-Bertani (LB)-agar plates containing 50 µg/ml ampicillin and incubated at 37 °C overnight. On the next day one single colony of bacteria was picked with a sterile Eppendorf tip, transferred to 100 ml ampicillin-containing (50 µg/ml) LB-medium and incubated with 225 rpm overnight. On the next morning 700 µl of the media was supplemented with 300 µl 50 % glycerol and immediately stored at -80 °C.

Plasmid purification using the Qiafilter^{TM}Plasmid Maxi Kit is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. Plasmid DNA was then washed with 70% ethanol and centrifuged for 10 min at 15000 g. The pellet was air-dried and redissolved in 1 ml of distilled water. Plasmid DNA concentration was determined by UV spectrophotometry at 260 nm.

Small interference RNA

Small interference RNA (siRNA) are small (21-23 nucleotides) double-stranded RNAs that are homologous to a target gene and are used to silence gene expression in animals and plants (Bantounas et al. 2004; Elbashir et al. 2001; Fire et al. 1998). Gene silencing through siRNA requires two main steps: First, double-stranded RNA is recognised by an enzyme named Dicer (member of RNase III nucleases) and cleaved into small double-stranded molecules (siRNA). Second, the siRNAs are bound by the RNA-induced silencing complex (RISC). The RISC is a multi-protein complex that guides the targeted RNA to degradation. Activation of RISC is accompanied by the unwinding of the siRNA duplex and one strand of the siRNA directs RISC to the target mRNA. Nuclease activity of RISC cleaves the target mRNA. In this study synthetic siRNAs homologous to genes encoding AC 3 and 5 were transfected into HEK 293 cells 48 h prior the experiment. Two µg of siRNA were applied to transfect confluent HEK 293 in 34 cm diameter culture plates with coverslips.

3.2.3. Calcium Imaging Experiments

This technique is used for measuring calcium signals in cultured cells. Because of its very strong concentration gradient across the plasma membrane (intracellular Ca^{2+} concentration ~ 30-150 nm; extracellular Ca^{2+} concentration ~ 10000 times higher), very short bursts of calcium entry will generate relatively large signals and can play an important role as a communicator and regulator of cell functions and activities. Calcium indicators can be classified into three groups: 1) phosphoproteins are luminescent indicators that emit light in a Ca^{2+}- dependent manner (e.g. aequorin); 2) fluorescent dyes change their spectral properties in response to binding of calcium ions and 3) fluorescent protein Ca^{2+} indicators are conjugates between calmodulin and fluorescent proteins resulting in conformational changes and altered fluorescent properties upon Ca^{2+} binding. Fluoerescent dyes can be subdivided into ratio-metric (e.g. Fura-2 and Indo-1) and single-wavelength dyes (e.g. Fluo-4). Ratio-metric dyes change either their excitation or emission spectra in response to calcium (Barreto-Chang and Dolmetsch 2009; Brownlee 2000). In the current work the ratio-metric fluorescent dye Fura-2 was used. It has an emission peak at 505 nm and changes its excitation peak from 340 nm to 380 nm following calcium binding. The intracellular calcium concentration is derived by calculating the ratio of fluorescence emission or excitation at distict wavelengths. Calcium imaging data were analysed with Tillvision software (Till Photonics, Gräfeling, GER).

For the first part of the study, HEK 293 cells were cultured in 34 mm culture plates with coverslips and transfected as shown in Tab. 1. To investigate the activity of wildtype or mutant TRPV1 (wt/m TRPV1) during opioid withdrawal, cells were treated for at least 6 h with 10 µM morphine which was then withdrawn by a strong wash out (withdrawal group). Wt/m TRPV1 activity of the withdrawal group was compared to a control group of HEK 293 cells transfected with wt/m TRPV1 and MOR, but without opioid treatment and withdrawal (CTRL). To examine the involvement of AC, siRNAs of AC 3 and 5 were co-transfected with wt TRPV1 and MOR. Twenty-four to 48 h after transfection medium was rinsed off by washing the cells twice with 700 µl Calcium Imaging Buffer (CIB) and cells were loaded with 3 µM Fura-2/AM for 30 min at 37 °C. Cells were washed 3 times with 1 ml CIB to remove extracellular Fura-2/AM and incubated for 10 min with 10 µM FSK and 2 mM IBMX in CIB to stimulate the production of cAMP and to inhibit the degradation of cAMP by phosphodiesterases. Thereafter, cells were placed in a recording chamber containing CIB. Pairs of images were collected every second at alternating exposures of 340 nm and 380 nm

(exposure time 100 ms) using a Polychrome V monochromator and a CCD camera (SensiCam). Coverslips were perfused with CIB and TRPV1 was activated by application of 1 µM capsaicin. CIB and capsaicin were removed with a pump (SCI Q 323, Watson Marlow). The change of the fluorescence ratio at 340 nm and 380 nm was calculated following subtraction of background fluorescence.

For the second part of the study HEK Tet-On cells were cultured in 34 mm culture plates with coverslips and transfected as shown in Tab. 1. Expression of the cDNA of interest was induced by addition of 1 µM doxycycline 12 h prior the experiment. Cells were washed and loaded as described above, but without incubation with FSK/IBMX. To examine the change in activity of TRPV1 with and without mustard oil pre-incubation, two groups of TRPV1/TRPA1 expressing HEK Tet-On cells were generated. Cells of the control group were loaded with Fura-2/AM, placed in the recording chamber and the increase of intracellular calcium was evoked by addition of 100 nM - 1 µM capsaicin. Cells of the second group were loaded with Fura-2/AM and also placed in the recording chamber. However, prior to the experiment, 20 µM mustard oil (MuO) was added for 2-3 min. TRPV1 was activated by capsaicin and the change of the fluorescence ratio was measured.

3.2.4. Electrophysiology

Patch Clamp experiments

This technique allows the study of single or multiple ion channels in membranes and can be applied to a wide variety of cells. It is especially useful to study exitable cells such as neurons, cardiomyocytes, muscle fibres and pancreatic beta cells. The patch clamp technique is a refinement of the voltage clamp and was developed by Neher in 1976. This discovery made it possible to record currents of single ion channels for the first time, proving their involvement in fundamental cell processes such as action potential conduction. In 1991 the establishment of the patch clamp technique by Neher and Sakmann was honoured with the Nobel Prize in Physiology and Medicine.

In this study, the experimental setup was arranged as follows: an inverse microscope with a micromanipulator, pre-amplifier, electrode holding and an automatized perfusion system was placed on a vibration-cushioned metal table. The setup was located in a Faraday cage to facilitate electrical isolation. The pre-amplifier was connected with the main-amplifier (EPC-10, serial 520136-E, HEKA, Lambrecht) and signals were digitalized with an analog/digital

converter (AD/DA-converter). Recording and analysis of the signals were realized with the Pulse-software of HEKA, Lambrecht. Additionally it was possible to transmit voltage commands to the amplifier. Measuring and reference electrodes were made of silver wire (Ag/AgCl electrode), which were chlorided with potassium chloride periodically. A flexible plastic tube was affixed to the electrode holding to produce low or high pressures at the pipette tip during the experiment. The micromanipulator was necessary to position the pipette close to the cell. Patch pipettes were made of borosilicate glass capillaries with filament (Nr. 1103240, $Ø_{outside}$: 1.5 mm, Øinside: 0.87 mm, Hilgenberg, Malsfeld) using a pipette puller. Pipette resistance, which gives information about the diameter of the pipette tip, was 3-6 MΩ.

The whole cell patch clamp technique was used to analyze TRPV1 specific ion currents in cultured DRG neurons or transfected HEK 293/Tet-On cells. Experiments were performed 12-24 h after DRG culture or transient transfection of HEK 293/Tet-On cells (see Tab. 1 and 2). Cells which were not in contact with other cells were chosen preferably and perfused with extracellular buffer (ECS, see 3.1.3.) during the experiment. The pipette was filled with intracellular solution (ICS, see 3.1.3.) and all experiments were performed at room temperature. The whole-cell-mode was established according to Current Protocols (Gerfen 2001). The cell-attached configuration is achieved after formation of the gigaseal. After a short suck the cell membrane perforates and the cell-attached mode transforms into the whole-cell configuration. This configuration allows the measurement of all ion currents in the patched cell.

The holding potential was set at -60 mV after switching to the whole cell mode and cells were directly activated by capsaicin (100 nM - 1 µM). For “withdrawal experiments” FSK (10 µM) and IBMX (2mM) were applied through the bath solution. The cell-permeable AC inhibitor SQ 22,536 (100 µM) was added 20 minutes, whereas morphine (10µM) and the protein kinase A inhibitor H89 (10 µM) were added for at least 6 h prior to the experiment. Opioid withdrawal was induced by a strong wash out of morphine.
For the “TRPV1/TRPA1-interaction” experiments mustard oil (20 µM) was added to the cells 2-3 min before activating TRPV1 with capsaicin. The resulting inward current was compared to DRG neurons or transfected cells, which were not pre-treated with mustard oil.
To simulate calcium free conditions, cells were incubated and measured in Ca^{2+}-free bath solution complemented with EGTA (100 µM) (ECS Ca^{2+}-free). PKA was inhibited by the addition of H89 (10 µM) for at least 6 h prior to the experiment.

3.2.5. Radioligand receptor binding studies

To determine the expression and functionality of TRPV1 during opioid withdrawal (hypothesis 1) and interaction with TRPA1 (hypothesis 2) radioligand receptor binding studies were performed. The law of mass action (Motulsky and Christopoulos, 2003; formula 1) describes the feature of a ligand (L) to interact with a specific receptor (R). The terms k^{+1} and k^{-1} represent rate constants for association and dissociation, respectively. The dissociation constant K_D [mol/l] denotes the concentration of a ligand at which 50 % of the receptors are occupied and defines the equilibrium of the ligand-receptor interaction. Equilibrium is reached when the rates of forward and backward reactions are equal. By use of progressively increasing amounts of radioactively labelled ligands (L_R, radioligand) in saturation experiments, it is possible to identify the maximum number of receptors (B_{max}) and the K_D (Formula 2). The K_D represents the affinity of a ligand to its receptor. Langmuir summarized the isotherm in formula 3.

$$[R] + [L] \underset{k^{-1}}{\overset{k^{+1}}{\longleftrightarrow}} [RL] \qquad \textbf{Formula 1}$$

$$K_D = \frac{k_{-1}}{k_{+1}} \qquad \textbf{Formula 2}$$

$$[RL] = \frac{B_{max} * [L_R]}{K_D + [L_R]} \qquad \textbf{Formula 3}$$

After transfection (see Tab. 4 and 5), HEK 293 and HEK HEK Tet-On cells were washed three times with ice cold binding buffer (Trizma® Pre-set crystals added with 0.25 mg/ml BSA), harvested, homogenized at maximimum speed 3 times for 10 sec and centrifuged at 42000 g at 4°C for 20 min. The pellet was resuspended in binding buffer, homogenized and centrifuged again. Then the pellet was dissolved in 2 ml binding buffer and prepared for protein quantification using the Bradford assay (Bradford 1976). Between 100 µg and 500 µg of protein, diluted in 400 µl binding buffer, were used for binding experiments. TRPV1 expression in HEK 293 and HEK Tet-On cells was determined with the radioactively labelled high affinity TRPV1 agonist resiniferatoxin ([^{3}H]RTX) (K_D = 84 nM; Szallasi et al., 1999). Fifty µl of [^{3}H]RTX in increasing concentrations from 100 pM to 2.4 nM were incubated with 400 µl membrane suspension and 50 µl binding buffer. Unspecific binding was defined by

using 10 µM cold (not radioactively labelled) RTX. The binding reaction was initiated by transferring the assay mixtures into a 37 °C water bath and terminated after 60 min incubation by cooling the tubes on ice. Additionally, non-specific RTX binding was reduced by adding bovine α_1-acid glycoprotein (100 µg per tube) after the binding reaction was stopped. Membrane-bound RTX was separated from the α_1-acid glycoprotein-bound RTX by pelleting the membranes in a centrifuge at 3600 rpm for 60 min following a resuspension of the pellet in 200 µl binding buffer. Separation of membrane-bound and free radioactivity was achieved by vacuum filtration (Brandel-Harvester) through polyethylenimin (0.1 % in 50 mM Tris) soaked glass fiber filters.
Tritium-labelled ligands emit low-energy beta rays detectable indirectly via scintillation spectroscopy (Yamamura and Hulme, 1992). Filters were placed in tubes and filled with 3 ml scintillation solution which contains a primary and a secondary scintillator. The emission spectrum of the secondary scintillator is detectable by a photomultiplier and measured with the aid of a scintillation counter as decay/counts per min (CPM). Only a very small part (3-6 %) of the energy of the radioactive rays is detected due to the high number of energy transfer processes.

Twenty-four h after filtration the radioactivity of the sample was measured with a liquid scintillation counter. Its efficiency amounted to 69 % (counter efficiency = $CPM_{specific}$ x 100 / decays/min [DPM]). Specific binding was calculated substracting the CPM of unspecific binding from the CPM of total binding:
$CPM_{specific} = CPM_{total} - CPM_{unspecific}$

The specific activity of the ligand was assessed with aid of the specific radioactivity. One Ci/mmol is equal to 2.2 x 10^{12} DPM:
Specific activity [cpm/fmol] =
specific radioactivity [Ci/mmol] x 2.2 x 10^{12} x counter efficiency / 10^{12} [fmol]

B_{max} was obtained using following equation:
B_{max} [fmol/mg] = $CPM_{specific}$ / specific activity [cpm/fmol] x protein content [mg]

The K_D and the hyperbole saturation curve were calculated with GraphPad Prism 5 (San Diego, USA) using nonlinear regression.

3.2.6. Immunoprecipitation / Co-immunoprecipitation

Immunoprecipitation (IP) is used to precipitate a protein of interest using a specific antibody raised against this protein. After protein/antibody binding, beads coated with the immunoglobulin-binding protein G are added to the mixture, resulting in the binding of the protein/antibody complex to the beads by interaction with the protein G. After centrifugation, the beads bound to the protein/antibody complex settle down as a pellet (Fig. 3.1).

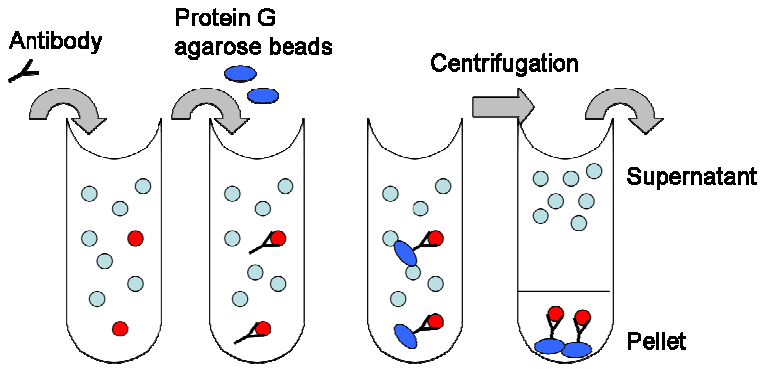


Fig. 3.1. General principle of immunoprecipitation.

Co–immunoprecipitation allows the precipitation of an intact protein complex. A specific antibody detects a protein that is known to be a member of the protein complex. After pulling down this protein, it is assumed that the whole protein complex is precipitated. Co-immunoprecipitation enables the detection of unknown members of an intact protein complex and is a powerful tool to detect physical protein-protein interactions.

Cells were cultured in 87 mm plates and transfected as described in Tab. 4 and 5. Prior to lysis, cells were washed twice with 10 ml PBS containing 10 mM sodiumvanadate and incubated with 750 µl lysis buffer (Ripa) for 10 min at 4°C. Cells were then collected with a cell scrapper, transferred to 1.5 ml eppendorf tubes and centrifuged at 13000 g at 4°C for 20 min. Thereafter protein concentrations were determined using the Bradford assay. An antibody raised against the protein of interest was added to 1-1.5 mg of protein sample and rotated for 2-3 h at 4°C. Thirty µl of protein G agarose beads were washed three times with lysis buffer and added to the protein-antibody solution. The whole mixture was rotated at 4°C overnight. The next day samples were centrifuged and washed three times. Finally, 30 µl of 2x sample buffer was added and the protein-antibody-beads-complex was heated at 95°C for

5 min to denature the protein. Subsequently, samples were ready for SDS gel-electrophoresis (described in the following chapter).
All IP-antibodies and their applied concentrations are listed in Tab. 6.

3.2.7. Western Blot analysis

Western Blot analysis is used for the detection, identification and quantification of proteins out of a complex protein mixture.
By use of gel electrophoresis, native or denatured proteins are separated by their molecular weight (denatured proteins) or by their 3 dimensional structures (native or denatured proteins). After electrophoresis, proteins are transferred to a nitrocellulose or polyvinylidene fluoride (PVD) membrane, which makes proteins accessible to antibody detection. After addition of the specific antibody against the protein of interest (primary antibody), the membrane is incubated with a secondary antibody conjugated with horseradish peroxidase that cleaves a chemiluminescent reagent. The reaction product emits luminescence correlated positively with the amount of protein. Exposure of the membrane to a photosensitive film reveals the conjugated secondary antibody bound to the primary antibody which binds the protein of interest (Broome and Gilbert 1978; Burnette 1981; Hawkes et al. 1982; Towbin et al. 1979) .
In my experiments, the SDS-polyacrylamide gel (resolving gel 8 %: 5.3 ml H_2O; 2 ml 40 % acryl amide mix; 2.5 ml 1.5 M Tris pH 8.8; 0.1 ml 10 % SDS; 0.1 ml 10 % APS; 0.006 ml TEMED and stacking gel: 1.5 ml H_2O; 0.25 ml 40 % acryl amid mix; 0.25 ml 1 M Tris pH 6.8; 0.02 ml 10 % SDS; 0.02 ml 10 % APS; 0.002 ml TEMED) was placed in a electrophoresis chamber filled with running buffer. Prestained protein standards and samples were carefully loaded into the gel wells. Electrophoresis was started at 60 mV until samples reached the resolving gel. Then, the voltage was raised up to 150 mV until proteins were completely separated (as seen in the protein standards well). The gel was then transferred into blotting solution. For transfer, gel was placed on top of a blotting paper and a blotting sponge and covered with a PVDF membrane which was also covered by a blotting paper and blotting sponge. All layers were clamped in a blotting chamber and transfered from the gel to the membrane at 350 mA for 1 h. Immediately after transfer, the membrane was incubated with blocking solution (2.5 - 5 % BSA and 2.5 - 5 % low-fat milk, respectively) for 1 h at room temperature (RT) at gentle agitation followed by incubation of the primary antibody overnight at 4 °C. On the next day the membrane was washed 3 times with TBS-Tween (3 x 10 min at room temperature under gentle agitation) and the secondary antibody was applied for 1 h at

RT under gentle agitation. Then the membrane was washed twice with TBS-Tween, a third time with TBS and covered with enhanced chemiluminescence solution. Horse radish peroxidase (coupled to the secondary antibody) catalyzes the conversion into a sensitized reagent which produces an excited carbonyl via oxidation by hydrogen peroxide. As a result, light is emitted and can be detected with a photosensitive film to give a quantitative image of a protein. The film is developed using the Fuji X-Ray Film Processor RG II. All primary and secondary antibodies, their applied concentrations and their related blocking solutions are listed in Tab. 6.

Table 6. Primary and secondary antibodies for (co-) immunoprecipitations and western blot analysis.

Primary antibody	Concentration	Secondary antibody	Concentration	Blocking solution
PhosphoSerines (abcam)		Anti-rabbit	1:5000	3 % BSA
PhosphoSerine (Quiagen)	1:200	Anti-mouse	1:5000	3 % BSA
PhosphoThreonine (abcam)	1:125	Anti-rabbit	1:5000	3 % BSA
ADCY 3 (abcam)	1:500	Anti-rabbit	1:5000	5 % low-fat milk
A cyclase V/VI (santa cruz)	1:200	Anti-rabbit	1:5000	5 % low-fat milk
GFP (abcam)	1:1000	Anti-mouse	1:5000	5 % low-fat milk
VR1 (neuromics)	1:500-1:1000	Anti-rabbit	1:5000	5 % low-fat milk
ANKTM1 (santa cruz)	1:200	Anti-goat	1:5000	5 % low-fat milk
TRPA1 (Prof. Heppenstall)	1:200	Anti-rabbit	1:5000	5 % low-fat milk

3.2.8. cAMP Enzyme-linked Immunosorbant Assay (ELISA)

The principle of cAMP-EIA is the competition between free cAMP molecules (from the sample) and peroxidase coupled cAMP molecules for binding with a specific cAMP antibody. Bound peroxidase-cAMP molecules oxidize the dye tetramethylbenzidine (TMB) into a blue derivate. The reaction is stopped with sulphuric acid. The acidification turns the blue-TMB derivate into a yellow compound which can be detected with an ELISA-photometer at 450 nm.

HEK Tet-On cells were cultured in a poly-L-lysine coated 96-well plate and transfected as mentioned in Tab. 3. At least 12 h prior to experiments, protein expression was induced by

application of 1 µM doxycycline. Cells were stimulated by 2 mM IBMX and 10 µM forskolin to avoid cAMP-degradation by phosphodiesterases and to exite cAMP-production by AC. 20 µM mustard oil was added 5 min prior to membrane preparation. To investigate the role of calcium in the accumulation of cAMP, calcium was removed from one sample group. The following groups of transfected HEK Tet-On cells were investigated:

TRPV1/TRPA1:

- stimulated with FSK/IBMX (w/o MuO)
- stimulated with FSK/IBMX and pretreated with 20 µM MuO (MuO)
- stimulated with FSK/IBMX in calcium free medium (w/o MuO; w/o Ca^{2+})
- stimulated with FSK/IBMX and pretreated with 20 µM MuO in calcium free medium (MuO; w/o Ca^{2+})

TRPV1/Ptre2 (mock):

- stimulated with FSK/IBMX (w/o MuO mock)
- stimulated with FSK/IBMX and pretreated with 20 µM MuO (MuO mock)

EIA experiments were performed following manufacturer's instructions using the non-acetylation protocol. After stimulation transfected HEK Tet-On cells were lysed and 100 µl of the lysate were transferred to an antigen-coated (donkey anti-rabbit IgG) ELISA plate. Simultaneously, a cAMP standard curve and blanks were prepared. All samples were arranged in duplicates and incubated for 2 h with an antiserum (rabbit anti-cAMP) followed by an 1 h incubation with cAMP peroxidase. Afterwards the plate was washed and TMB substrate was added. Approximately 20 min later a blue dye was developed which changed to a yellow colour by addition of H_2SO_4. The intensity of the colour was detected with an ELISA-reader at λ=450 nm. Data were analyzed using SOFTmax® Pro software (Molecular Devices, Sunnyvale, USA). The cAMP content was calculated with the aid of a standard curve, where the fluorescence was plotted versus the concentration in a semi logarithmic manner.

3.2.9. Statistical analysis

Statistical and graphical analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, USA), Microsoft Excel (Microsoft Corporation, Unterschleißheim, Germany) and Image J. Statistical significance was determined with Student´s unpaired t-test, 1 way ANOVA with Dunnet post-hoc test or 2 way ANOVA with Bonferroni post-hoc test. To

calculate K_D and B_{max} non-linear regression curve fits were performed.Statistical significance was denoted as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

4. Results

4.1. Interaction of TRPV1 and μ-opioid receptor during opioid withdrawal

It was previously shown that the activity of TRPV1 is modulated by acutely applied opioids via MOR and the cAMP/PKA pathway (Endres-Becker et al. 2007).
To examine possible sensitizing mechanisms of TRPV1 during opioid withdrawal, the activity and expression of wild type and mutant TRPV1 was analyzed using whole cell patch clamp, calcium imaging and radioligand binding studies. Phosphorylation of the receptor was studied by immunoprecipitations and western blot analysis. The role of AC was tested by inhibition and silencing using siRNA. Finally, behavioural experiments were performed to determine the relevance of TRPV1 mediated hyperalgesia during opioid withdrawal.

4.1.1. Wild type TRPV1 activity and expression during opioid withdrawal

After incubation of HEK 293 cells with 10 μM morphine for at least 6 h, withdrawal was induced by a strong washout. All cells (CTRL and withdrawal group) were pretreated with 10 μM FSK and 2 mM IBMX for 10 min to activate AC and elevate intracellular cAMP. Patch clamp experiments were performed in HEK 293 cells transiently transfected with wild type (wt) TRPV1 and wt MOR. In morphine-treated cells the mean inward current after morphine withdrawal was 3.82 fold higher than in control cells (Fig. 4.1.1.). This difference was statistically significant (unpaired t-test; *, $p<0.05$).

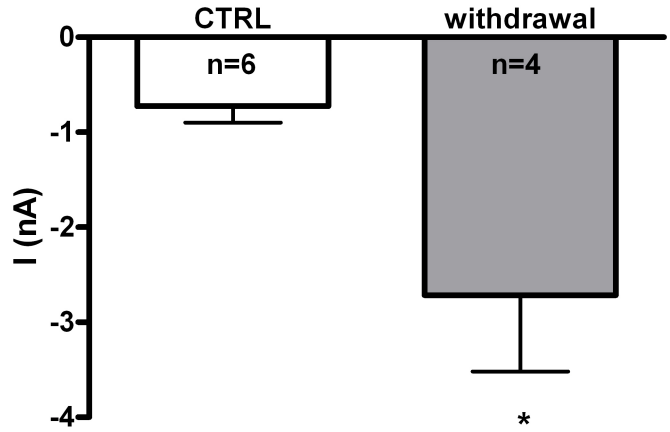


*Fig. 4.1.1. Capsaicin (1μM) induced TRPV1 currents of HEK 293 cells transiently transfected with TRPV1 and MOR during opioid withdrawal (withdrawal, grey bar) and without any treatment (CTRL white bar) any treatment. Withdrawal group showed a significant increased capsaicin induced current compared to CTRL cells. Bars are presented as mean ± SEM (unpaired t-test, *, p<0,05).*

Calcium imaging experiments confirmed our electrophysiological data (Fig. 4.1.2.). We measured a stronger increase of fura ratio (i.e. intracellular calcium concentration) after capsaicin stimulation during opioid withdrawal compared to control cells (Fig. 4.1.2.). The maximum fura ratio in control cells (0.82 ± 0.03; n = 111) was significantly lower than in cells withdrawn from morphine (1.08 ± 0.02; n = 209) (unpaired t-test, ***, p<0,001).

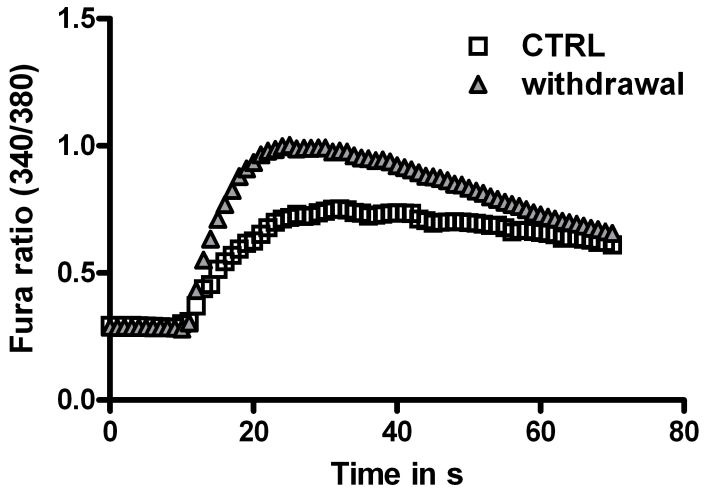


Fig. 4.1.2.Capsaicin (1 µM) induced change of the fura ratio over time in HEK 293 cells transiently transfected with cDNA of TRPV1 and MOR during morphine withdrawal and under control conditions.

Radioligand binding assays (CTRL: n = 4; withdrawal: n = 5) were performed to investigate whether differences in the functional assays were related to an increase in TRPV1 expression. Binding sites of TRPV1 (and MOR) expressing HEK 293 cells without treatment (CTRL) and cells pretreated with morphine (at least for 6 h) followed by a strong washout were quantified using [^{3}H]-RTX. Opioid pretreatment and withdrawal did not change TRPV1 expression (B_{max}: CTRL = 126.9 ± 31.36 fmol/mg; withdrawal = 137.2 ± 49.13 fmol/mg; n.s., unpaired t-test, p>0.05) or affinity (K_D: CTRL = 2.1 ± 0.35 nM; withdrawal = 1.77 ± 0.24 nM; n.s., unpaired t-test, p>0.05) (Fig. 4.1.3.).

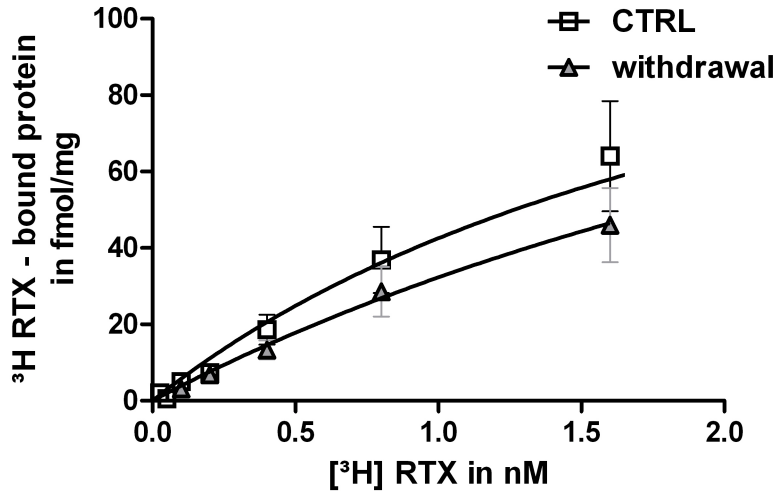


Fig. 4.1.3. TRPV1 expression in HEK 293 cells transiently transfected with TRPV1 and MOR during opioid withdrawal compared to untreated cells. Saturation curves of ³[H]-RTX binding at TRPV1 with and without opioid withdrawal.

Phosphorylation of TRPV1 during opioid withdrawal

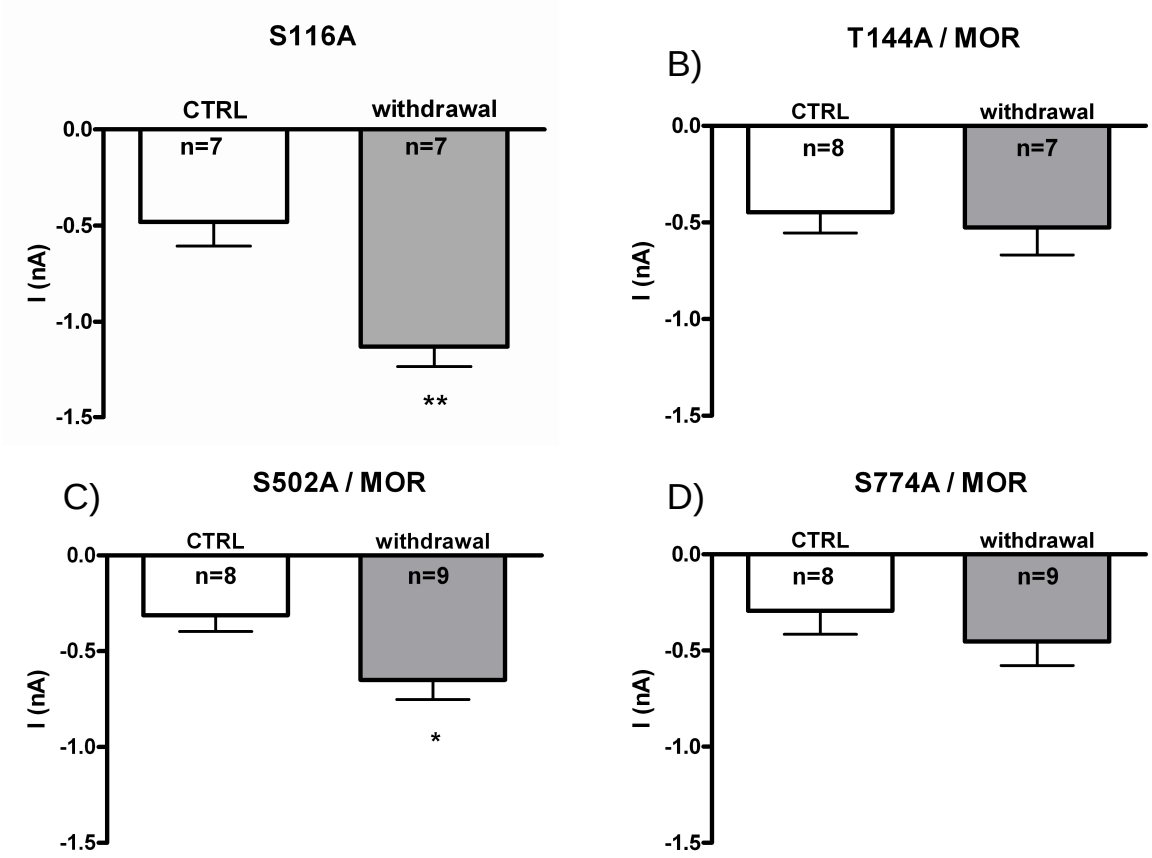
HEK 293 cells transiently expressing wild type TRPV1 and wild type MOR after opioid withdrawal or without treatment (controls) were examined. After lysis, electrophoresis, blotting and blocking, cell membranes were incubated overnight with antibodies against phosphorylated serine (PhosphoSerine, Qiagen, dilution of 1:200 following manufacture´s instruction) and phosphorylated threonine (PhosphoThreonine, Abcam, dilution of 1:125 following manufacture´s instruction) residues. I expected a band at 121 kDa (~ 95 kDa + 26 kDa YFP). Incubation of the PVD membrane with phosphoserine residue detecting antibody as well as phosphothreonine residue detecting antibody detected stronger bands at the prediced TRPV1 size in cells of the withdrawal group compared to the control group. The average band intensity was increased by 119 ± 8.5 % (unpaired t-test, n. s., $p>0.05$, n=3) and 130.5 ± 12 % (unpaired t-test, *, $p<0.05$, n=4), respectively.

4.1.2. Mutant TRPV1 activity during opioid withdrawal

Since we detected a stronger phosphorylation of TRPV1 during opioid withdrawal, we investigated specific PKA phosphorylation sites of TRPV1. Previous studies have identified a series of potential PKA phosphorylation sites within the TRPV1 sequence (Bhave et al. 2002; Mohapatra and Nau 2003). According to these findings, we selected serine 116 (S116), threonine 144 (T144), serine 502 (S502) and serine 774 (S774) for our experiments. Mutants

of these posphorylation sites were kindly provided from Prof. C. Zöllner. In these mutants serine or threonine were replaced by alanine residues (A) which cannot be phosphorylated by PKA.

HEK 293 cells were transiently transfected with mutant TRPV1 (S116A, T144A, S502A and S774A) and wild type MOR and cultured for whole cell patch clamp and calcium imaging experiments, as described in 3.2.2. The culture plate was incubated for at least 6 h with 10 µM morphine. Prior to the experiment, morphine was strongly washed out and transfected cells were incubated with FSK/IBMX 10 min prior to activation of TRPV1 with 1µM capsaicin. In S116A- and S502A-TRPV1 mutants, opioid withdrawal showed a significant increase in capsaicin induced TRPV1 currents compared to control cells without morphine pretreatment (Fig. 4.1.4.A and C).



*4.1.4. Capsaicin (1µM) induced TRPV1 currents in HEK 293 cells transiently transfected with mutant TRPV1 and wt MOR during opioid withdrawal and under control conditions without morphine pretreatment. Graphs show mean currents ± SEM of TRPV1 mutants A) S116A (unpaired t-test, **, p<0.01), B) T144A (unpaired t-test, n. s., p>0.05), C) S502A (unpaired t-test, *, p<0.05) and D) S774A (unpaired t-test, n. s., p>0.05). Increased capsaicin induced TRPV1 current during opioid withdrawal was seen in S116A and S502A but not in mutants T144A and S774A.*

In T144A- and S774A-TRPV1 mutants opioid withdrawal did not induce a significant increase in capsaicin mediated TRPV1 activity (Fig. 4.1.4.). Similar results were obtained using calcium imaging experiments (Fig. 4.1.5.): S116A and S502A TRPV1 mutants showed a significant increase in capsaicin-induced $[Ca^{2+}]_i$ increase during opioid withdrawal compared with control cells. In T144A- and S774A-TRPV1 mutants, no significant increase in capsaicin–induced $[Ca^{2+}]_i$ increase was detected during opioid withdrawal in comparison to CTRL cells.

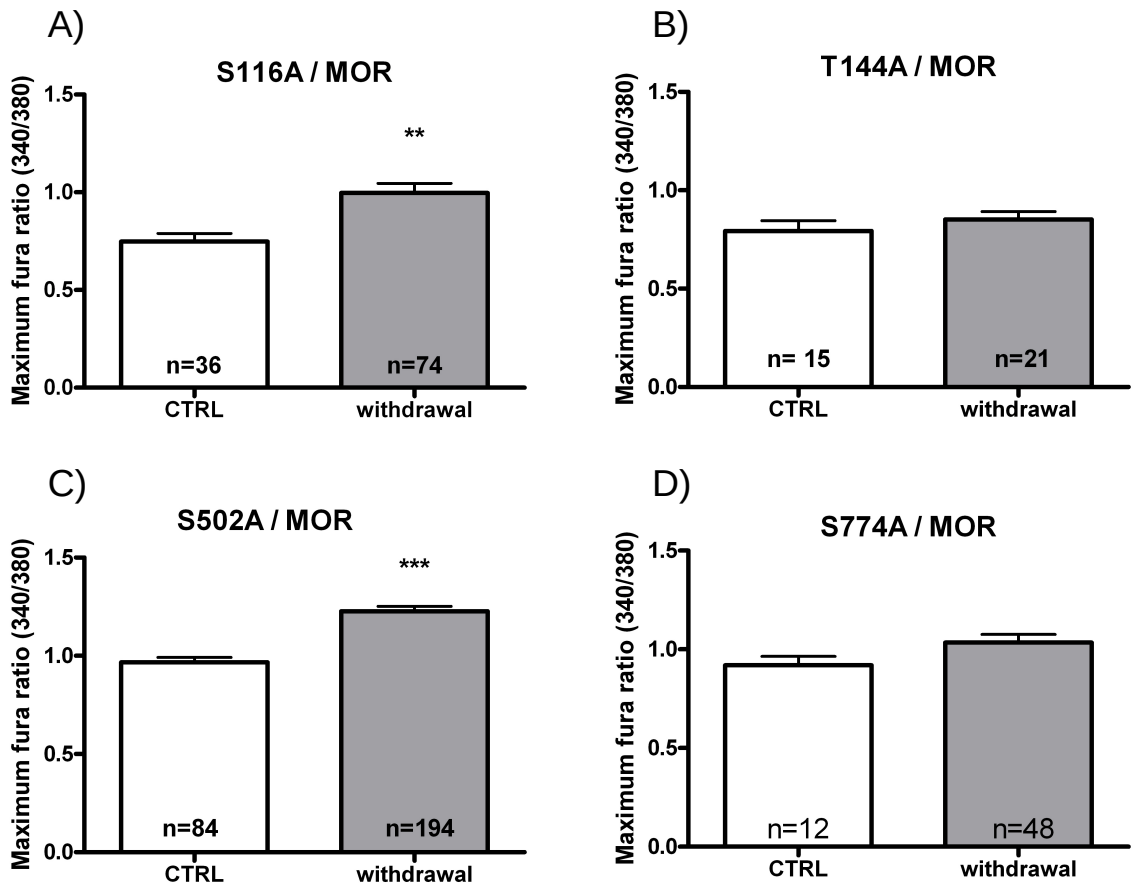


*Fig. 4.1.5. Capsaicin (1µM) induced maximum fura ratio in HEK 293 cells transfected with mutant TRPV1 and wt MOR under control conditions without morphine pretreatment and during opioid withdrawal. A) S116A vs. CTRL (unpaired t-test, **, p<0,01), B) T144A vs.CTRL (unpaired t-test, n. s., p>0,05), C) S502A vs.CTRL (unpaired t-test, ***, p<0,001) and D) S774A vs.CTRL (unpaired t-test, n. s., p>0,05).*

4.1.3. Role of adenylylcyclases 3 and 5 during opioid withdrawal

Opioid pretreatment induces a compensatory upregulation of AC activity that becomes manifest during withdrawal (Nestler and Aghajanian 1997). Previous studies in our laboratory also showed a significantly elevated mRNA level of AC isoforms 3 and 5 (Endres-Becker 2007) in DRG during opioid withdrawal. The nonselective AC inhibitor SQ 22,536 was applied (for 20 min) to block the activity of AC 1 - 9. Figure 4.1.6. shows the capsacin-induced TRPV1 current in HEK 293 cells expressing wt TRPV1 and MOR with and without SQ 22,536 pretreatment with and without morphine treatment. The enhanced capsaicin-induced TRPV1 current during opioid withdrawal was reversed in the presence of the AC inhibitor.

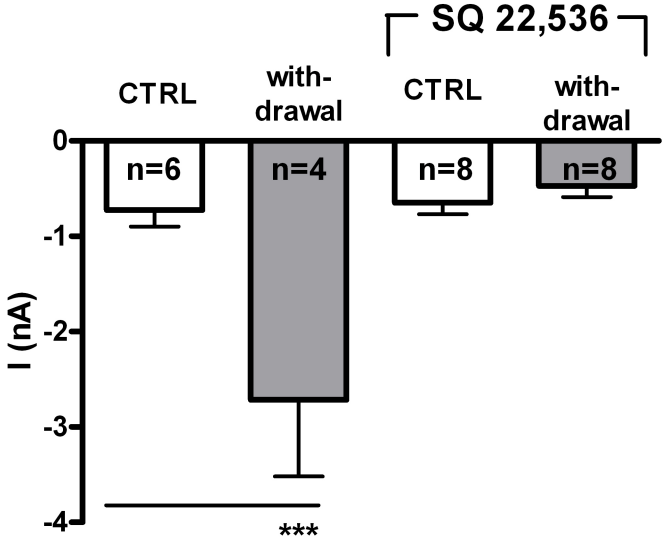


*Fig. 4.1.6. Capsaicin (1µM) induced TRPV1 currents in HEK 293 cells transiently transfected with wt TRPV1 and wt MOR without (CTR;, withdrawal) and with (CTRL SQ 22,53, withdrawal SQ 22,536) SQ 22,535 treatment . Graphs show mean ± SEM of capsaicin-induced (1µM) TRPV1 currents (1way ANOVA, Dunnet post hoc test, CTRL versus withdrawal, ***, p<0,001; CTRL versus CTRL SQ 22,536, n. s., p>0,05; CTRL versus withdrawal SQ 22,536, n. s., p>0,05). Pretreatment with SQ 22,536 reversed the increased TRPV1 current during withdrawal.*

To further delineate the role of different AC in opioid withdrawal we investigated the role of AC 3 and 5. Using small interference RNA (siRNA) we silenced AC 3 and 5 gene expression in HEK 293 cells transiently transfected with TRPV1 and MOR.

Downregulation of AC 3 or 5 expression was verified by western blot analysis. Band intensity of AC 3 in siRNA AC 3 transfected cells was 45.87 ± 7,41 % of the band intensity of untreated HEK 293 cells (unpaired t-test, **, p<0.01, n=3). Band intensity of AC 5 in

downregulated cells achieved 71.09 ± 8,14 % of band intensity of untreated HEK 293 cells (unpaired t-test, n. s., p>0.05, n=2). Thus, we were not able to detect a significant silencing of AC 5, probably since the antibody is also able to detect AC 6.

For calcium imaging experiments HEK 293 cells were transiently transfected with siRNA AC 3 or siRNA AC 5 48 h before the experiment. Opioid withdrawal was induced as mentioned above and $[Ca^{2+}]_i$ after capsaicin stimulation was measured. Fura ratios in cells treated with and without opioid withdrawal are presented in Figure 4.1.7.

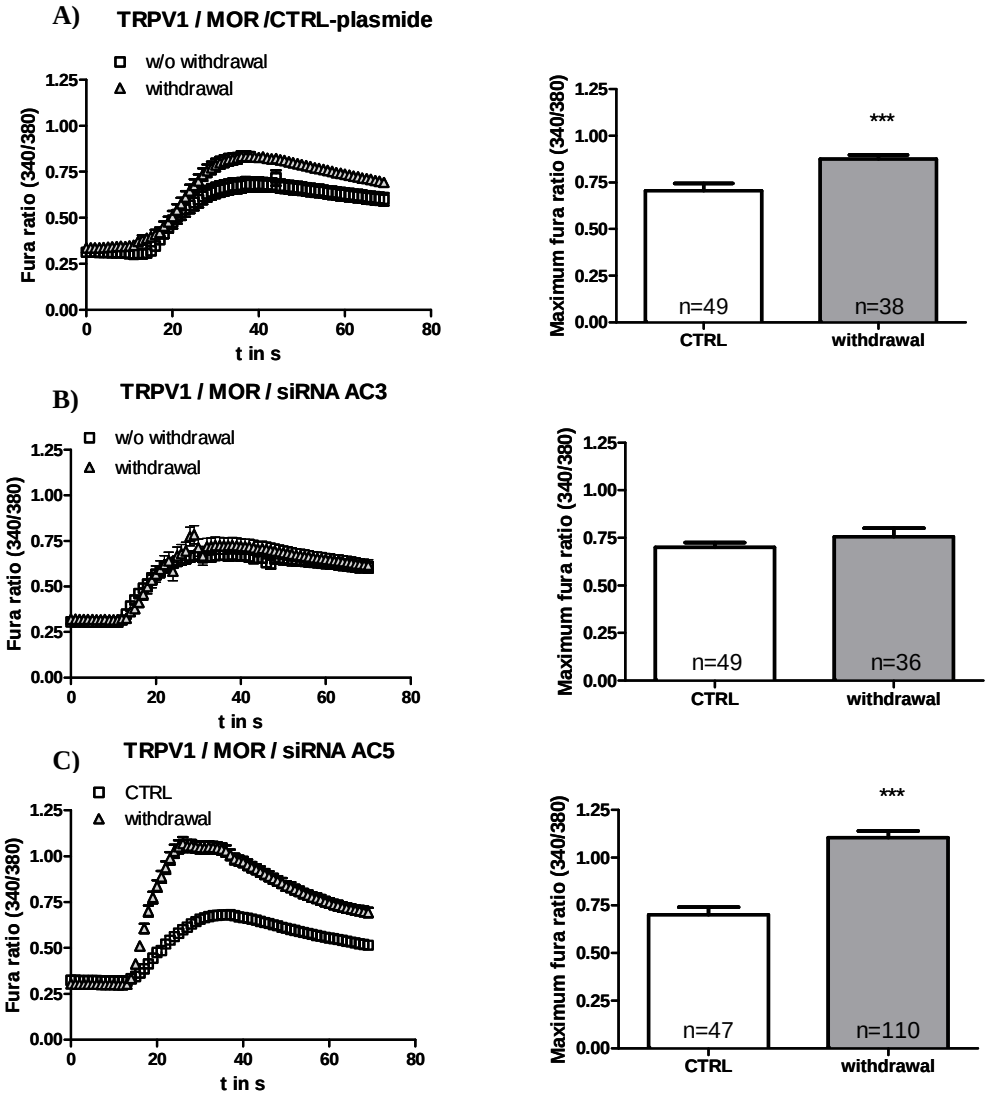


Fig. 4.1.7. Capsaicin induced change of the fura ratio in HEK 293 cells transiently transfected with wt TRPV1, wt MOR and CTRL plasmid (A) or siRNA AC 3(B) or AC 5 (C) with and without opioid withdrawal (left panel) and mean of maximum fura ratio (right panel). siRNA for AC 3 abrogated opioid withdrawal induced TRPV1 sensitization.

Cells expressing TRPV1, MOR and siRNA control plasmid showed a significantly enlarged maximum fura ratio after capsaicin application during opioid withdrawal in comparison to transfected cells without opioid treatment (unpaired t-test, ***, p<0.001) (Fig. 4.1.7. upper panel). This effect was abolished when AC 3 was silenced (unpaired t-test, n. s., p>0.05) (Fig. 4.1.7. middle panel). In contrast, downregulation of AC 5 did not abolish opioid withdrawal induced upregulation of TRPV1 activity (t-test, ***, p<0.001) (Fig. 4.1.7. lower panel).

4.1.4. Effects of opioid withdrawal *in vivo*

Thermal hypersensitivity during opioid withdrawal

The functional relevance of opioid withdrawal was investigated using the paw withdrawal latency (PWL) behavioural test (Hargreaves test). In Figure 4.1.8. A) PWL of rats pretreated with different concentrations of i. pl. fentanyl (0.05 - 0.9 µg in 20 µl H_2O) is illustrated. The upper line (black squares) represents the PWL of rats, which obtained intraplantar (i. pl.) fentanyl (0.05 - 0.9 µg) 3 times every 10 minutes. 5 min after the last injection, PWL was measured. After a maximum PWL of 35 seconds, the experiment was stopped to avoid tissue damage. In a second experimental setup the PWL was determined 1 h after the last fentanyl injection. PWL, measured 1 h after the last fentanyl injection was significantly decreased in a concentration dependent manner compared to baseline, where saline was injected instead of fentanyl (two way ANOVA, Bonferroni post hoc test, p<0,001). Figure 4.1.8. B) shows that the decreased PWL during opioid withdrawal is reversed by the addition of the opioid antagonist naloxone (NLX) (one way ANOVA, Dunnet post hoc test, ***, p<0,001). Naloxone (10 µg i.pl.) was injected simultaneously with fentanyl (0.7 µg). PWL was examined 1 hour after the last injection.

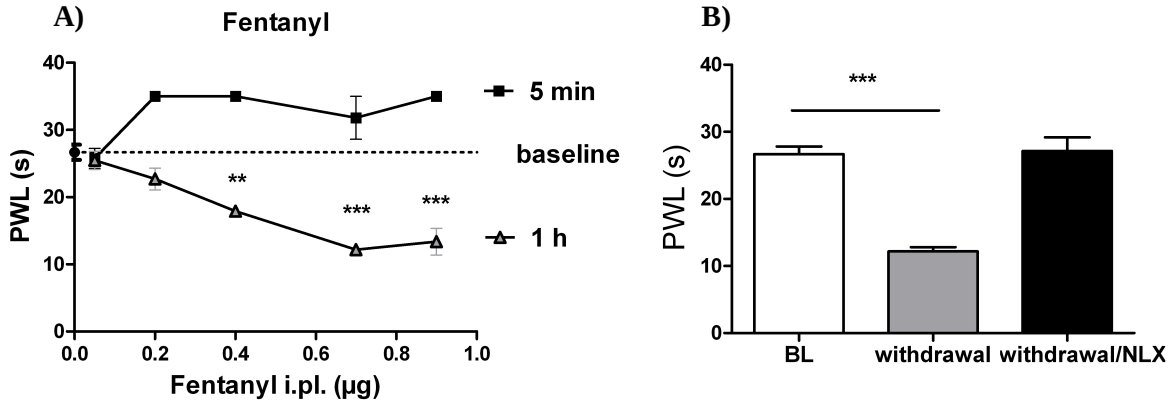


Fig. 4.1.8. Thermal hypersensitivity during opioid withdrawal. A) Upper line shows the PWL measured 5 min after the last fentanyl injection (increased PWL compared to baseline represents analgesic effects of fentanyl). Lower line shows the PWL 1 h after the last fentanyl injection (2way

*ANOVA, ***, P<0,001). B) demonstrates the PWL measured 1 h after the last saline (BL) or fentanyl (0.7 µg) (withdrawal) or fentanyl + naloxone (withdrawal/NLX) injection.. Thermal hypersensitivity during opioid withdrawal was abolished by simultaneous administration of the opioid antagonist naloxone (1way ANOVA, Dunnet post hoc test, ***, p<0,001).*

The decreased PWL during withdrawal from fentanyl (0.7 µg 3 times every 10 minutes) was also abrogated in a concentration dependent manner when H89 (PKA inhibitor) and capsazepine (TRPV1 antagonist) was simultaneously injected i. pl. (Fig. 4.1.9.).

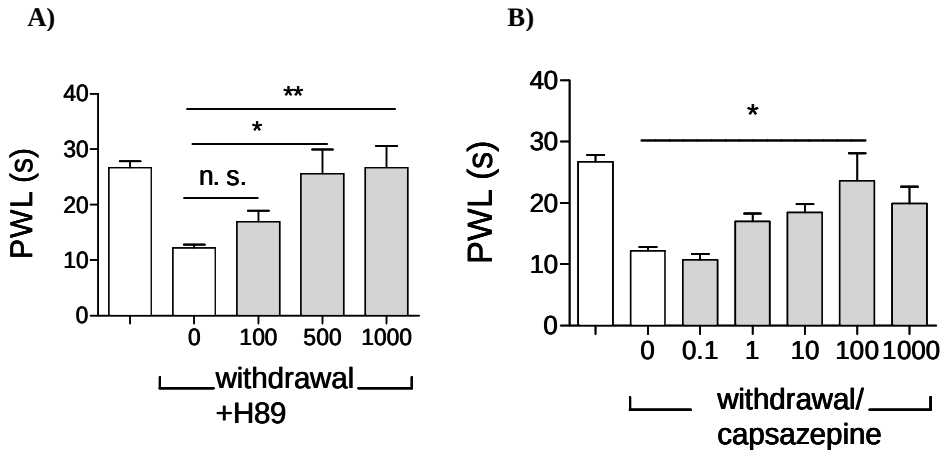


*Fig. 4.1.9. Concentration dependent reversal of withdrawal induced thermal hypersensitivity by addition of A) H89 and B) capsazepine (1way ANOVA, Dunnet post hoc test, *, p<0,05, **, p<0,01).*

Nocifensive behaviour during opioid withdrawal

Nocifensive behaviour (i.e. protecting and flinching of the paw) was measured 5, 10, 15 and 20 min after i.pl. capsaicin (30 µg) injection. Opioid withdrawal was induced by i. pl. injection of fentanyl (0.7 µg) 3 times in an interval of 10 minutes. One hour after the last opioid injection, capsaicin was applied and nocifensive behaviour was measured over time. The control group received i.pl. saline instead of fentanyl. Figure 4.1.10 A) and B) illustrates that both protecting and flinching behaviour were significantly increased during opioid withdrawal (protecting behaviour 5 min after capsaicin injection, 2 way ANOVA, Bonferroni post hoc test, *, p<0.05; flinching behaviour 10 min after capsaicin injection, 2 way ANOVA, Bonferroni post hoc test, ***, p<0.001). This increase was completely abolished by the i.pl. application of the cell permeable PKA inhibitor H89 (Fig. 4.1.10. C) and D).

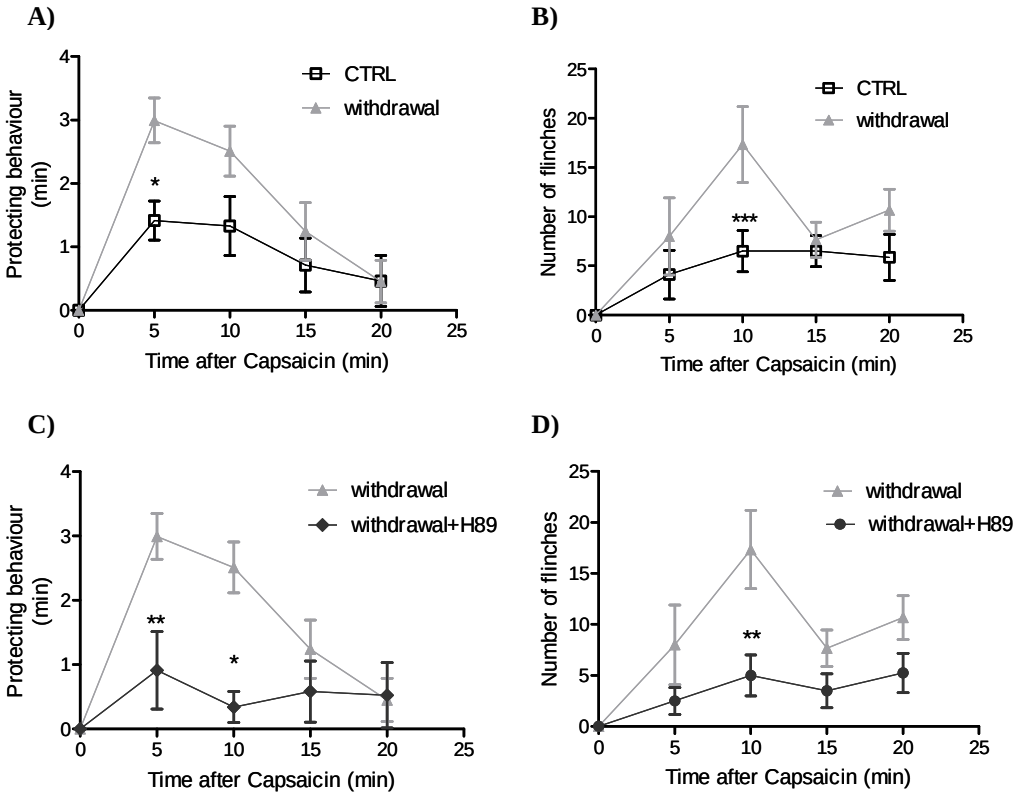


*Fig. 4.1.10. Increased protecting (A) and fliching (B) behaviour during opioid withdrawal was reversed by the addition of H89 (C) and (D) (2way ANOVA and Bonferroni post hoc test, *, p<0.05, **, p<0.01, ***, p<0.001).*

4.2. Interaction of TRPV1 and TRPA1

The second part of my thesis investigated TRPV1 sensitization after stimulation of cells with TRPA1 ligands.

4.2.1. Direct physical interaction of TRPV1 and TRPA1

The expression of TRPV1 ion channels after activation of TRPA1 was investigated using radioligand binding assays with [^{3}H] labelled resiniferatoxin ([^{3}H]-RTX). HEK Tet-On cells were transiently transfected with wild type TRPV1 and wild type TRPA1, or with wt TRPV1 and the empty vector Ptre2 (mock transfection). In Figure 4.2.1 saturation curves of [^{3}H]-RTX binding are presented. No changes in the number of TRPV1 binding sites or their dissociation constants (K_D) were detected in the presence of TRPA1 (TRPV1/TRPA1: B_{max} = 283.1 ± 61.54 fmol/mg, K_D = 2.77 ± 0.94 nM, n = 7; TRPV1/Ptre2: B_{max} = 315.9 ± 133.6 fmol/mg, K_D = 4.2 ± 2.51 nM, n = 5, unpaired t-test, n. s., p>0.05).

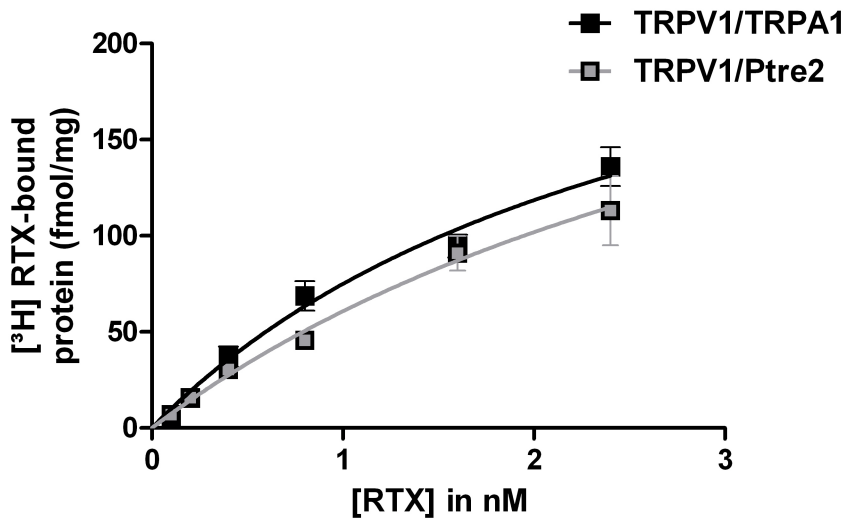


Fig. 4.2.1. [^{3}H]-RTX binding at TRPV1 in the presence (TRPV1/TRPA1) and absence (TRPV1/Ptre2) of TRPA1 in HEK Tet-On cells.

We then tested whether TRPA1 activation with 20 µM mustard oil (MuO) changes the number of TRPV1 binding sites on HEK293 cells. MuO was applied for 1 h or 16 h. Control cells were not stimulated with MuO. Radioligand binding experiments measured the number of TRPV1 binding sites. No significant differences in TRPV1 binding sites were detected after MuO stimulation (Fig. 4.2.2., 1 way ANOVA, Dunnet post hoc test, n. s., p>0.05).

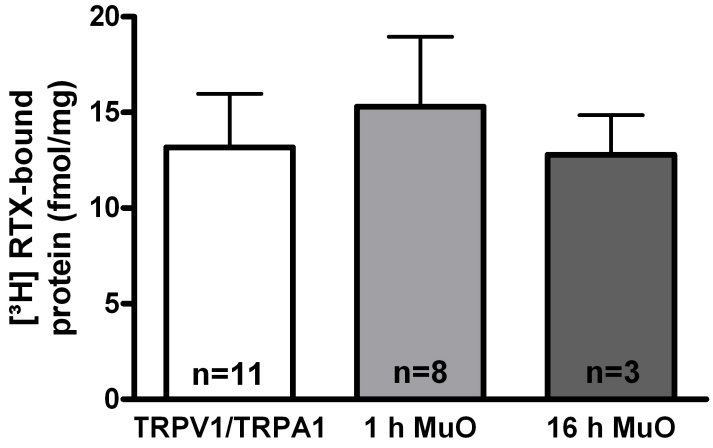


Fig. 4.2.2. TRPV1 binding sites on HEK Tet-On cells expressing wt TRPV1 and wt TRPA1 treated without (TRPV1/TRPA1) or with MuO for 1h or 16 h. No significant differences in the number ofTRPV1 binding sites were detectable (1 way ANOVA, Dunnet post hoc test, n. s., p>0.05).

To directly show a possible physical interaction between both channels, co-immunoprecipitations were performed. HEK Tet-On cells were transiently transfected with wt

TRPV1 and wt TRPA1. Membranes were prepared as described in 3.2.6. and separated in two fractions. One fraction was stored for western blot analysis and in the other one, TRPA1 was immunoprecipitated using a TRPA1 antibody. Figure 4.2.3. presents results of western blot analysis of TRPV1/TRPA1 expressing membranes that were not immunoprecipitated (left lane) and TRPA1 immunoprecipitated membranes (right lane). TRPV1-YFP was detected using a GFP-antibody. No TRPV1 band was detected in the TRPA1 immunoprecipitated fraction. Thus, no apparent physical interaction occurred between TRPV1 and TRPA1 in our expression system.

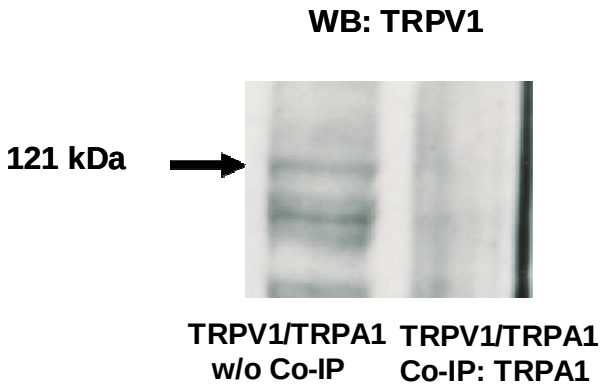


Fig. 4.2.3. Western blot analysis of HEK Tet-On cell transfected with TRPV1 and TRPA1. The first lane was loaded directly with TRPV1/TRPA1 containing cell lysate. In the second lane TRPA1 was immunoprecipitated from TRPV1/TRPA1 expressing HEK Tet-On cells. After gel electrophoresis, blotting and blocking, both fractions were incubated with GFP antibody detecting TRPV1-YFP.

4.2.2. Interaction of TRPV1 and TRPA1 via signalling pathways

A potential interaction of TRPV1 and TRPA1 by PKA signalling pathways was investigated in this part of my thesis. The second messenger cAMP is responsible for the transfer of the inactive state of protein kinase A (PKA) into its active state. I investigated whether the activation of TRPA1 by MuO might increase PKA activity and subsequently sensitize TRPV1. The activity of wt TRPV1 after stimulation of wt TRPA1 with mustard oil (MuO) was examined performing whole cell patch clamp and calcium imaging experiments in heterologous expression systems and native cultured dorsal root ganglion (DRG) neurons. Furthermore, the intracellular cAMP content of HEK Tet-On cells transfected with wt TRPV1 and wt TRPA1, with or without MuO-evoked TRPA1 stimulation was measured, as well as the capsaicin-induced mutant TRPV1 activity after wt TRPA1 activation. The phosphorylation of TRPV1 after TRPA1 activation was determined using immunoprecipitation and western blot analysis.

Modulation of wt TRPV1 activity after MuO-induced TRPA1 stimulation

To explore the modulation of capsaicin-induced $[Ca^{2+}]_i$ increase by the activation of TRPA1, HEK Tet-On cells were first transiently transfected either with wt TRPV1 or with wt TRPA1. Experiments in Figure 4.2.4 indicate that MuO is a specific ligand for TRPA1 and capsaicin a specific ligand for TRPV1 because neither ligand was active in the complementary assay.

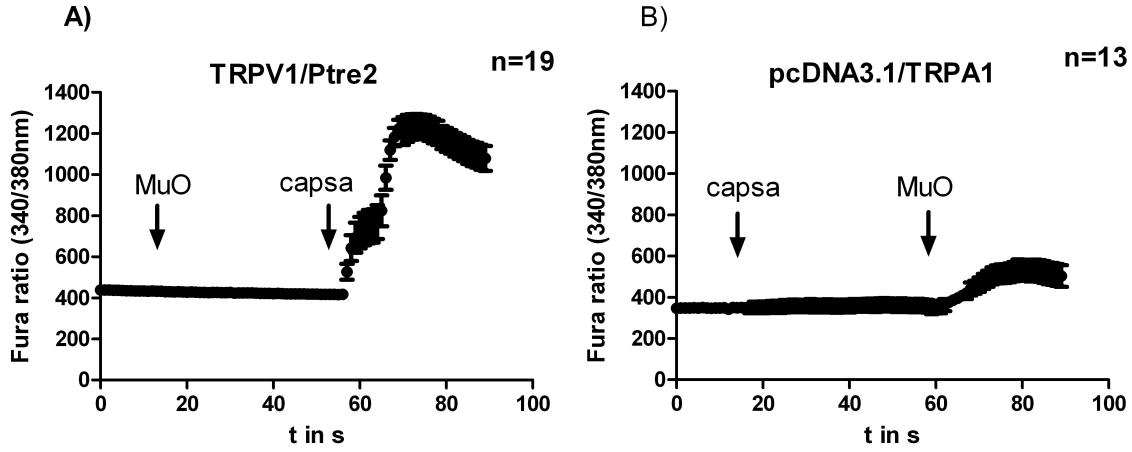


Fig.4.2.4. Fura ratio of A) HEK Tet-On cells only expressing TRPV1. MuO did not induce any response in TRPV1 expressing cells. B) HEK Tet-On cells only expressing TRPA1 did not respond to capsaicin stimulation but did so to MuO.

However, in cells transfected with both TRP channels, MuO pretreatment significantly increased capsaicin responses (t-test, $p<0.001$; Fig. 4.2.5).

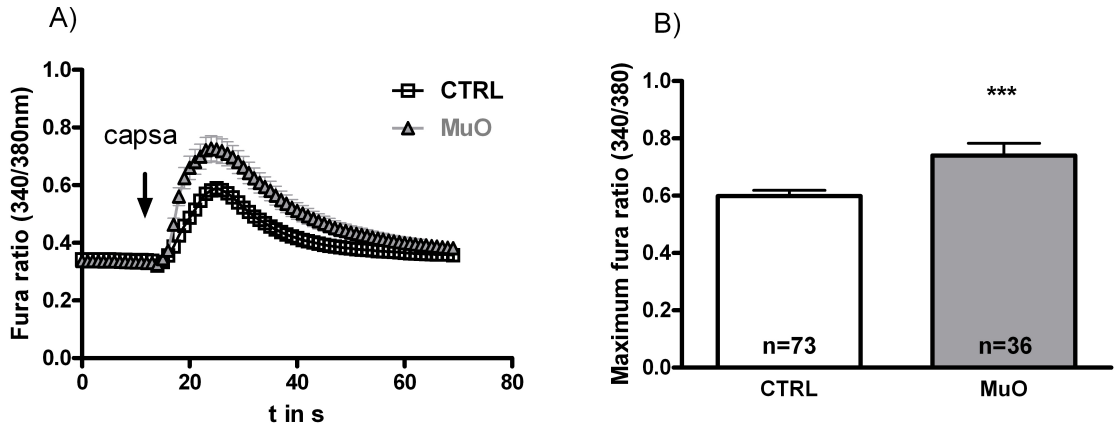


*Fig. 4.2.5. Capsaicin (100 nM) induced change of the fura ratio over time (A) and maximum change of the fura ratio (B) of wt TRPV1 and wt TRPA1 expressing HEK Tet-On cells without (CTRL) or with MuO pretreatment (20 µM, 2 min)(MuO). MuO pretreatment significantly increased fura ratio (unpaired t-test, ***, p<0,001).*

Whole cell patch clamp experiments confirmed these results. MuO pretreatment significantly increased TRPV1 currents after capsaicin stimulation (CTRL: I = 0.73 ± 0.09 nA; MuO: I = 2.38 ± 0.52 nA). In the absence of TRPA1 this increase was not detectable (Fig. 4.2.6).

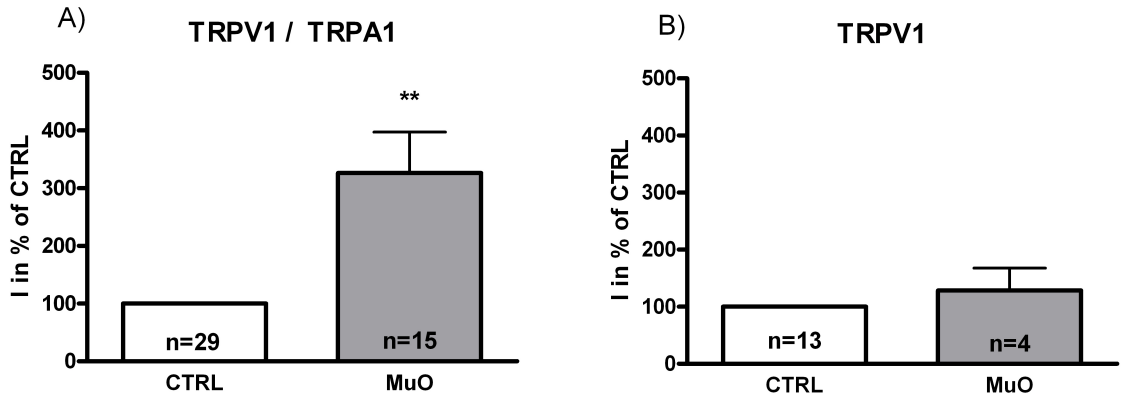


*Fig. 4.2.6.A) Capsaicin-induced TRPV1 currents of TRPV1 / TRPA1 expressing HEK Tet-On cell without (CTRL) and with pretreatment (MuO) by MuO (20 µM for 2 min) (t-test, **, p<0,01). B) MuO pretreatment did not increase capsaicin responses in the absence of TRPA1 (mock transfection: TRPV1/Ptre2) (t-test, n. s., p>0,05).*

In the absence of extracellular calcium, MuO pretreatment did not increase capsaicin-induced currents at TRPV1 (Fig. 4.2.7. A). An increase in capsaicin-induced TRPV1 currents after MuO stimulation was blocked by addition of a cell permeable PKA-inhibitor (H89) (Fig. 4.2.7. B).

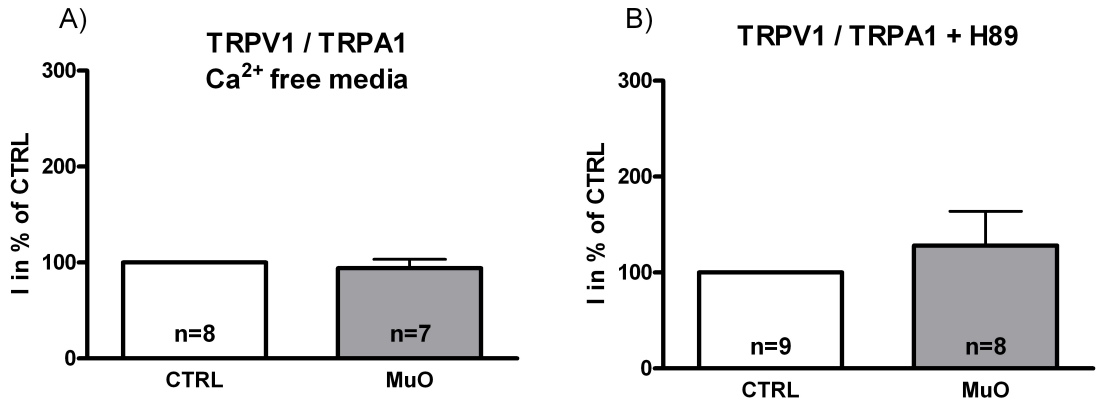


Fig. 4.2.7. TRPV1 and TRPA1 co-expressing cells were investigated in the absence of extracellular calcium (A) and in the presence of H89 (B). MuO pretreatment did not increase capsaicin responses at TRPV1 in the absence of calcium (A) and in the presence of H89 (B).

Change of the intracellular cAMP concentration after TRPA1 activation

The amount of cAMP was measured after MuO stimulation using an enzyme linked immunosorbant assay (ELISA). The accumulation of cAMP was normalized to cells without MuO pretreatment. cAMP increased significantly after MuO pretreatment. However, this increase was not detectable in the absence of calcium or TRPA1 (Fig. 4.2.8.).

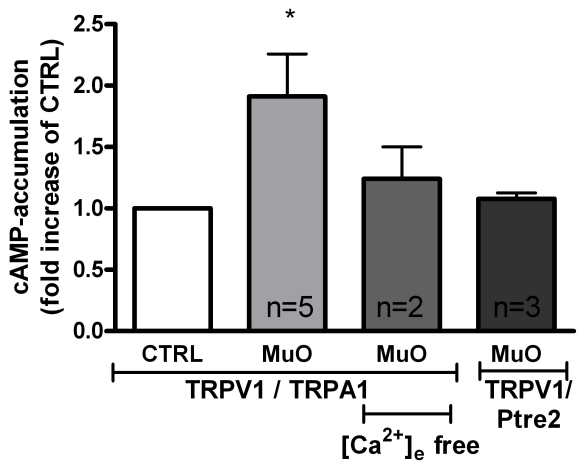


*Fig. 4.2.8. Increase of cAMP in TRPV1/TRPA1 and TRPV1/Ptre2 expressing cells after MuO pretreatment. cAMP increased significantly after MuO stimulation, however, not without extracellular calcium or TRPA1 (1way ANOVA, Dunnet post hoc test, *, P<0.05).*

Phosphorylation of TRPV1

An increase in cAMP, as shown in hypothesis 1, increases PKA activity and subsequently sensitizes TRPV1 via specific PKA phosphorylation sites. Therefore, phosphorylation of TRPV1 was investigated using immunoprecipitation and western blot analyses. In the presence and absence of MuO treatment, cultured and transfected cells were lysed and immunoprecipitated with an antibody directed against TRPV1. Western blots were incubated with antibodies directed against phosphorylated serine and threonine residues. Band intensity of MuO pretreated cells incubated with the Phosphoserine antibody showed an increase compared to control cells (Fig. 4.2.9 A). Band intensity of MuO pretreated cells incubated with the Phosphothreonine antibody increased significantly compared to control cells (unpaired t-test, $p<0.01$, n=3) (Fig. 4.2.9 B) indicating a stronger phosphorylation of TRPV1.

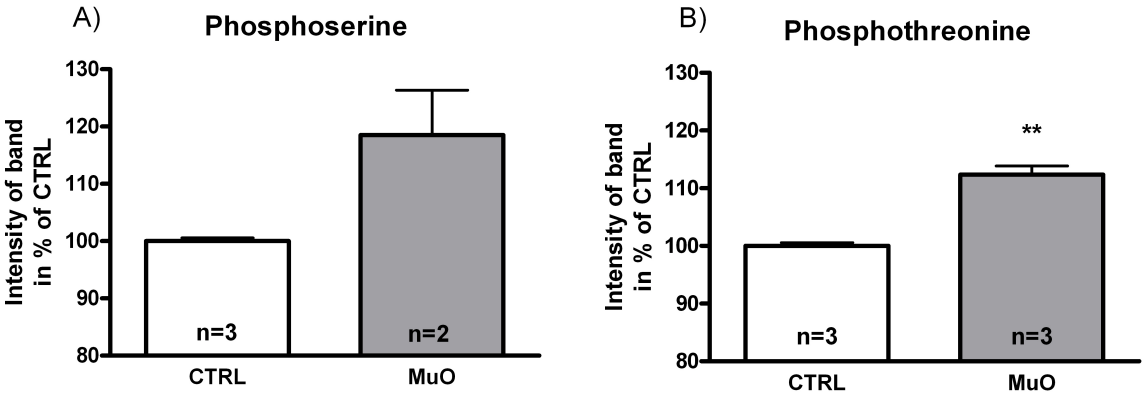


Fig.4.2.9. Band intensities of TRPV1 immunoprecipitated TRPV1/TRPA1 expressing cells without (CTRL) and with MuO pretreatment (MuO) using antibodies against phosphorylated serine (A) and threonine (B) residues.

Modulation of mutant TRPV1 activity after MuO pretreatment

To further delineate the TRPV1 amino acid residues involved, the capsaicin-induced activity of mutant TRPV1 was investigated using calcium imaging and whole cell patch clamp experiments. HEK Tet-On cells were transiently co-transfected with mutant S116A-, T144A-, S502A- or S774A-TRPV1 and wild type TRPA1. The capsaicin-induced increase of intracellular calcium, indicated by the maximum increase of the fura ratio, is shown in Fig. 4.2.10. MuO pretreatment significantly increased capsaicin-induced TRPV1 activity in T144A and S502A mutants but not in S116A and S774A mutants (Fig. 4.2.10).

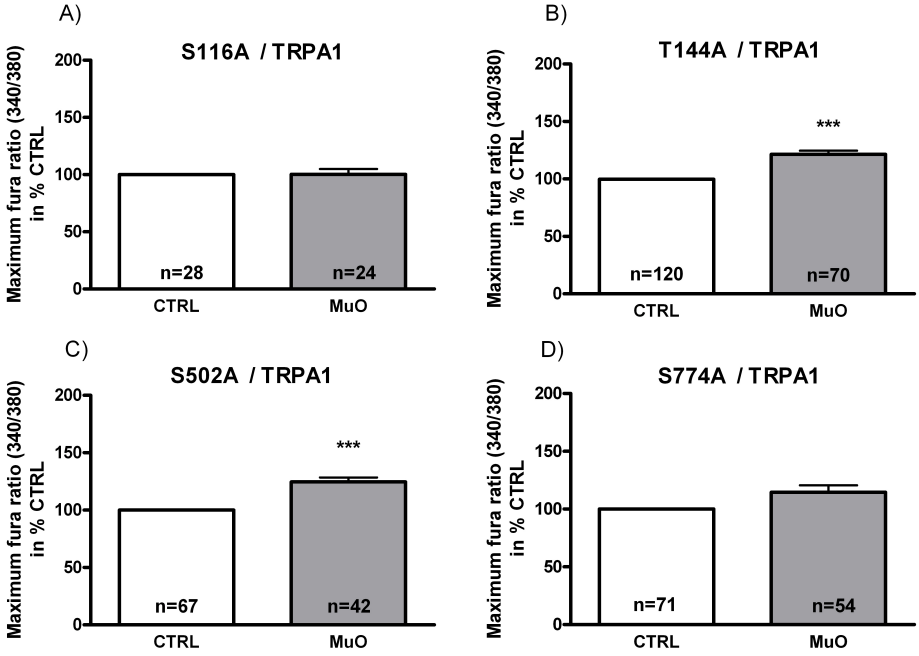


*Fig. 4.2.10. Capsaicin-induced maximum fura ratio in % of CTRL of cells transfected with mutant TRPV1 (S116A (A), T144A (B), S502A (C) and S774A (D) and wt TRPA1) after MuO pretreatment.Unpaired t-test, n. s., $p>0.05$ (A and D) and ***, $p<0.001$ (B and C).*

In whole cell patch clamp experiments, MuO pretreatment increased capsaicin-induced TRPV1 activity significantly in T144A mutants but not in S116A, S502 and S774A mutants (Fig. 5.2.11).

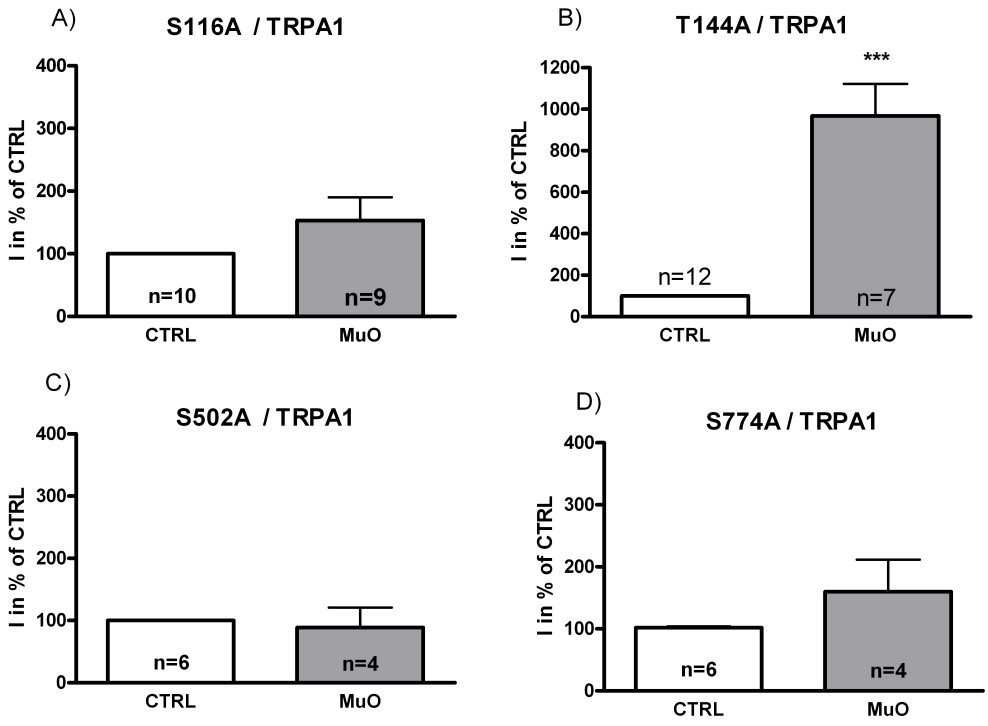


*Fig. 4.2.11.Capsaicin-induced TRPV1 currents in cells co-transfected with mutant TRPV1 (S116A (A), T144A (B), S502A (C) and S774A (D)) and wt TRPA1. MuO pretreatment significantly increased capsaicin-induced TRPV1 activity in T144A mutants (unpaired t-test, ***, P<0.001), however not in S116A, S502A and S774A mutants.*

Modulation of TRPV1 activity after MuO pretreatment in native sensory neurons

Whole cell patch clamp experiments were performed to determine the relevance of these TRPV1 sensitizing mechanisms in cells endogenously expressing TRPV1 and TRPA1. Without MuO pretreatment, 100 nM capsaicin induced a mean TRPV1 current of I = -0.43 ± 0.15 nA. MuO pretreatment significantly increased capsaicin-induced TRPV1 activity (I = -1.28 ± 0.4 nA) (unpaired t-test, *, $p<0.05$). This effect was blocked by removal of extracellular calcium or addition of the PKA inhibitor H89 (Fig. 4.2.12).

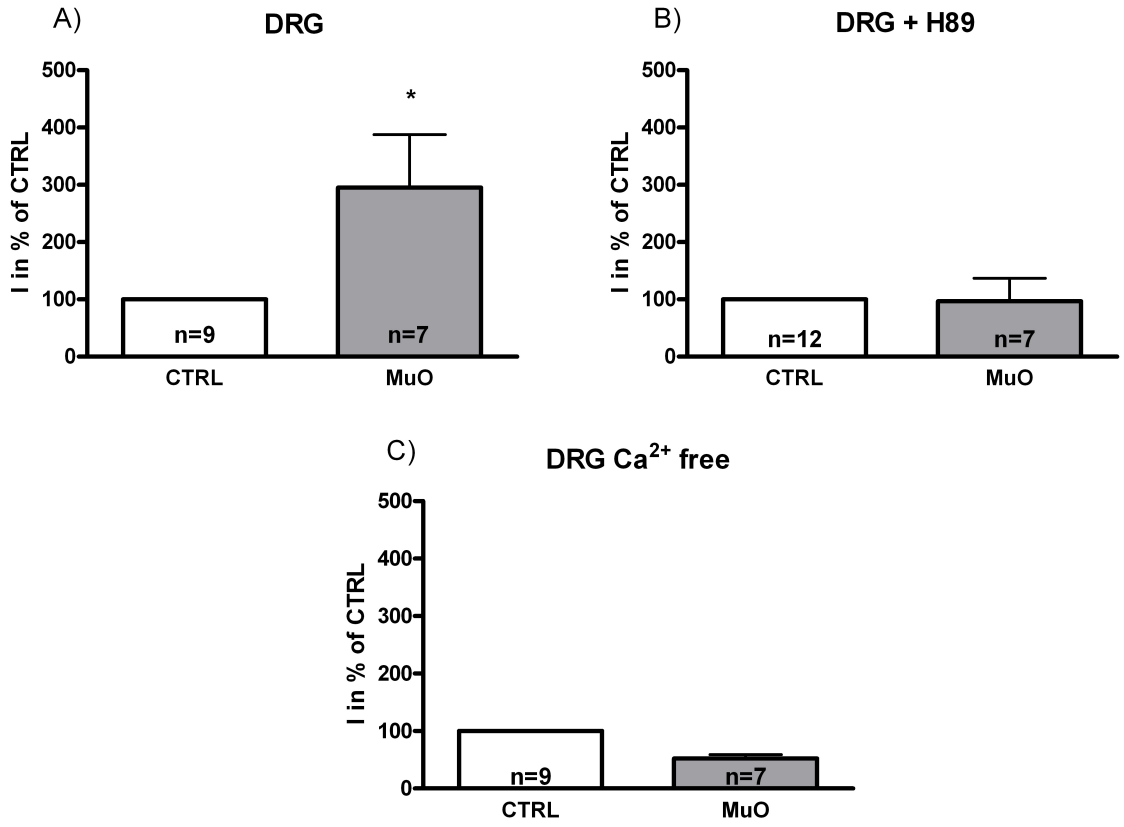


Fig. 4.2.12.Capsaicin-induced TRPV1 currents after MuO pretreatment in sensory neurons (A), in the presence of PKA inhibitor H89 (B) and in the absence of extracellular calcium (C). MuO increased TRPV1 activity. This increase was blocked by H89 and removal of extracellular calcium.

5. Discussion

The TRPV1 channel plays a pivotal role in the excitation of peripheral sensory neurons during painful injury and inflammation of peripheral tissues. In the present studies I investigated two different scenarios leading to the sensitization of this channel: opioid withdrawal and interaction with TRPA1. The main findings were:

Hypothesis 1: Opioid withdrawal sensitizes TRPV1 in a cAMP/PKA dependent manner.

1. I found a significant increase of capsaicin-induced TRPV1 activity during opioid withdrawal.
2. Opioid withdrawal did not change the expression of TRPV1.
3. Phosphorylation of serine and threonine residues at TRPV1 was enhanced during opioid withdrawal. Phosphorylation sites include threonine 144 and serine 774 but not serine 116 and serine 502.
4. Inhibition of adenylylcyclases and downregulation of isoform 3, but not 5, reversed the enhanced TRPV1 activity during opioid withdrawal.
5. Both opioid withdrawal- and capsaicin-induced hyperalgesia were apparently mediated by TRPV1 and PKA in vivo.

Hypothesis 2: TRPA1 stimulation sensitizes TRPV1 in a cAMP/PKA dependent manner.

6. TRPA1 activation increased capsaicin-induced TRPV1 activity in a cAMP/PKA dependent manner.
7. An increase in cAMP/PKA after TRPA1 stimulation resulted in an increase in TRPV1 phosphorylation.
8. Phosphorylation sites at TRPV1 include serine 116 and serine 774 but not threonine 144.
9. Similar mechanisms underly TRPV1 sensitization in transfected HEK cells and in native sensory neurons.

5.1. Hypothesis 1: Opioid withdrawal sensitizes TRPV1 in a cAMP/PKA dependent manner.

5.1.1. Increased TRPV1 activity during opioid withdrawal

In the first part of my thesis, I was able to show a significant increase in capsaicin induced TRPV1 activity during opioid withdrawal using whole cell patch clamp and calcium imaging experiments. We identified enhanced phosphorylation of TRPV1 during opioid withdrawal using immunoprecipitation and western blot analysis, and detected a crucial role for PKA phosphorylation of residues threonine 144 and serine 774 at TRPV1. Out of 9 known adenylylcyclase isoforms I found isoform 3 to be the most important in our expression system. Behavioural experiments revealed the physiological relevance of TRPV1 sensitization during opioid withdrawal in vivo.

Administration of opioids is common for treatment of moderate to severe pain. Paradoxically, the chronic intake of opioids may lead to a phenomenon termed “opioid induced hyperalgesia (OIH)”. However, this term has caused confusion in the literature. What has mostly been described is, in fact, a long known phenonemon in animals (Celerier et al. 2000; Laulin et al. 1998; Mao et al. 2002a, 2002b), volunteers (Angst and Clark 2006; Angst et al. 2003; Compton et al. 2003; Hood et al. 2003; Koppert et al. 2003) and patients (Davis et al. 2007; Joly et al. 2005; Singla et al. 2007) occurring during withdrawal from opioids. The earliest reports appeared during the nineteenth century. It was recognized that a potent analgesic such as morphine could actually cause an enhancement of pain (Rossbach 1880). This was replicated in numerous pre-clinical and clinical studies (Baron and McDonald 2006; Celerier et al. 2001; Celerier et al. 2000; Compton et al. 2001; Guignard et al. 2000; Mao 2002; Mao et al. 1995). Although the precise molecular mechanisms are not yet understood, hyperalgesia is generally thought to result from neuroplastic changes in the peripheral and central nervous systems that lead to sensitization of pronociceptive pathways. Examples are changes of the central glutaminergic system, increased levels of spinal dynorphin, elevated release of excitatory neuropeptides such as CGRP from primary afferents and activity of a subset of neurons within the rostral ventromedial medulla that facilitate spinal nociceptive processing (Chu et al. 2008; Gardell et al. 2002; Mao et al. 1994; Mao et al. 2002a; Morgan et al. 1992; Narita et al. 2001; Zeitz et al. 2001).

Opioid induced hyperalgesia is still discussed controversially. So far pain hypersensitivity was only measured either in the presence of extraordinarily high opioid concentrations or

after removal of opioids (opioid withdrawal) (Davis et al. 2007; Fishbain et al. 2009; Pud et al. 2006; Singla et al. 2007). We found hyperalgesia in behavioural animal experiments only during opioid withdrawal, not in the presence of an opioid. Therefore, in the current thesis this phenonemon is termed opioid withdrawal-induced hyperalgesia (OWIH).

Abrupt termination of chronic opioid use produces several intense withdrawal syndromes in animal models and humans. Besides hyperalgesia, withdrawal syndroms include tachycardia, hypertension, nausea, vomiting, hyperthermia, diarrhoea, piloerection and dysphoria. In extreme cases some of them can be life-threatening.

Potential mechanisms of OWIH include an upregulation of PKC and subsequent increased activity of NMDA-receptors (Mao et al. 1994) or release of spinal dynorphin, which can cause the activation of pronociceptive signalling pathways (Vanderah et al. 2000) as well as phosphorylation and sensitization of α_2-adrenergic and adenosine receptors (Aley et al. 1995; Aley and Levine 1997c). Opioid withdrawal might also reveal a compensatory upregulation of AC activity (Nestler 1992; Sharma et al. 1975). Whereas acute opioid exposure inhibits AC activity resulting in a reduction of intracellular cAMP, repeated or prolonged opioid exposure leads to a compensatory upregulation of the cAMP pathway, including increased concentrations of ACs and PKA. Although this has not been shown so far, it is hypothesized that an increased PKA activity might counteract the inhibitory effect of acute opioid application or might increase pain sensitivity. A potential mechanism to explain enhanced cAMP levels after opioid withdrawal has been postulated by Chakrabarti and colleagues: a shift of MOR-coupled signalling from predominantly $G_{\alpha i}$-inhibitory to $G_{\beta\gamma}$-stimulatory might mitigate the persistent inhibition of AC by chronic opioid treatment. These authors also showed that chronic morphine augmented association of protein phosphatase 2A and $G_{\alpha s}$, resulting in a dephosphorylation of the endogenous phosphoprotein $G_{\alpha s}$. Dephosphorylated $G_{\alpha s}$ increases the association of G_s with MOR leading to an enhanced availability of $G_{\beta\gamma}$, which in turn activates $G_{\beta\gamma}$ sensitive AC isoform resulting in an elevated cAMP synthesis and subsequent phosphorylation of proteins via PKA (Chakrabarti and Gintzler 2007; Gintzler and Chakrabarti 2006).

Up-regulation of the cAMP pathway effects cell signalling in several ways for example the transcription factor cAMP response element binding protein (CREB). Many genes consists of consensus cAMP response element (CRE) sites in their promotors to which CREB dimers bind, including neuropeptides, neurotransmitter synthesizing enzymes, neurotransmitter receptors, signalling proteins, and other transcription factors (Lonze and Ginty 2002; Mayr and Montminy 2001; Chao and Nestler 2004).

The response to repeated morphine and its withdrawal was extensively investigated in the locus coeruleus (LC) (Nestler 2001). Upon chronic opioid administration and withdrawal, CREB expression was increased in the LC, resulting in an enhanced expression of AC 8 and tyrosine hydroxylase (Lane-Ladd et al. 1997; Chao et al. 2002). Tyrosine hydroxylase catalyzes the conversion of L-tyrosine to dihydroxyphenylalanine (DOPA), a precursor for dopamine, which is in turn a precursor for noradrenaline and adrenaline (Kaufman 1995).

Another, opioid withdrawal affected and investigated area in the brain is the nucleus accumbens (NA), which is thought to play an important role in the reward system. Up-regulation of cAMP and CREB in this region may also contribute to states of dysphoria seen in early withdrawal (Hyman and Malenka 2001; Nestler 2001), because a target of CREB in the NA is dynorphin, whose release is contributed to dysphoria through a negative feedback loop to ventral tegmental area dopamine neurons (Hyman and Malenka 2001; Shippenberg and Rea 1997).

Immediate early genes, which encode transcription factors, also play a role in addiction and opioid withdrawal. The Fos family include ΔFosB, which acts as either a transcriptional inducer or repressor (Chao and Nestler 2004). ΔFosB isoforms are very stable and their levels gradually accumulate with repeated drug exposure and persist for weeks after the drug is withdrawn (Andersson et al. 2003; Chen et al. 1997; Kelz and Nestler 2000; Moratalla et al. 1996). Overexpression of ΔFosB using transgenic mice showed increased AMPA glutamate receptor subunit GluR2 expression (Kelz et al. 1999).

Despite the abundant information of central neuronal modulations during opioid withdrawal, very little is known about modulations in the peripheral nervous system. Since we know that OWIH is associated with different forms of hyperalgesia (e.g. burning sensation), that TRPV1 can mediate hyperalgesia and that TRPV1 is a target for cAMP/PKA mediated phosphorylation (Bhave et al. 2002; Mohapatra and Nau 2003; Rathee et al. 2002; Vetter et al. 2008), we postulated an increased TRPV1 activity during opioid withdrawal.

5.1.2. TRPV1 expression and opioid withdrawal

Reasons for increased TRPV1 activity during opioid withdrawal might include enhanced expression of the protein on the membrane surface, rapid translocation of inactive monomers to the membrane or sensitization of the channel via phosphorylation. Chen et al. found increased TRPV1 mRNA levels in the spinal cord and sciatic nerve in rats after prolonged treatment with morphine. However, total TRPV1 protein levels were unaltered (Chen et al. 2008). This was similar in my experiments. TRPV1 binding sites expressed on cell

membranes did not change during opioid withdrawal. However, Chen and others demonstrated that chronic morphine treatment can result in an increase in the phosphorylation of p38, ERK and JNK in DRG neurons (Cui et al. 2006; Ma et al. 2001) which can subsequently regulate gene products such as c-fos, brain derived neurotrophic factor (BDNF), neurokinin 1, and CGRP through phosphorylation of CREB (Lonze and Ginty 2002; McClung and Nestler 2003). Thus, an upregulation of TRPV1 gene products without an increase in TRPV1 protein expression at the cell surface is still conceivable. Future experiments should exclude the possibility that intracellular stores of inactive TRPV1 monomers might increase during chronic opioid therapy. However, as our experiments have shown, opioid withdrawal seems to not increase the number of functionally active TRPV1 channels at the cell membrane.

5.1.3. Increased phosphorylation of TRPV1 during opioid withdrawal

Sensitization of ion channels can occur rapidly by posttranslational regulation via phosphorylation (Woolf and Costigan 1999). TRPV1 is known to have multiple phosphorylation sites for several protein kinases including PKA, PKC, CaMKII, Src kinase and Cyclin-dependent kinase 5 (Cdk5). Potential target sites of Src kinase and Cdk5 are Y200 and T407, respectively (Pareek et al. 2007; Zhang et al. 2005). CAMKII is reportedly involved in TRPV1 phosphorylation at S502 and T704 (Jung et al. 2004). PKC is implicated in phosphorylation of T370, S502, T704 and S800, and PKA in phosphorylation of S6, S116, T144, S502, S774 and S820 (Bhave and Gereau 2004; Bhave et al. 2002).

In this study, I concentrated on PKA phosphorylation sites because the cAMP/PKA pathway is known to be upregulated during prolonged opioid treatment. I was able to identify enhanced phosphorylation of serine and threonine residues of TRPV1 using immunoprecipitation and western blot analysis during opioid withdrawal. These findings are consistent with studies exploring ^{32}P-orthophosphate incorporation by TRPV1 following pre-incubation with 8-Br-cAMP (a membrane-permeable activator of PKA) (Jeske et al. 2008) and in vivo phosphorylation (Lee et al. 2005). This and another group identified the scaffolding protein A-kinase Ankyrin Protein 150 (AKAP150) as indispensable for PKA translocation to TRPV1 on the plasma membrane, resulting in phosphorylation and sensitization of TRPV1 (Jeske et al. 2008; Schnizler et al. 2008). They also found that AKAP150 co-localized with regulatory subunit II of PKA (RII) and TRPV1, consistent with a mechanism first proposed in 2002 by Rathee and colleagues, who demonstrated that administration of AKAP-inhibitors attenuated FSK-stimulated translocation of the PKA

catalytic subunit to the plasma membrane in DRG neurons (Jeske et al. 2008; Rathee et al. 2002). Thus, my findings are consistent with the notion that prolonged opioid treatment upregulates cAMP/PKA and subsequent PKA-mediated phosphorylation of TRPV1 at the cell membrane.

5.1.4. Mutation of threonine 144 and serine 774, but not serine 116 and serine 502, resulted in a loss of increased TRPV1 activity during opioid withdrawal

So far, posttranslational modulations of TRPV1 resulting in sensitization were mainly attributed to phosphorylation by PKC (Bhave et al. 2003; Cesare and McNaughton 1996; Crandall et al. 2002; Liang et al. 2001; Numazaki et al. 2002; Sugiura et al. 2002; Vellani et al. 2001), while phosphorylation by PKA was thought to underly prevention or reduction of TRPV1 desensitization. Capsaicin-induced TRPV1 currents exhibit complex desensitization patterns with several kinetic components. Acute desensitization occurs during sustained capsaicin application, whereas decreasing currents with multiple capsaicin applications have been termed tachyphylaxis (Bhave et al. 2002; Koplas et al. 1997; Liu and Simon 1996). It was postulated that prevention of acute desensitization is accomplished through phosphorylation by CamKII and hindrance of tachyphylaxis by PKA (Bhave et al. 2002; Jung et al. 2004; Mohapatra and Nau 2003). Additionally, phosphorylation at serine 116 was mentioned as the major event preventing tachyphylaxis. However, Mohapatra and colleagues also identified threonine 370 as an important site regarding prevention of tachyphylaxis, which was inconsistent with findings of Bhave and colleagues (Bhave et al. 2002; Mohapatra and Nau 2003).

Although, phosphorylation of TRPV1 is usually linked with prevention of desensitization, several studies also described a PKA mediated sensitization of TRPV1 at different phosphorylation sites such as threonine 144, threonine 370 and serine 502 (Lopshire and Nicol 1998; Rathee et al. 2002; Pitchford and Levine 1991). Since we found different phosphorylation residues of TRPV1 during opioid withdrawal, we investigated mutations where serine 116, threonine 144, serine 502 and serine 774 were replaced by alanins. We chose S116, T144 and S502 because numerous studies provided evidence that these are functional PKA phosphorylation sites. T144 and S502 are thought to be involved in potentiation of heat currents and S116 in prevention of tachyphylaxis. Since less is known about the functional role of S774 we also decided to include it in our investigations.

Using whole cell patch clamp and calcium imaging experiments, we found an impaired TRPV1 activity in TRPV1 mutants T144A and S774A during opioid withdrawal compared to wild type TRPV1. This indicates that both PKA phosphorylation sites are involved in sensitization during opioid withdrawal. However, it does not exclude the possibility that additional phosphorylation sites, e.g. T370 or T704, play a role as well. The involvement of T144, T370 and S502 in PKA mediated sensitization was shown by Rathee and colleagues (Rathee et al. 2002). We were unable to show a functional role of S502 in our assay. This might be related to different isoforms of PKA activated by opioid withdrawal. Another explanation might be that Rathee et al. replaced S502 not only by alanine but also by aspartate, which represents a constitutively phosphorylated form of TRPV1. Additionally, they used heat as TRPV1 activator. Other studies reported phosphorylation of S502 by PKC and CaMKII (Bhave et al. 2003; Jung et al. 2004) or detected T144 and T370 as target residues for calcineurin, a phosphatase responsible for dephosphorylation of TRPV1 (Jeske et al. 2006).

We also found a functional role for S774 during opioid withdrawal. This is the first demonstration of involvement of S774 in PKA-mediated sensitization of TRPV1, albeit other studies described its role in prevention of tachyphylaxis and acute desensitization (Bhave et al. 2002; Mohapatra and Nau 2003). So far, phosphorylation of S116 by PKA was not reported to contribute to TRPV1 sensitization. Consistently, we found enhanced capsaicin-evoked S116A TRPV1 activity during opioid withdrawal, suggesting that S116 is not directly involved in TRPV1 sensitization.

In summary, TRPV1 is apparently phosphorylated under basal conditions (autophosphorylation) and dephosphorylated via calcium dependent activation of calcineurin upon activation. Rephosphorylation of TRPV1 by PKA, PKC and CaMKII not only prevents desensitization, but also sensitizes TRPV1 and reduces its activation thresholds (Lee et al. 2005). We identified PKA phosphorylation sites T144 and S774 as important targets mediating the sensitization of TRPV1 during opioid withdrawal.

5.1.5. Downregulation of adenylylcyclase isoform 3, but not 5, reverses the enhanced TRPV1 activity during opioid withdrawal

Nine AC isoforms have been described and are classified into 4 groups. Group 1 (AC 1, 3 and 8) are stimulated by calmodulin in a calcium-dependent manner. Group 2 (AC 2, 4 and 7) are conditionally stimulated by $G_{\beta\gamma}$-subunits and activated by PKC. Group 3 (AC 5 and 6) are stimulated by $G_{\alpha s}$ and inhibited by $G_{\alpha i}$-subunits, PKA and calcium. Group 4 (AC 9) is

relatively insensitive to FSK (Hacker et al. 1998) and inhibited by calcineurin and PKC (Cumbay and Watts 2004; Paterson et al. 2000).

Using the the nonspecific AC inhibitor SQ 22,356 we were able to block an increase in capsaicin-induced TRPV1 activity during opioid withdrawal. Previous experiments in our laboratory have shown that mRNA transcripts of AC 3 and 5 are significantly up-regulated during opioid withdrawal (Endres-Becker 2007). In calcium imaging experiments, increased TRPV1 activity during opioid withdrawal was prevented by down-regulating AC 3 but not AC 5. This may be due to a lower expression of isoform 5 during opioid withdrawal. Hence, AC 3 might play a more prominent role than AC 5 in the sensitization of TRPV1 during opioid withdrawal. Indeed, in our prior experiments mRNA levels of AC 3 were 4-fold increased compared to a 1.7-fold increase of AC 5 transcripts. Expression levels of isoforms 2, 6 and 9 were unchanged and AC 1, 4, 7 and 8 were not detectable (Endres-Becker 2007).

Emerging evidence suggests that cAMP superactivation can be caused by enhanced $G_{\alpha s}$-receptor coupling, G-protein dissociation and $G_{\alpha s}$-AC interactions (Chakrabarti and Gintzler 2007; Shy et al. 2008; Watts and Neve 2005). However, the molecular mechanisms still remain unclear. Recent evidence suggests that signalling via G-proteins can be regulated by receptor independent accessory proteins (Takesono et al. 1999). A member of this protein class is an activator of G-protein signalling 3 (AGS3). AGS3 binds to $G_{\alpha i}$-GDP, enhances unbound free $G_{\beta\gamma}$ stimulation of AC 2 and 4, and/or diminishes $G_{\alpha i}$-GTP inhibition of AC (Blumer et al. 2007; De Vries et al. 2000; Kimple et al. 2002; Takesono et al. 1999; Yao et al. 2005). Knockdown of AGS3 expression or inhibition of $G_{\beta\gamma}$ resulted in a blockade of morphine-induced cAMP/PKA signalling in primary nucleus accumbens/striatal neurons (Yao et al. 2005).

A possible shift from inhibitory to stimulatory G-proteins during opioid withdrawal can also result in an activation of the PLC/PKC pathway. Several AC isoforms including AC 3 (weak) and 5 are positively regulated by PKC (Watts and Neve 2005). Furthermore, PKC is known to activate voltage-dependent calcium channels, leading to an augmentation of intracellular calcium which may activate calcium-sensitive AC such as AC 3 but is known to inhibit AC 5. PKA activity is able to enhance PKCε activity via PLC and its anchoring protein εRACK (Yao et al. 2008), promoting PKCε stimulation of AC 5 and AC 7, resulting in a positive feedback in cAMP production.

In contrast to our results, a prevention of withdrawal-induced cAMP-superactivation was shown using siRNA against AC 5 (Fan et al. 2009). However, we did not directly investigate cAMP-superactivation but TRPV1 activity as a downstream effect. Moreover, Fan and

colleagues investigated nucleus accumbens/striatal neurons, where different AC isoforms are expressed. In other cell types AC 1, 5, 6 and 8 (Avidor-Reiss et al. 1997; Nevo et al. 1998), AC 6 and 7 (Bie et al. 2005) or AC 4 and 7 (Rivera and Gintzler 1998) were upregulated during prolonged opioid treatment or withdrawal. Thus, expression and regulation of AC isoforms seem to strongly depend on the expression system (tissue type, cell line, species), duration of treatment and opioid ligand used (Defer et al. 2000; Hanoune and Defer 2001).

5.1.6. Nociceptive behaviour during opioid withdrawal

To delineate the physiological relevance of TRPV1 sensitization during opioid withdrawal in vivo, we investigated the paw withdrawal latency (PWL) evoked by a radiant heat source, and capsaicin-induced nocifensive behaviour in rats. We measured a significantly decreased PWL during opioid withdrawal (thermal hyperalgesia), which was prevented in a concentration dependent manner by the MOR antagonist naloxone (NLX), the PKA inhibitor H89 and the TRPV1 antagonist capsazepine. Furthermore, opioid withdrawal significantly enhanced and H89 abolished capsaicin-induced flinching and protecting behaviour. Thus, consistent with my in vitro studies, opioid withdrawal apparently induced TRPV1-mediated hyperalgesia via PKA activation in vivo.

Numerous clinical and experimental studies identified that the development and magnitude of opioid withdrawal induced hyperalgesia differ for different types of pain, including the development of thermal hyperalgesia (reviewed in Angst et al. (Angst and Clark 2006)). Lipman and colleagues observed that study patients undergoing withdrawal had a significantly lower tolerance to heat-evoked pain (outside the 95% confidence interval) compared with healthy volunteers (Lipman and Blumenkopf 1989). In the early seventies several animal studies followed underlining the formation of thermal hyperalgesia during opioid withdrawal which is line with results of our behavioural experiments however the involvement of peripheral TRPV1 was not investigated until now (Kayan et al. 1971; Mao et al. 1994; Tilson et al. 1973; VonVoigtlander and Lewis 1983). Mao et al. carried out one of the first and most complete studies investigating the role of spinal cord in the genesis of thermal hyperalgesia during opioid withdrawal, which involve both NMDA/non-NMDA receptors and PKC translocation/activation (Mao et al. 1994). Animal studies exploring the development of hyperalgesia during opioid withdrawal in the periphery delineate the development of mechanical hyperalgesia, which was also dependent on PKC activity as well as the activation of guanosine triphosphate binding proteins (Aley et al. 1995; Aley and Levine 1997a, 1997b, 1997c; Arts et al. 1991; Khasar et al. 1995).

In my docotoral thesis I could show that the thermo-sensitive unselective cation channel TRPV1 might play an important role in the development of thermal hyperalgesia during opioid withdrawal. These findings might play a role in the future in the development of new therapeutic strategies to prevent or to treat opioid withdrawal induced hyperalgesia using e.g. specific antagonists against TRPV1.

5.2. Hypothesis 2: TRPA1 stimulation modulates TRPV1 activity in a cAMP/PKA dependent manner

Approximately 30-50 % TRPV1 expressing small to medium sized peripheral sensory neurons co-express TRPA1 and almost all TRPA1 positive neurons co-express TRPV1 (Hjerling-Leffler et al. 2007; Kobayashi et al. 2005; Linte et al. 2007; Story et al. 2003). Furthermore, currents induced by the TRPA1 agonists MuO and WIN55,212 were almost exclusively detected in TRPV1 positive cells (Diogenes et al. 2007; Jordt et al. 2004; Story et al. 2003) and both channels are activated by compounds which cause a pungent burning sensation. Thus, I hypothesized that TRPV1 sensitization can result from physical and/or functional interactions of both channels.

Numerous studies identified physical interactions of different members of the TRP ion channel family through formation of heteromultimeric complexes, whereas the most complexes are made up of monomers from different members of the same TRP channel subfamily (Bai et al. 2008; Dietrich et al. 2005; Hellwig et al. 2005; Park et al. 2008; Schilling and Goel 2004). However, there is recent evidence that members of different TRP channel subfamilies are able to form heteromultimeric complexes forming a new channel with new biophysical properties and modes of activation distinct from that of the individual channel (Bai et al. 2008; Park et al. 2008).

Additionally, there is the possibility of interactions between TRPV1 and TRPA1 via signalling pathways. Several studies were published with some inconsistent results. One group identified inhibition of TRPV1 activity by TRPA1 stimulation via calcineurin-mediated desensitization of TRPV1 (Akopian et al. 2007; Jeske et al. 2006). Calcium-dependent sensitization of TRPV1 can also occur through activation of CAMKII and PKC (Jung et al. 2004; Mandadi et al. 2004) or via activation of calcium sensitive ACs and subsequent PKA activation. Anand and colleagues detected enhanced capsaicin responses after pre-incubation with low-concentrations of the TRPA1 agonist cinnamaldehyde (CA) at 225 µM which is consistant to my results but not at higher concentrations (Anand et al. 2008).

5.2.1. TRPA1 stimulation does not alter the expression of TRPV1

Akopian and colleagues showed that TRPA1 desensitization is influenced by TRPV1 co-expression in sensory neurons and transfected CHO cells (Akopian et al. 2007). They explained this phenomenon with a stabilizing effect of TRPV1 on the membrane expression

of TRPA1. Internalization of membrane-bound channels can be suppressed by interactions with other proteins, including subunits of the channels (Bernstein and Jones 2007). Another possible mechanism for the modulation of functional TRPV1 tetramers is the cAMP-dependent release and translocation of TRPV1 monomers from intracellular pools to the cell membrane (Vetter et al. 2008). Given that TRPA1 activation can stimulate calcium-sensitive ACs and intracellular concentration of cAMP, we examined TRPV1 membrane expression after TRPA1 stimulation of co-transfected HEK293 cells. However, neither the affinity nor the number of TRPV1 binding sites changed during TRPA1 activation. These experiments indicate that TRPV1 is not recruited from intracellular pools to the cell membrane or internalized in the presence of a TRPA1 agonist in our assay.

5.2.2. TRPV1 and TRPA1 do not form complexes in transfected HEK Tet-On cells

One of the most interesting properties of the TRP ion channel family is the ability to form heteromultimeric complexes. Recently, heteromeric complexes between members of different TRP channel subfamilies were identified (Bai et al. 2008; Kobori et al. 2009; Park et al. 2008; Zhang et al. 2009). These heteromeric complexes form a channel with unique and new biophysical properties

A heteromeric formation between members of the same TRP channel subfamily was shown for the TRPV subfamily. Heteromeric channel formation have been proposed for TRPV1, TRPV3, TRPV5 and TRPV6 channel subunits (Hoenderop et al. 2003; Kedei et al. 2001; Smith et al. 2002). A study investigating homo- and heteromeric assembly of HEK 293 cells transfected with TRPV1-6 channels identified heteromeric formation of TRPV1 and TRPV2 subunits and TRPV5 and TRPV6, using Fluorescent Resonance Energy Transfer (FRET) and co-immunoprecipitation (Hellwig et al. 2005). However, heteromeric assembly of TRPV1 and TRPV2 seem to be without physiological relevance, since both channels are expressed in different tissues and cell types (Birder et al. 2002; Caterina et al. 1999; Caterina et al. 1997; Tominaga et al. 1998). TRPV1 is also thought to form heteromeric complexes with TRPV3 monomers based on the observation that TRPV3 is transcribed from a gene adjacent to TRPV1, is co-expressed in DRG neurons with TRPV1, co-precipitates with TRPV1 in heterologous expression systems and may reduce TRPV1 responsiveness to capsaicin (Smith et al. 2002). They also mentioned that except TRPV5 and TRPV6, TRPV channel subunit preferentially form homomeric pore complexes (Hellwig et al. 2005). A very recent study showed physical interaction of TRPV1 and TRPA1 subunits. These differing results might be

caused by the distict TRPA1 origin because Staruschenko *et al.* used cDNA encoding for mouse TRPA1 (Staruschenko et al. 2010).

One accepted method to prove biophysical interactions is co-immunoprecipitation. Using this method we failed to detect potential heteromeric complexes between TRPV1 and TRPA1 monomers. Together with our binding affinity studies, which did not indicate differences in the binding affinities if both proteins were expressed, we conclude that there is no direct physical interaction between TRPV1 and TRPA1.

5.2.3. TRPA1 stimulation increases TRPV1 activity in a calcium and cAMP dependent manner

Using calcium imaging and whole cell patch clamp experiments, we detected a significantly increased TRPV1 activity after pre-stimulation of the cells with MuO. We hypothesized that TRPA1 activation with MuO causes an influx of cations including calcium ions. This would elevate the activity of calcium sensitive AC, resulting in an increase of cAMP and subsequent PKA stimulation, to eventually phosphorylate and sensitize TRPV1. Indeed, the increased TRPV1 activity was not apparent in the absence of TRPA1 and extracellular calcium, or when PKA was inhibited by H89.

These findings were not in agreement with results of another group. They discovered that TRPV1 (co-expressed with TRPA1 in CHO cells or natively expressed in trigeminal ganglion neurons) was cross-desensitized through activation of TRPA1. In their studies TRPA1 activation resulted in a recruitment of the calcium-dependent calcineurin pathway, whereby calcineurin mainly dephosphorylated threonine 144 and 370 at TRPV1, causing desensitization of the channel (Akopian et al. 2007; Akopian et al. 2008; Jeske et al. 2006; Ruparel et al. 2008; Patwardhan et al. 2006).

On the other hand, MuO is known to induce neurogenic inflammation resulting in hypersensitivity to mechanical and thermal stimuli in vivo (Caterina et al. 2000). Additionally, in vivo and in vitro studies showed thermal sensitization by an elevation of intracellular calcium (Distler et al. 2003; Guenther et al. 1999; Kress and Guenther 1999). A recent study measured enhanced capsaicin responses of rat DRG neurons pre-incubated with low concentrations of cinnamaldehyde (CA) (225 µM), while high concentrations (450 µM and 2mM) caused inhibition (Anand et al. 2008). These findings are in line with our results showing that low concentrations of TRAP1 ligands increased TRPV1 activity. These effects seem to be concentration-dependent and higher concentrations of TRPA1 agonists apparently result in a switch from sensitizing PKA-mediated TRPV1 phosphorylation to desensitizing

calcineurin-mediated dephosphorylation of TRPV1. Additionally, calcium-dependent sensitization of TRPV1 can also occur through other kinases like CaMKII and PKC (Jung et al. 2004; Mandadi et al. 2004). The balance between calcium-stimulated kinase and phosphatase activities seems to result in a tightly regulated system responsible for modulating TRPV1 activity. Thus, future studies will have to determine the crucial concentrations of TRPA1 agonists and the duration of the agonist application eliciting this switch. These findings are interesting for the clinical applications since highly concentrated TRPA1 agonists might have antinociceptive effects while lower concentrations might increase pain.

The activation of TRPA1 by MuO leads to an increase of intracellular calcium since TRPA1 is a non-selective cation channel. Our laboratory has shown that calcium directly activates TRPA1 through an intracellular EF-hand domain (Zurborg et al. 2007), possibly resulting in an (auto)amplification of TRPA1 activity. We found that in the absence of extracellular calcium, TRPA1 activation failed to induce TRPV1 sensitization. In line with our findings, Distler and colleagues have shown that rat sensory neurons expressAC 1 and AC 8, which were activated by an elevation of intracellular calcium leading to an increase of cAMP and translocation of the catalytic PKA subunit. This subsequently increased TRPV1 phosphorylation (Distler et al. 2003). We also measured increased cAMP levels after MuO pre-stimulation, which were abrogated in the absence of extracellular calcium and of TRPA1 (Fig. 4.2.8.).

5.2.4. TRPV1 is phosphorylated after TRPA1 stimulation

Using immunoprecipitation of TRPV1, we detected higher band intensities in MuO pre-stimulated TRPV1/TRPA1 expressing cells utilizing phosphoserine and phosphothreonine specific antibodies (Fig. 4.2.9.). This supports the hypothesis that TRPA1 activation can increase TRPV1 phosphorylation and subsequently sensitize TRPV1. Studies of another group showed contrary results. They identified dephosphorylated TRPV1 after TRPA1 stimulation with cannabinoids investigating sensory neurons and transfected CHO cells with autoradiography and ^{32}P-incorporation assays (Jeske et al. 2006; Patwardhan et al. 2006). The differing results may be explained by the different expression systems and the longer duration of TRPA1 stimulation (10 min versus 2 min in our experiments). The latter point may be particularly important because the amount of calcium entering the cell is dependent on the duration of TRPA1 channel activation. Thus, intracellular calcium may switch from sensitizing to desensitizing concentrations. Other reasons may be the varying expression levels of calcium sensitive AC isoforms in sensory neurons, CHO cells and HEK Tet-On

cells. Future experiments will have to validate the presented phosphorylation results using another method, for example radioimmunoassay, and the investigation of PKA inhibitors.

5.2.5. Mutation of TRPV1 phophorylation sites reversed the increased TRPV1 activity after TRPA1 activation

To identify potential PKA phosphorylation sites at TRPV1 after TRPA1 stimulation we used TRPV1 mutants S116A, T144A, S502A and S774A. We found an abrogation of the increased TRPV1 activity in mutants S116A and S774A. Mutant T144A showed even stronger capsaicin-induced currents after MuO stimulation compared to wild type TRPV1. An explanation may be that this phosphorylation residue is an important target for calcineurin such that its mutation negatively influences the dephosphorylation of the receptor. This was already shown in a study delineating an important role for T144 regarding dephosphorylation by phosphatases (Jeske et al. 2006).

Results of calcium imaging and whole cell patch clamp experiments in serine 502 mutants were inconsistent in our study. I still measured an increased mutant TRPV1 activity after MuO pre-treatment using calcium imaging experiments, which was not detectable in whole cell patch experiments. A possible explanation may be the difference between the two experimental procedures. Calcium imaging depicts the entry of calcium into the cytosol not only from the extracellular space but also from intracellular stores. This is an indirect indicator for the activity of the stimulated membrane channel and it does not exclude that additional channels are involved in the calcium entry or that calcium is released from the endoplasmatic reticulum (ER). In a recent study the stimulation of ectopic but functional TRPV1 channels on the ER lead to an increase of cytosolic calcium (Gallego-Sandin et al. 2009). This issue needs to be clarified in further experiments in our assay. Whole cell patch clamp experiments are a more direct method for the investigation of membrane channel activities, since they directly record changes of currents after agonist administration.

Replacing serine 116 by alanine abolished the enhanced TRPV1 activity after MuO pre-treatment in both calcium imaging and whole cell patch clamp experiments. This is in accordance with previous studies that identified S116 as a fundamental PKA phosphorylation site for PKA-dependent TRPV1 sensitization (Bhave et al. 2002; Mohapatra and Nau 2005).

To our knowledge an important role for serine 774 as PKA phosphorylation site in TRPV1 sensitization was shown here for the first time. Previous investigations using ESI mass spectroscopy and tandem MS/MS sequencing delineated S774 as a PKA phosphorylation site,

but did not assign functional effects completely consistent with phosphorylation (Bhave et al. 2002).

Our studies concerning PKA phosphorylation sites at TRPV1 may be extended by use of phosphorylation assays or the incorporation of ^{32}P in the future. Additionally, TRPV1 double or triple mutants should be designed and investigated.

5.2.6. TRPA1 stimulation enhanced TRPV1 currents in native DRG neurons in a calcium and PKA-dependent manner

To corroborate our results obtained in transfected cell lines, we decided to investigate natively TRPA1 and TRPV1 expressing DRG neurons. We also found a significant increase of capsaicin-induced TRPV1 activity after TRPA1 activation in DRG neurons. As mentioned above, contrasting results were obtained by the group of Hargreaves, investigating cross-desensitization of TRPA1 and TRPV1 in trigeminal sensory neurons pre-stimulated with TRPA1 agonists (Akopian et al. 2007; Akopian et al. 2008; Jeske et al. 2006; Ruparel et al. 2008; Patwardhan et al. 2006). Again, the differing experimental procedures, AC expression patterns and the tightly (calcium concentration dependent) balanced system concerning calcium-dependent kinase-mediated sensitization or calcium-dependent calcineurin-mediated desensitization of TRPV1 may account for the divergent results.

5.3. Limitations, future prospects and clinical relevance

My thesis examined the sensitization of TRPV1 in two independent settings: first during opioid withdrawal and second via activation of TRPA1. Both scenarios share the same intracellular signalling pathway, i.e. activation of AC, increased cAMP levels, subsequent translocation and activation of PKA, and phosphorylation of TRPV1 at several residues (Figs. 5.1. and 5.2.). Thermal and inflammatory hyperalgesia also involves phosphorylation and sensitization of TRPV1, e.g. PGE_2 or PGI_2 - and bradykinin-receptor signalling (Sugiura et al. 2002). Thus, modulation of TRPV1 via phosphorylation apparently play a central role in sensitization, regardless of the triggering events.

My initial results were obtained in cell lines and were then confirmed in native neurons and in vivo experiments. The latter indicate an important role of TRPV1 phosphorylation in the enhanced pain sensitivity occurring during opioid withdrawal. The ultimate goal is to translate these findings into clinical studies and to derive novel therapeutic approaches.

Many in vivo experiments and clinical examinations of opioid addicts described the clinical relevance of hyperalgesia associated with the use and withdrawal of opioids (reviewed in (Chu et al. 2008). However, the experimental and/or clinical settings and methodologies were very heterogeneous, e.g. investigations of (i) former opioid addicts on methadone maintenance therapy (Compton 1994; Compton et al. 2001; Doverty et al. 2001; Schall et al. 1996), (ii) perioperative exposure to opioids in patients undergoing surgery (Chia et al. 1999; Cooper et al. 1997; Guignard et al. 2000), (iii) healthy human volunteers after acute opioid exposure using human experimental pain testing (Angst et al. 2003; Koppert et al. 2001; Koppert et al. 2003; Troster et al. 2006; Chu et al. 2006), or (iv) prospective observational studies in opioid-naïve pain patients undergoing initiation of chronic opioid therapy (Chu et al. 2006). About 90 publications describe OWIH leading to thermal or mechanical hyperalgesia in various animal models (Angst and Clark 2006). So far, most studies support the notion that neural plasticity in the central nervous system is most important during opioid withdrawal.

Some studies investigated peripheral mechanisms of OWIH/OIH. By using small peripherally acting doses of the mu-opioid agonist DAMGO they observed the development of tolerance and mechanical hyperalgesia. Adenosine A1 and A2 agonists lead to similar findings (Aley and Levine 1997c). Later the involvement of PKC and ACs in modulating this phenomenon was reported (Aley and Levine 1997a, 1997b) as well as the involvement of β2-adrenergic receptors (Liang et al. 2006).

As already mentioned above the participation of NMDA and non-NMDA excitatory amino acid receptors and also PKC was mostly contributed to mediate OWIH/OIH on the spinal level, which is until now the main explanation for OWIH/OIH (Dunbar and Pulai 1998; Mao et al. 1994). Due to these findings numerous studies were designed investigating the pharmacologic modulation of OWIH/OIH targeting spinal NMDA receptors (reviewed in (Chu et al. 2008).

My thesis provides a new mechanism underlying OWIH at the level of the peripheral nervous system. These findings indicate that both centrally and peripherally acting opioids bear the potential to induce hyperalgesia during withdrawal. Thus, even if new opioid drugs become available which act peripherally avoiding central side effects, abrupt withdrawal of such drugs must be avoided since we showed that peripheral TRPV1 receptors are sensitized and contribute to thermal hyperalgesia (Fig. 5.1.). Importantly, however, my studies have not considered the influence of an inflammatory environment, which is a major determinant for the efficacy of peripherally acting opioids (Zollner and Stein 2007). Further studies need to

examine whether other signalling pathways are involved in sensitization of TRPV1 during opioid withdrawal and how inflammation modulates TRPV1 during opioid withdrawal. Moreover, it should be clarified whether the activation of other opioid receptor types (δ-and κ-opioid receptors) alter the activity of TRPV1 in the periphery.

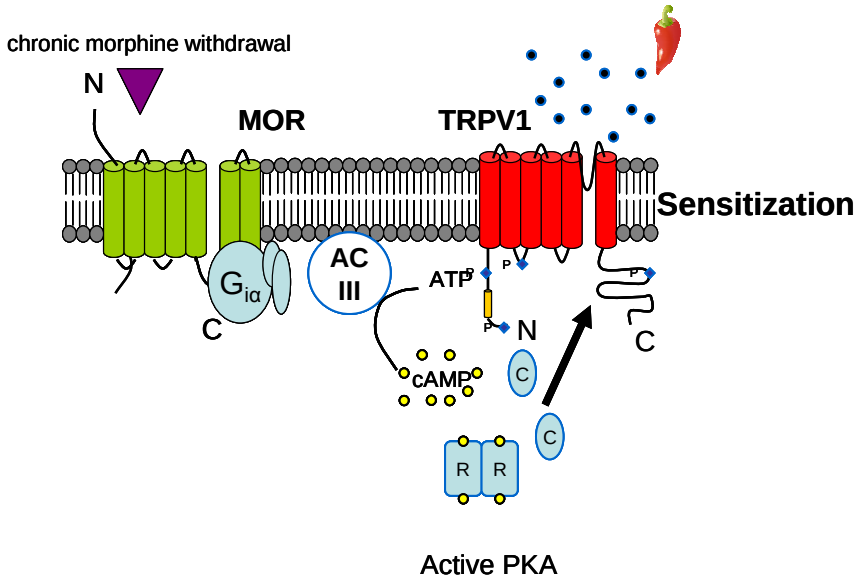


Fig. 5.1. Schematic presentation of sensitizing mechanisms of TRPV1 during opioid withdrawal in the periphery.

In the second part of my studies I showed that TRPV1 sensitization is also accomplished by functional interactions with TRPA1. Additional experiments are necessary to understand the molecular mechanisms underlying sensitizing and desensitizing effects on TRPV1 after TRPA1 stimulation. It would be most important to identify the concentrations of TRPA1 agonists, which evoke the switch from sensitizing to desensitizing actions, especially for the development and use of new analgesic therapeutics, which act at peripheral TRPV1 or TRPA1. The role of PKA phosphorylation sites of TRPV1 needs to be elucidated in more detail. Because this mechanism strongly depends on intracellular calcium concentrations, other calcium-dependent signalling pathways (e. g. PLC/PKC pathway) should be taken into account as well as the upregulation of AC isoforms after TRPA1 activation. Finally, in vivo experiments of wild type and knock out TRPV1 and/or TRPA1 mice could be helpful in understanding thermal hyperalgesia after MuO pre-stimulation. Fig. 5.2. presents a schematic description of the TRPA1 mediated TRPV1 sensitization.

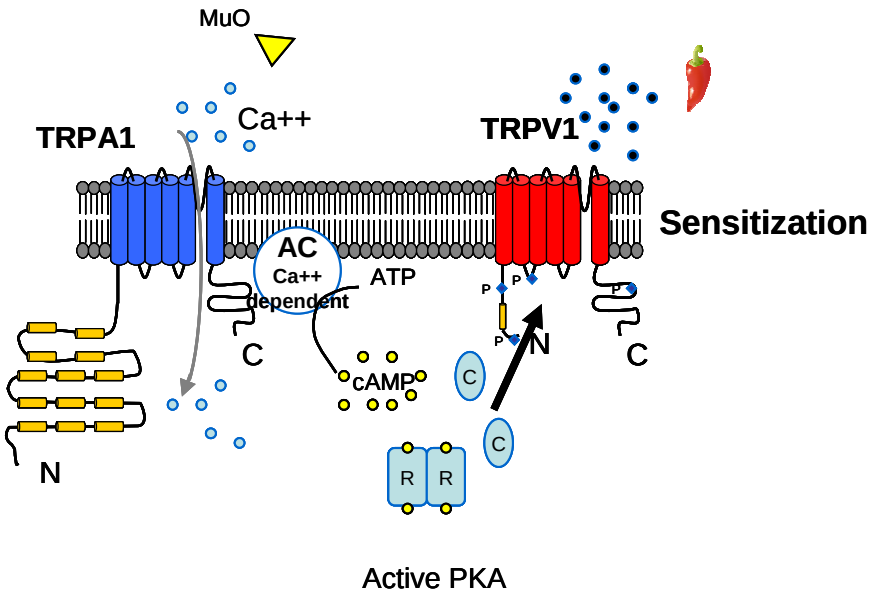


Fig. 5.2. Schematic presentation of sensitizing mechanisms of TRPV1 after TRPA1 stimulation.

6. Summary

Transient Receptor Potential Vanilloid 1 (TRPV1) is a ligand-gated ion channel expressed on sensory nerves that responds to noxious heat, protons, and chemical stimuli such as capsaicin. TRPV1 plays a critical role in the development of tissue injury, inflammation or nerve lesions. Transient Receptor Potential Ankyrin 1 (TRPA1), strongly co-expressed with TRPV1, is also activated by compounds that cause a pungent burning sensation. TRPA1 functioned as a downstream target for components of the inflammatory milieu that elicit nociceptor excitation and pain hypersensitivity.

Opioids such as morphine have been used widely for the treatment of many types of acute and chronic pain. Application of morphine leads to a dissociation of G-proteins and causes a reduced activity of adenylylcyclases (AC), resulting in a lower amount of cAMP. However, opioid withdrawal following chronic activation of the μ opioid receptor (MOR) induces AC superactivation and subsequently an increase in cAMP and Protein Kinase A (PKA) activity.

The aims of this doctoral thesis were, first, to investiagate the modulation of the activity of TRPV1 during opioid withdrawal and second, the influence of TRPA1 stimulation on TRPV1 activity.

The activity of TRPV was significantly increased during opioid in a cAMP and PKA sensitive manner. This sensitization was reversed by inhibiting ACs, silencing AC 3 and mutating PKA phosphorylation sites threonine 144 and serine 774. Furthermore, the paw withdrawal latency of male Wistar rats was significantly decreased and the nocifensive behaviour was significantly enhanced during opioid withdrawal. These results indicate a new mechanism underlying hyperalgesia during opioid withdrawal in the peripheral nervous system, which might offer new possibilities to treat withdrawal-induced painful sensations in patients.

In the second part of the study, it was proven that TRPA1 activation sensitizes TRPV1 activity in a calcium and cAMP/PKA dependent manner. TRPA1 stimulation enhanced TRPV1 phosphorylation and the involvement of PKA phosphorylation sites serine 116 and serine 774 was shown. Finally, we also detected a calcium sensitiv increased TRPV1 activity after TRPA1 activation in dorsal root ganglion neurons.

In summary, the current study delineates the sensitization of TRPV1 by two independent experimental and clinical settings. First, the sensitization of TRPV1 during opioid withdrawal and second, the sensitization of TRPV1 via activation of TRPA1. Both topics share the same intracellular signalling pathway: The action of AC, subsequent translocation and activation of

PKA due to increased cAMP levels and phosphorylation of TRPV1 at several PKA phosphorylation residues.

Zusammenfassung

Der Ionenkanal *Transient Receptor Potential Vanilloid 1* (TRPV1) ist auf sensorischen Nervenfasern exprimiert, welche auf Hitze, Protonen und chemische Reize wie Capsaicin reagieren. TRPV1 ist entscheidend an der Entstehung von Schmerz und der thermalen Hyperalgesie unter Entzündungsbedingungen beteiligt. Der Ionenkanal *Transient Receptor Potential Ankyrin* 1 (TRPA1) ist mit TRPV1 stark ko-exprimiert und wird ebenfalls durch Stoffe aktiviert, die als scharf und brennend empfunden werden. Er ist sowohl an der Nozizeptorerregung als auch an der Schmerzsensitivierung während einer Entzündung beteiligt.

Der Einsatz von Opioiden wie Morphin wird vielfach zur Linderung akuter und chronischer Schmerzsyndrome eingesetzt. Opioide bewirken die Aktivierung der Opioidrezeptoren, was die Dissoziation des gekoppelten G – Proteins hervorruft. Nachfolgend werden die Adenylatzyklasen (AC) inhibiert, die cAMP-Bildung gehemmt und demzufolge die cAMP-abhängige Protein Kinase A (PKA) nicht aktiviert. Die PKA ist entscheidend an der Sensitivierung und Resensitivierung des TRPV1 beteiligt. Ihre Hemmung resultiert in einer geringeren Aktivität des TRPV1. Andererseits induziert der Entzug von chronisch applizierten Opioiden eine „Superaktivierung“ der AC und somit einen Anstieg des cAMP-Gehaltes und Protein Kinase A (PKA) Aktivität.

In dieser Dissertation sollte untersucht werden, ob und wie 1) die Aktivität von TRPV1 während des Opioidentzugs moduliert wird und 2) ob die TRPA1 Stimulation einen Einfluss auf die TRPV1 Aktivität hat.

Es wurde gezeigt, dass die Aktivität von TRPV1 während des Opioidentzug signifikant erhöht ist. Dieser Mechanismus war cAMP und PKA abhängig. Die Sensitivierung konnte durch das Inhibieren der AC Aktivität insbesondere der Isoform 3 aufgehoben werden. Des Weiteren führte die Mutation der PKA Phosphorylierungsstellen Threonin 144 und Serin 774 zu einer Aufhebung der Sensitivierung. Durch Verhaltensexperimente an männlichen Wistar Ratten konnte weiterführend gezeigt werden, dass TRPV1 eine fundamentale Rolle an der peripheren Schmerzüberempfindlichkeit während des Opioidentzugs spielt.

Im zweiten Teil der Arbeit wurde gezeigt, dass die TRPA1 Aktivierung zu einer TRPV1 Sensitivierung führt. Die Aktivierung des TRPA1 führt zu einem Einstrom von insbesondere Kalziumionen, die kalzium-sensitive AC aktivieren können. Die erhöhte AC Aktivität resultierte in einem erhöhten cAMP-Spiegel, somit zur Translokation der PKA und schlussendlich zur Phosphorylierung und Sensitivierung des TRPV1.

Zusammenfassend beschreibt die vorliegende Arbeit die Sensitivierung von TRPV1 in zwei unabhängigen experimentellen und klinischen Situationen. Erstens, die Sensitivierung von TRPV1 während des Opioidentzugs und zweitens, die Sensitivierung des TRPV1 nach TRPA1 Aktivierung. Beide Themengebiete teilen den gleichen intrazellulären Signalweg: Die Aktivität der AC mit anschließender Translokation and Aktivierung der PKA, welche TRPV1 an bestimmten PKA Phosphorylierungsstellen phosphoryliert und somit sensitiviert. Diese Beobachtungen zeigen neue Erklärungsmöglichkeiten zur Entstehung thermaler Hyperalgesie im peripheren Nervensystem während des Opioidentzugs und nach TRPA1 Stimulation.

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8. Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

9. Publications and presentations

9.1. Publications

Spahn V, Endres-Becker J, Fischer O, Stein C and Zöllner C. Opioid withdrawal increases TRPV1 activity in a PKA dependent manner. *In preparation.*

Spahn V, and Zöllner C. TRPV1 activity is modulated by interaction with TRPA1 via PKA signalling pathways. *In preparation.*

9.2. Presentations

Poster presentations

Spahn V, Endres-Becker J, Fischer O, Schäfer M, Stein C and Zöllner C. Interaction of Transient Receptor Potential Vanilloid 1 (TRPV1) with G-protein coupled receptors and TRP ion channels. 13th World Congress On Pain, 28th August - 3rd September, 2010, Montréal, Canada

Spahn V, Fischer O, Endres-Becker J, Stein C, Schäfer M, and Zöllner C. Modulation of TRPV1 activity during opioid withdrawal. 38th annual meeting of the Society for Neuroscience, November 15-19, 2008 Washington D.C., USA

Spahn V, and Zöllner C. Opioid withdrawal increases TRPV1 activity in a PKA dependent manner. Berlin Neuroscience Forum 2008, June 5-7, 2008, Liebenwalde, Germany

Abstracts

Spahn V, Endres-Becker J, Fischer O, Stein C and Zöllner C. Interaction of Transient Receptor Potential Vanilloid (TRPV1) with G-protein coupled receptors and TRP ion channels. Presentation-No PH 158. Abstract Viewer/ Itenary Planner, Montreal: 13th World Congress On Pain, 2010. Online

Spahn V, Fischer O, Endres-Becker J, Stein C, Schäfer M, and Zöllner C. Modulation of TRPV1 activity during Opioid withdrawal. Programm-No 265.9. Abstract Viewer/Itenary Planner. Washington, DC: Society for Neuroscience, 2008. Online

Spahn V, and Zöllner C. Opioid withdrawal increases TRPV1 activity in a PKA dependent manner. Programm-No 95. Abstract USB-stick. Liebenwalde, Germany: Berlin Neuroscience Forum, 2008.

Oral presentations

Spahn V. Interaction of Transient Receptor Potential Vanilloid 1 (TRPV1) with G-protein coupled receptors and TRP ion channels. Modeling of Pain Switches-Meeting. 17. August 2009, Cottbus, Deutschland.

Spahn V . Aktuelle Ergebnisse einer industrieanthropologischen Körperbautypologie für das junge Erwachsenenalter. 7. Kongress der Gesellschaft für Anthropologie e. V.: Eine Wissenschaft in der Öffentlichkeit. September 10-14, 2007, Freiburg im Breisgau, Germany

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Selbstständigkeitserklärung

Ich versichere, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe. Stellen, die anderen Werken im Wortlaut oder Sinn entnommen sind, wurden durch Quellenangaben kenntlich gemacht. Dies gilt ebenfalls für bildliche Darstellungen.

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