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Klinik für Anästhesiologie mit Schwerpunkt operative Intensivmedizin CBF
Arbeitsbereich Experimentelle Anästhesiologie
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Habilitationsschrift

Sensitization and Inhibition of Pain

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Table of contents

Abbreviations

AC	adenylyl cyclase
AKAP	protein-A kinase anchoring protein
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ARMS	ankyrin-rich membrane spanning protein
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
DOR	δ- opioid receptor
DRG	dorsal root ganglion
FDA	US Food and Drug Administration
FF3	(±)-N-[1-(2-fluoro-2-phenylethyl)piperidine-4-yl]-N-phenyl propionamide
G-protein	heterotrimeric guanine nucleotide–binding protein
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GIRK	G protein-coupled inwardly-rectifying potassium
GPCR	G-protein coupled receptor
GRK	G-protein-coupled receptor kinase
GTP	guanosine triphosphate
IASP	International Association for the Study of Pain
MOR	μ- opioid receptor
mRNA	messenger ribonucleic acid
NFEPP	(±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide
NMDAR	N-methyl-D-aspartate receptor
NSAID	non-steroidal anti-inflammatory drug
KOR	κ- opioid receptor
pK_a	negative base-10 logarithm of the acid dissociation constant
PKA	protein kinase A
PKC	protein kinase c
S	serine
T	threonine

TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1

1. Introduction

1.1. Pain

The International Association for the Study of Pain (IASP) defines pain as "*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.*" (IASP, 2011). The European Commission stated that in the European countries, there are approximately 150 million people who suffer from different types of pain during their life. Furthermore, pain due to musculoskeletal disorders causes 50-60 % of inability to work resulting in combined direct and indirect costs about 240 billion € per year (SIP, 2018). Therefore, the adequate treatment of pain has high humanitarian and societal impact. To adequately approach this problem, mechanisms underlying pain development/sensitization in response to pathologic conditions and strategies of pain inhibition with reduced side effects have to be investigated.

Pain can be broadly divided into physiological and pathological pain. Physiological pain - also termed nociceptive pain - functions as a warning system and protects from further tissue damage. Pathological pain – for example chronic neuropathic pain – may develop from a primary lesion or disease of the somatosensory system and is usually influenced by a variety of psycho-social factors (Treede, 2015a; Stein, 2018).

Nociception is *"the neural process of encoding noxious stimuli."* (IASP, 2011). Nociception includes the transduction of noxious stimuli into electrical signals, the transmission of these signals and finally the perception of these stimuli as pain (Basbaum et al., 2009). The transduction of a harmful stimulus is initiated by the activation of peripheral sensory neurons (nociceptive neurons) whose endings are equipped with many receptors/ion channels that are activated by stimuli like temperature, pressure or chemicals (IASP, 2011). Painful thermal stimuli are transduced by several members of the transient receptor potential (TRP) ion channel family (see chapter 1.2.1.). Chemical stimuli such as protons activate e.g. acid-sensing ion channels and/or the transient receptor potential vanilloid 1 (TRPV1) (reviewed in (Gangadharan and Kuner, 2013). Mechano-transduction in mammals is still not fully understood. However, the involvement of the potassium channel family K_{2P} (TREK-1, TREK-2 and TRAAK) and of PIEZO1 and PIEZO2 channels was shown. Members of the sodium channel family DEG/ENaC and other TRP channels were identified in invertebrates but not confirmed in vertebrates, and their roles are still unclear (reviewed in (Ranade et al., 2015).

Nociceptive neurons can be separated into unmyelinated C-fibers and thinly myelinated Aδ-fibers. The cell bodies of these neurons are located in the dorsal root ganglia (DRG), and they project to the dorsal horn of the spinal cord, which is arranged in distinct laminae. From there, ascending pathways such as the spinothalamic and spinoreticulothalamic tracts transmit painful signals to the thalamus and brainstem. Finally, these signals are transmitted to the cortex where they are perceived as pain (Basbaum et al., 2009).

1.2. Sensitization/Hyperalgesia

Increased pain upon application of a noxious stimulus is called hyperalgesia (IASP, 2011). This sensitization can occur at peripheral (primary hyperalgesia), spinal (secondary hyperalgesia) and supraspinal (tertiary hyperalgesia)) levels of the pain pathway (Sandkuhler, 2009; Gangadharan and Kuner, 2013). The activation threshold of nociceptive neurons is reduced in response to injury or inflammation due to the sensitization of their peripheral endings. Injury of tissue leads to a change of the chemical milieu due to mast cell degranulation, secretion of mediators by inflammatory cells, and/or the activation of enzymes like cyclooxygenase-2 (Woolf and Ma, 2007). This so called “inflammatory soup” consists of protons, adenosine triphosphate (ATP), kinins, prostaglandins, neuropeptides, histamine and lipids, and activates the respective receptors on the peripheral nerve endings. In consequence, numerous neuronal intracellular signaling cascades are activated resulting in increased activity of kinases like protein kinase A (PKA), protein kinase C (PKC), phosphoinositide-3-kinase, mitogen-activated protein kinases and c-Jun N-terminal kinases. These events lead to increased phosphorylation and functional expression of TRP and sodium channels. All these mechanisms reduce thresholds of neuronal activation (reviewed in (Woolf and Ma, 2007; Gangadharan and Kuner, 2013). Peripheral sensitization is usually restricted to the site of injury (Sandkuhler, 2009). Central sensitization at the spinal level may outlast initial stimuli resulting in pain chronification and/or amplification (Sandkuhler, 2009). The activation of different ion channels (e.g. N-methyl-D-aspartate receptor (NMDAR)) contribute to the development and maintenance of central sensitization. Membrane depolarization due to sustained release of excitatory mediators (e.g. glutamate, substance P and calcitonin gene-related peptide (CGRP)) from primary afferent nociceptive neurons activates calcium inward currents that maintain central sensitization (Latremoliere and Woolf, 2009). In addition, intracellular calcium increases via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), voltage-gated calcium channels and due to release from intracellular stores. Several metabotropic receptors such as B2 (bradykinin), NK1R (substance P), CGRPR (CGRP), TrkB (BDNF) can activate calcium-

sensitive kinases that phosphorylate NMDARs and AMPARs (reviewed in (Latremoliere and Woolf, 2009).

1.2.1. TRPV1

The TRPV1 channel belongs to the TRP superfamily, which consists of seven subfamilies namely TRPC ("classical" or "canonical"), TRPV ("vanilloid"), TRPM ("melastatin"), TRPN ("NOMPC"), TRPA ("ankyrin"), TRPP ("polycystic"), and TRPML ("melastatin like"). The TRP family is heterologous group of cation channels that can detect thermal, chemical, and/or mechanical stimuli involved in the perception of pain, taste, and changes in temperature (Darre and Domene, 2015). They were initially identified in *Drosophila melanogaster* (Montell and Rubin, 1989; Patapoutian et al., 2009).

Six members of the TRP family are summarized as thermo-TRPs responding to temperatures from noxious cold to painful heat. The TRPV1 is one of them and is mainly expressed in peptidergic and nonpeptidergic C- and in Aδ-fibres in the dorsal root, trigeminal, nodose and sympathetic ganglia. This channel was also detected in sensory neurons innervating bladder, lung and cochlea (reviewed in (Brito et al., 2014; Mickle et al., 2015). In the central nervous system, TRPV1 is expressed in laminae I and II of the dorsal horn of the spinal cord, in the brainstem, olfactory bulb and in various brain nuclei (e.g. solitary tract, caudalis, ambiguous, parabrachial). Furthermore, TRPV1 can be found in nonneuronal cells such as dermal cells (Stander et al., 2004) as well as in dental tissue, ovary and testis (reviewed in (Mickle et al., 2015; Gouin et al., 2017). The structure of TRPV1 was determined in 2013 using electron cryo-microscopy and confirmed the similarity to voltage gated ion channels (Clapham et al., 2001;

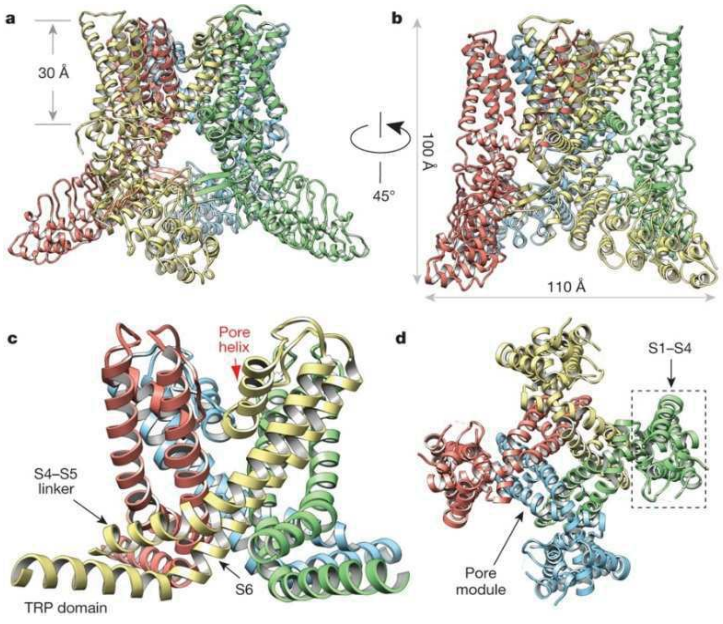


Figure 1: Ribbon diagram of TRPV1's atomic model showing the tetrameric structure (Liao et al., 2013). **a and b)** Views from the side. **c)** Side view of S5-P-S6 pore with TRP domain. **d)** Bottom view of TRPV1. Reprinted by permission from Copyright Clearence Center: Springer Nature, Nature, Structure of the TRPV1 ion channel determined by electron cryo-microscopy, M. F. Liao, E. H. Cao, D. Julius, Y. F. Cheng, 2013.

Montell, 2005). Four symmetric TRPV1 monomers form a tetramer with a central ion pore (Figure 1).

Each monomer displays 6 transmembrane α-helices (S1-6) spanning the lipid bilayer, 6 ankyrin repeats followed by a linker domain and the pre-S1 helix. Transmembrane 4 and 5 are connected by a S4-S5 linker. The pore helix and the pore loop are flanked by S5 and S6 followed by the TRP domain (Figure 2) (Cao et al., 2013; Liao et al., 2013).

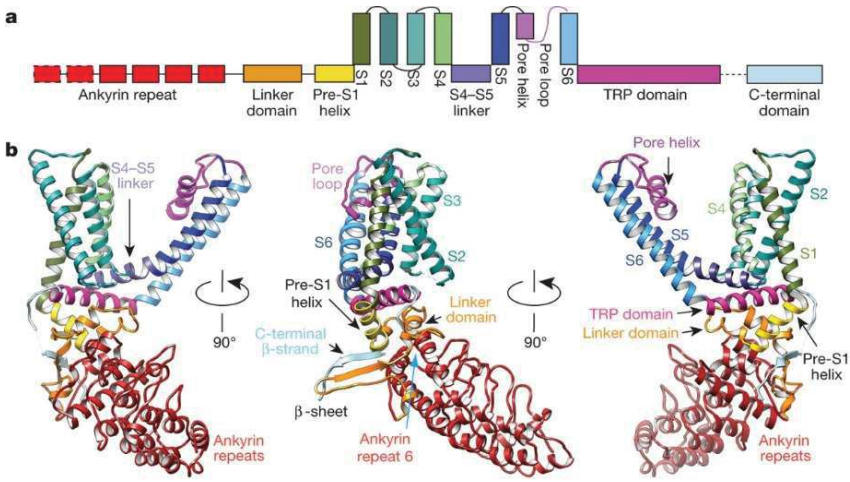


Figure 2: Detailed structure of a TRPV1 monomer (Liao et al., 2013). **a)** Linear diagram with major domains of a TRPV1 monomer. **b)** Ribbon diagram presenting three different views of a monomer. Reprinted by permission from Copyright Clearence Center: Springer Nature, Nature, Structure of the TRPV1 ion channel determined by electron cryo-microscopy, M. F. Liao, E. H. Cao, D. Julius, Y. F. Cheng, 2013.

Capsaicin, the pungent ingredient of chili pepper, temperatures above 43° C and protons are the most prominent examples for TRPV1 agonists (Caterina et al., 1997; Tominaga et al., 1998; Mickle et al., 2015). TRPV1 activation increases intracellular calcium concentrations resulting in substance P and CGRP release, which leads to edema and neurogenic inflammation (reviewed in (Gouin et al., 2017). Furthermore, the channel plays an important role in the development of thermal hyperalgesia in response to inflammation or injury and is activated and sensitized by substances that are released from damaged tissues or the sensory neuron (reviewed in (Tominaga, 2006; Szallasi and Sheta, 2012; Vay et al., 2012; Gouin et al., 2017)).

TRPV1 can be modulated by several mediators: Notably, prostaglandins, adenosine, serotonin, bradykinin, and adenosine ATP bind to neuronal receptors (mostly heterotrimeric guanine nucleotide–binding protein (G-protein) coupled receptors (GPCRs)) that in turn activate kinases via intracellular signaling cascades (Tominaga, 2006). Several serine and threonine kinases such as PKA, PKC, calcium/calmodulin-dependent protein kinase II and Src kinase can phosphorylate TRPV1 resulting in enhanced responses to capsaicin and reduced activation thresholds in response to temperature and capsaicin (reviewed in (Vay et al., 2012)).

In addition, TRPV1 is modulated by numerous other molecules like the protein-A kinase anchoring protein (AKAP) 79, which binds PKA and PKC and forms a signaling complex with

the C-terminus of TRPV1 leading to its phosphorylation and sensitization (Zhang et al., 2008; Jeske et al., 2009; Vay et al., 2012; Gouin et al., 2017). PKA and Src kinase activity not only sensitize TRPV1 via the phosphorylation of intracellular residues, but also increase the expression of functional TRPV1 tetramers on the neuronal plasma membrane (Zhang et al., 2005; Vetter et al., 2008). Continuous or repeated TRPV1 activation results in decreased TRPV1 responsiveness (desensitization and tachyphylaxis) due to calcineurin and calmodulin activity leading to TRPV1 dephosphorylation or direct inhibition by binding to the calmodulin bindings sites at the N-and C-termini of the channel (reviewed in (Tominaga, 2006; Gouin et al., 2017).

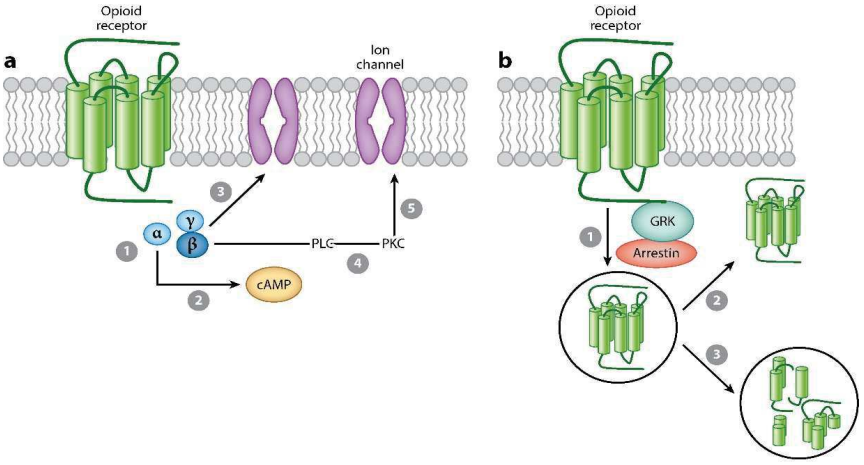
1.3. Pain inhibition

Nociceptor stimulation (e.g. in response to inflammation) activates several endogenous pathways in the peripheral and central nervous system that result in inhibition of pain. These include the release of opioid peptides and gamma-aminobutyric acid (GABA) and the activation of inhibitory noradrenergic and serotonergic mechanisms. Most of these mediators stimulate GPCRs. In sensory neurons, this leads to a reduction of excitatory neurotransmitter release and the opening of potassium and chloride channels resulting in hyperpolarizing inhibitory potentials. During later phases of tissue injury, anti-inflammatory mediators are released that counteract pain and inflammation (reviewed in (Stein, 2016)). Clinical pain treatment targets many proteins like GPCRs, ion channels and regulatory enzymes (Dray, 2009). Currently available classes of pain medication include opioids (which activate opioid receptors), non-steroidal anti-inflammatory drugs (NSAIDs) (which inhibit prostaglandin synthesis), antidepressants (which inhibit serotonin- and noradrenalin reuptake), and anticonvulsants (which block calcium and/or sodium channels) (Lynch and Watson, 2006). Among them, opioids are the most effective pain therapeutics (Dray, 2009).

1.3.1. Opioid receptors

There are three classical opioid receptor types namely the μ- (MOR), δ- (DOR), and κ- (KOR) opioid receptor. These are activated by their endogenous ligands β-endorphin, enkephalin, and dynorphin, they are encoded by three different genes (Oprm1, Oprd1, and Oprk1) and are expressed in peripheral and central neurons, in neuroendocrine, immune, and ectodermal cells (Evans et al., 1992; Kieffer et al., 1992; Meng et al., 1993; Wang et al., 1993; Mollereau et al., 1994; Stein, 2016). An additional receptor, the nociceptin receptor, is encoded by Oprl1 and

endogenously activated by nociceptin (Toll et al., 2016; Corder et al., 2018) The nociceptin receptor is not activated by low concentrations of classical opioids



Stein C. 2016.
Annu. Rev. Med. 67:433–51

Figure 3: Opioid receptor signaling (Stein, 2016). **a)** Agonist induced G-protein dissociation of opioid receptors. **b)** Agonist-induced opioid receptor desensitization via GRK-induced phosphorylation and subsequent arrestin binding. Republished with permission of ANNUAL REVIEWS, from "Opioid Receptors, C. Stein, 67, 2016"; permission conveyed through Copyright Clearance Center, Inc.

(Toll et al., 2016; Stein, 2018). The crystal structures of these receptors were resolved, giving insights into receptor/ligand interactions (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012; Huang et al., 2015; Che et al., 2018). All four receptors belong the class A subgroup of GPCRs and show 60 % homology among their genes. Upon ligand binding, conformational changes lead to a coupling of inhibitory G-proteins at the receptors' C-terminus. Thereafter, the guanosine diphosphate (GDP) bound to the α-subunit of the G-protein trimer is replaced by guanosine triphosphate (GTP), resulting in dissociation into $G_{\alpha i}$- and $G_{\beta\gamma}$- subunits (Figure 3a). The subunits exert separate downstream effects: $G_{\alpha i}$ inhibits adenylyl cyclases (ACs) leading to decreased intracellular cyclic adenosine monophosphate- (cAMP)-levels, which can lead to inhibition of excitatory membrane ion channels via activation of intracellular enzymes. $G_{\beta\gamma}$ directly activates potassium channels and inhibits voltage dependent calcium channels. The overall result of these events is suppression of the release of excitatory neurotransmitters (substance P, CGRP), hyperpolarization and inhibition of neuronal excitability, eventually leading to analgesia (Corder et al., 2018; Machelska and Celik, 2018; Stein, 2018).

All clinically relevant opioid analgesics bind to the MOR. However, they also induce several adverse side effects including respiratory depression, addiction, sedation, nausea, and constipation, which have contributed to the high morbidity and mortality in the context of the opioid crisis (Stein, 2018). Most side effects are mediated by MOR-induced G-protein activation of central or intestinal opioid receptors. Respiratory depression is due to $G_{\beta\gamma}$-

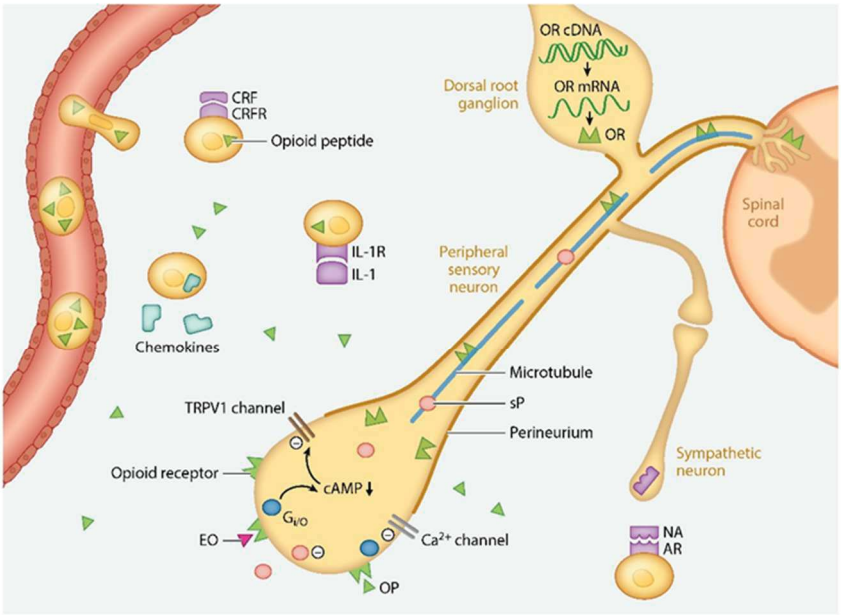
mediated activation of G protein-coupled inwardly-rectifying potassium (GIRK) channels in the respiratory center of the brainstem. Sedation and constipation are mediated by $G_{\beta\gamma}$-induced potassium channel activation and calcium channel inhibition in the hypothalamic arousal system and in the enteric nervous system, respectively. Nausea and vomiting are thought to be elicited by $G_{\alpha i/o}$- induced inhibition of the cAMP-PKA pathway and calcium channels in the vestibular system (reviewed in (Stein, 2016; Machelska and Celik, 2018).

Tolerance is the diminished magnitude of a given drug effect with repeated administration of the same dose, or the need for higher dosages to generate the same effect (Basbaum et al., 2009; Stein, 2018). Tolerance can develop to all opioid-induced effects like analgesia, respiratory depression, nausea, sedation, and constipation. The molecular mechanisms underlying tolerance are not completely elucidated so far. One explanation is ligand-induced MOR desensitization. This involves GPCR kinases (GRKs), which phosphorylate several serine (S) and threonine (T) residues at the cytoplasmatic loops and C-terminus, leading to arrestin binding and subsequent internalization of the receptor (Figure 3b). Two amino acid sequences were identified to be targets for GRKs and crucial for MOR desensitization, namely ^{354}TSST^{357} and ^{370}TREHPSTANT^{379} (Kliewer et al., 2019). Studies have shown that S375 and the hierarchical phosphorylation of the flanking residues T370, T376 and T379 by GRK2 play an important role resulting in β-arrestin 2 binding. This prevents further coupling of MOR to G-proteins and leads to clathrin-induced MOR internalization followed by either receptor recycling or degradation. However, MOR desensitization is only one mechanism of the multifaceted process of tolerance development (Bohn et al., 2000; Williams et al., 2013; Kliewer et al., 2019).

The selective activation of opioid receptors on peripheral sensory neurons lacks the centrally and intestinally mediated side effects, and can induce potent antinociception, particularly under pathological conditions: During tissue injury, numerous adaptations occur in the nervous, endocrine and immune systems (Rittner et al., 2008). For example, MOR messenger ribonucleic acid (mRNA) and protein expression is upregulated in a neuronal electrical activity and cytokine dependent manner, probably due to cytokine-induced increased opioid receptor transcription in the neurons innervating the inflamed tissue. Furthermore, the axonal transport of opioid receptors to peripheral nerve terminals and their expression on neuronal membranes is enhanced during injury. This was dependent on cytokines and nerve growth factor (Hassan et al., 1993; Jeanjean et al., 1995; Zollner et al., 2003; Mousa et al., 2007). The overall effect is an increased opioid receptor density and G-protein coupling in peripheral sensory neurons. In addition, the number of opioid receptor expressing neurons is augmented during injury (Stein

et al., 1990; Zhou et al., 1998; Zollner et al., 2003) and the disruption of the perineural barrier facilitates the access of opioid ligands to their receptors (Antonijevic et al., 1995; Rittner et al., 2012).

Furthermore, it was shown that the development of tolerance was reduced in inflammatory pain due to the continuous presence of endogenous opioid peptides and increased opioid receptor recycling (Stein et al., 1996; Zollner et al., 2008). Figure 4 summarizes opioid action in inflamed tissue. Endogenous opioid peptides (β-endorphin, enkephalin, dynorphin and endomorphin) are expressed in immune cells which extravasate and accumulate in inflamed



Stein C. 2016.
Annu. Rev. Med. 67:433–51

Figure 4: Peripheral opioid action (Stein, 2016). OP-opioid peptide, EO-exogenous opioid, OR-opioid receptor, NA-noradrenaline, AR-adrenalin receptor, IL-1-interleukin 1, IL-1R, IL-1 receptor, CRF-corticotropin-releasing-factor, CRFR-CRF receptor. Republished with permission of ANNUAL REVIEWS, from "Opioid Receptors, C. Stein, 67, 2016"; permission conveyed through Copyright Clearance Center, Inc.

tissue (Stein and Machelska, 2011). The gene expression of opioid peptide precursors and of their processing enzymes in immune cells is upregulated during tissue injury. Cytokines, stress and bacteria stimulate release of opioid peptides, which then bind to peripheral opioid receptors and induce analgesia (Rittner et al., 2009; Stein and Machelska, 2011) (reviewed in (Stein, 2016; 2018).

1.4. Aim of the present work

The overall aim of my work was to elucidate molecular mechanisms contributing to pain sensitization and to develop approaches to inhibit pain without inducing adverse side effects. I investigated the pain pathway from two perspectives:

1. In the first part, I focused on mechanisms underlying the initiation of pain and sensitization. I concentrated on mechanisms that sensitize TRPV1, since this excitatory ion channel plays a central role during tissue injury and inflammation.
2. In the second part, I focused on the opioid system under pathological conditions. The aim was to develop novel opioid analgesics, which are exclusively active in injured tissues and therefore do not induce common centrally or intestinally mediated side effects.

2. Results

2.1. TRPV1 sensitization via the cAMP and PKA pathway induces pain sensitization

TRPV1 plays a pivotal role in the sensitization to painful stimuli in pathological conditions (Caterina et al., 2000; Davis et al., 2000; Tominaga, 2006). We aimed to identify mechanisms that do not directly inhibit TRPV1 signaling but sensitize TRPV1, since it was previously shown that direct TRPV1 blockade can result in hyperthermia and skin burns (Woolf, 2010; Szallasi and Sheta, 2012; Vay et al., 2012).

2.1.1. Modulation of Transient Receptor Vanilloid 1 Activity by Transient Receptor Potential Ankyrin 1

In the following study, we investigated interactions of TRPV1 and TRPA1.

The following text is extracted from the abstract of Spahn V, Stein C and Zöllner C. Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. Mol Pharmacol. 2014 Feb;85(2):335-344. doi: https://doi.org/10.1124/mol.113.088997

> *"Transient receptor potential vanilloid 1 (TRPV1) is a nonselective ligand-gated cation channel responding to noxious heat, protons, and chemicals such as capsaicin. TRPV1 is expressed in sensory neurons and plays a critical role in pain associated with tissue injury, inflammation, and nerve lesions. Transient receptor potential ankyrin 1 (TRPA1) is coexpressed with TRPV1. It is activated by compounds that cause a burning sensation (e.g., mustard oil) and, indirectly, by components of the inflammatory milieu eliciting nociceptor excitation and pain hypersensitivity. Previous studies indicate an interaction of TRPV1 and TRPA1 signaling pathways. Here we sought to examine the molecular mechanisms underlying such interactions in nociceptive neurons. We first excluded physical interactions of both channels using radioligand binding studies. By microfluorimetry, electrophysiological experiments, cAMP measurements, and site-directed mutagenesis we found a sensitization of TRPV1 after TRPA1 stimulation with mustard oil in a calcium and cAMP/protein kinase A (PKA) dependent manner. TRPA1 stimulation enhanced TRPV1 phosphorylation via the putative PKA phosphorylation site serine 116. We also detected calcium-sensitive increased TRPV1 activity after TRPA1 activation in dorsal root ganglion neurons. The inhibition of TRPA1 by HC-030031 (1,2,3,6-tetrahydro-1,3- dimethyl-N-[4-(1-methylethyl)phenyl]-2,6-dioxo-7H-purine-7- acetamide, 2-(1,3-dimethyl-2,6-*

dioxo-1,2,3,6-tetrahydro-7Hpurin- 7-yl)-N-(4 isopropylphenyl)acetamide) after its initial stimulation (and the calcium-insensitive TRPA1 mutant D477A) still showed increased capsaicin-induced TRPV1 activity. This excludes a calcium-induced additive TRPA1 current after TRPV1 stimulation. Our study shows sensitization of TRPV1 via activation of TRPA1, which involves adenylyl cyclase, increased cAMP, subsequent translocation and activation of PKA, and phosphorylation of TRPV1 at PKA phosphorylation residues. This suggests that cross-sensitization of TRP channels contributes to enhanced pain sensitivity in inflamed tissues."

2.1.2. Ankyrin-rich membrane spanning protein as a novel modulator of TRPV1-function in nociceptive neurons

In the next study, we investigated an important putative interaction partner of TRPV1, namely ARMS. ARMS is a large adaptor protein with numerous protein-protein interaction motifs (Peter 2017) that is involved in neurotrophic signaling, neuronal development and homeostasis (Cesca 2018). Since TRPV1 and ARMS share the neurotrophic signaling pathway (Zhang et al., 2005; Neubrand et al., 2012) and they are co-expressed on the mRNA level in DRG neurons (Isensee et al., 2014), it was important to examine whether the two proteins are also co-expressed and whether this influences TRPV1 activity.

The following text is extracted from the abstract of Peter J*, Kasper C*, Buschow R, Hucho T, Stein, C Jordt SE, Brackmann M+ and Spahn V+ Ankyrin-rich membrane spanning protein as a novel modulator of TRPV1-function in nociceptive neurons. Eur J Pain. 2017 Jul;21(6): 1072-1086. doi: https://doi.org/10.1002/ejp.1008

> *"Background: The ion channel TRPV1 is mainly expressed in small diameter dorsal root ganglion (DRG) neurons, which are involved in the sensation of acute noxious thermal and chemical stimuli. Direct modifications of the channel by diverse signalling events have been intensively investigated, but little is known about the composition of modulating macromolecular TRPV1 signalling complexes. Here, we hypothesize that the novel adaptor protein ankyrin-rich membrane spanning protein/kinase D interacting substrate (ARMS) interacts with TRPV1 and modulates its function in rodent DRG neurons. Methods: We used immunohistochemistry, electrophysiology, microfluorimetry and immunoprecipitation experiments to investigate TRPV1 and ARMS interactions in DRG neurons and transfected cells.*
>
> *Results: We found that TRPV1 and ARMS are co-expressed in a subpopulation of DRG neurons. ARMS sensitizes TRPV1 towards capsaicin in transfected HEK 293 cells and in mouse DRG neurons in a PKA-dependent manner. Using a combination of functional imaging and immunocytochemistry, we show that the magnitude of the capsaicin response in DRG neurons depends not only on TRPV1 expression, but on the co-expression of ARMS alongside TRPV1.*

Conclusion: These data indicate that ARMS is an important component of the signalling complex regulating the sensitivity of TRPV1.

Significance: The study identifies ARMS as an important component of the signalling complex regulating the sensitivity of excitatory ion channels (TRPV1) in peripheral sensory neurons (DRG neurons) and transfected cells."

2.1.3. Opioid withdrawal increases TRPV1 activity in a protein kinase A-dependent manner

In this study, we aimed to explore the influence of opioid withdrawal on TRPV1 channel activity. Withdrawal is induced when an opioid agonist is suddenly removed or its dosage is significantly reduced. Clinically relevant symptoms of opioid withdrawal are lacrimation, piloerection, yawning, myalgia, nausea, vomiting, photophobia, insomnia, autonomic hyperactivity, and hyperalgesia. These symptoms might be due by AC superactivation and subsequent cAMP overshoot (Carcoba et al., 2011; Shah and Huecker, 2019). It was previously shown that acute opioid administration inhibited TRPV1 activity via the cAMP/PKA pathway in peripheral sensory neurons (Endres-Becker et al., 2007), and that opioid withdrawal induces AC superactivation and increases PKA activity in the central nervous system (Sharma et al., 1975; Nestler, 1992). Since PKA regulates and sensitizes TRPV1, we investigated the effects of opioid withdrawal on TRPV1 activity in peripheral neurons.

The following text is extracted from the abstract of Spahn V, Fischer O, Endres-Becker J, Schäfer M, Stein C and Zöllner C. Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A -dependent manner. Pain. 2013 Apr;154(4): 598-608. doi: http://dx.doi.org/10.1016/j.pain.2012.12.026

> *"Hyperalgesia is a cardinal symptom of opioid withdrawal. The transient receptor potential vanilloid 1 (TRPV1) is a ligand-gated ion channel expressed on sensory neurons responding to noxious heat, protons, and chemical stimuli such as capsaicin. TRPV1 can be inhibited via μ-opioid receptor (MOR)-mediated reduced activity of adenylyl cyclases (ACs) and decreased cyclic adenosine monophosphate (cAMP) levels. In contrast, opioid withdrawal following chronic activation of MOR uncovers AC superactivation and subsequent increases in cAMP and protein kinase A (PKA) activity. Here we investigated (1) whether an increase in cAMP during opioid withdrawal increases the activity of TRPV1 and (2) how opioid withdrawal modulates capsaicin-induced nocifensive behavior in rats. We applied whole-cell patch clamp, microfluorimetry, cAMP assays, radioligand binding, site-directed mutagenesis, and behavioral experiments. Opioid withdrawal significantly increased cAMP levels and capsaicin-induced TRPV1 activity in both transfected human embryonic kidney 293 cells and dissociated dorsal root ganglion (DRG) neurons. Inhibition of AC and PKA, as well as mutations of the PKA phosphorylation sites threonine 144 and serine 774, prevented the enhanced*

TRPV1 activity. Finally, capsaicin-induced nocifensive behavior was increased during opioid withdrawal in vivo. In summary, our results demonstrate an increased activity of TRPV1 in DRG neurons as a new mechanism contributing to opioid withdrawal-induced hyperalgesia."

2.2. Inhibition of injury-induced pain without side effects

While the detailed knowledge of sensitizing mechanisms may serve as the basis for the development of new therapeutics, it is equally important to consider the activation of the pain inhibiting system. The use of opioids is limited by adverse side effects like respiratory depression, addiction, sedation, nausea and constipation (Benyamin et al., 2008). Current strategies are the development of abuse deterrent formulations, allosteric modulators, bivalent ligands, biased opioid ligands and the augmentation of the endogenous opioid peptide system, but they did not yield clinically useful analgesics so far (reviewed in (Stein, 2018). The following studies present a novel way to address this question via the restriction of opioid receptor activation to the periphery.

2.2.1. A nontoxic pain killer designed by modeling of pathological receptor conformations

The following text is extracted from the abstract of Spahn V*, Del Vecchio G*, Labuz D, Rodriguez-Gaztelumendi A, Massaly N, Temp J, Durmaz V, Sabri P, Reidelbach M, Machelska, H, Weber M+ and Stein C+. A nontoxic pain killer designed by modeling of pathological receptor confirmations. Science. 2017 Mar 3;355(6328):966-969. doi: https://doi.org/10.1126/science.aai8636

> *„Indiscriminate activation of opioid receptors provides pain relief but also severe central and intestinal side effects. We hypothesized that exploiting pathological (rather than physiological) conformation dynamics of opioid receptor-ligand interactions might yield ligands without adverse actions. By computer simulations at low pH, a hallmark of injured tissue, we designed an agonist that, because of its low acid dissociation constant, selectively activates peripheral μ-opioid receptors at the source of pain generation. Unlike the conventional opioid fentanyl, this agonist showed pH-sensitive binding, heterotrimeric guanine nucleotide–binding protein (G-protein) subunit dissociation by fluorescence resonance energy transfer, and adenosine 3′,5′- monophosphate inhibition in vitro. It produced injury-restricted analgesia in rats with different types of inflammatory pain without exhibiting respiratory depression, sedation, constipation, or addiction potential."*

2.2.2. Analgesic effects of a novel pH-dependent μ-opioid receptor agonist in models of neuropathic and abdominal pain

We further investigated the selective activation of peripheral opioid receptors by our newly designed opioid receptor agonist (±)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (NFEPP) in other pathological conditions such as neuropathic and abdominal pain. Furthermore, we explored NFEPP binding in native tissue.

The following text is extracted from the abstract of Rodriguez-Gaztelumendi A, Spahn V, Labuz D, Machelska H, Stein C. Analgesic effects of a novel pH-dependent μ-opioid receptor agonist in models of neuropathic and abdominal pain. Pain. 2018 Nov;159(11):2277-2284. doi: http://dx.doi.org//10.1097/j.pain.0000000000001328

> *"Recently, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), a newly designed μ-opioid receptor (MOR) agonist with a low pKa, has been shown to produce injury-restricted analgesia in models of inflammatory and postoperative pain, without exhibiting typical opioid side effects. Here, we investigated MOR binding of NFEPP in brain and dorsal root ganglia, pH in injured tissues, and the analgesic efficacy of NFEPP compared with fentanyl in a chronic constriction injury model of neuropathic pain, and in the acetic acid–induced abdominal writhing assay in rats. Binding experiments revealed significantly lower affinity of NFEPP compared with fentanyl at pH 7.4. In vivo, pH significantly dropped both at injured nerves after chronic constriction injury and in the abdominal cavity after acetic acid administration. Intravenous NFEPP as well as fentanyl dose-dependently diminished neuropathy-induced mechanical and heat hypersensitivity, and acetic acid–induced abdominal constrictions. In both models, NFEPP-induced analgesia was fully reversed by naloxone methiodide, a peripherally restricted opioid receptor antagonist, injected at the nerve injury site or into the abdominal cavity. Our results indicate that NFEPP exerts peripheral opioid receptor–mediated analgesia exclusively in damaged tissue in models of neuropathic and abdominal pain."*

2.2.3. Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist

Next, we investigated another fentanyl-derivative with reduced pK_a. The aim of this study was to examine our concept that the pK_a of an opioid receptor ligand should be close to the pH of injured tissue to achieve analgesia selectively mediated by peripheral opioid receptors without producing adverse side effects.

The following text was extracted from the abstract of Spahn V, Del Vecchio G, Rodriguez-Gaztelumendi A, Temp J, Labuz D, Kloner M, Reidelbach M, Machelska H, Weber M, Stein C. Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. Sci Rep. 2018 Jun 12;8(1):8965. doi: https://doi.org/10.1038/s41598-018-27313-4

> *"Novel pain killers without adverse effects are urgently needed. Opioids induce central and intestinal side effects such as respiratory depression, sedation, addiction, and constipation. We have recently shown that a newly designed agonist with a reduced acid dissociation constant (pK_a) abolished pain by selectively activating peripheral μ-opioid receptors (MOR) in inflamed (acidic) tissues without eliciting side effects. Here, we extended this concept in that pK_a reduction to 7.22 was achieved by placing a fluorine atom at the ethylidene bridge in the parental molecule fentanyl. The new compound (FF3) showed pH-sensitive MOR affinity, [^{35}S]-GTPγS binding, and G protein dissociation by fluorescence resonance energy transfer. It produced injury-restricted analgesia in rat models of inflammatory, postoperative, abdominal, and neuropathic pain. At high dosages, FF3 induced sedation, motor disturbance, reward, constipation, and respiratory depression. These results support our hypothesis that a ligand's pK_a should be close to the pH of injured tissue to obtain analgesia without side effects."*

3. Discussion

"A life without pain is the basis for human well-being and pain therapy in the sense of pain reduction or inhibition is an essential human right" (Treede, 2015b). Although "a life without pain" is an unrealistic goal, it is certainly important to strive for sufficient pain relief without adverse side effects. Thus, both mechanisms of sensitization during injury and strategies to directly inhibit pain have to be investigated. My work addressed two components of the pain pathway: First, the mechanisms underlying TRPV1 sensitization, as this ion channel plays an important role in injury-induced hyperalgesia. Knowing these mechanisms in detail may be useful for the development of new pain therapeutics that reverse TRPV1 sensitization. Second, I investigated the development of novel opioids that exclusively activate opioid receptors at the site of tissue injury and do not induce side effects commonly associated with opioid treatment. Both strategies produced results that seem to be of clinical relevance.

The need for safe pain therapeutics is urgent. Epidemiological studies suggest that approximately 20 Mio. people in Europe suffer from pain, and their quality of life is dramatically reduced. In addition, direct and indirect societal costs due to pain are tremendous (SIP, 2018). Numerous pain medications are available, but they have many sometimes life threatening constraints. Opioids, for example, are limited by sedation, respiratory depression, nausea, addiction, tolerance, and constipation. The current opioid crisis/epidemic is at least partially a result of inappropriate prescription/use and undervalued side effects. On the other hand, cyclooxygenase inhibitors induce gastrointestinal and cardiovascular side effects, and anticonvulsants as well as antidepressants produce sedation, ataxia and arrhythmias (Coxib et al., 2013; Califf et al., 2016; Spahn et al., 2017).

3.1. Reversal of TRPV1 sensitization as a target for new pain medications

The TRPV1 channel has been a target for development of pain therapeutics since its discovery in 1997, because it is crucial for the transduction of painful stimuli (Szallasi and Sheta, 2012). In the studies presented here, we identified interaction partners, which lead to cAMP/PKA-dependent TRPV1 sensitization and subsequent hyperalgesia (Spahn et al., 2013; Spahn et al., 2014; Peter et al., 2017). This knowledge could serve as a basis for new therapeutics targeting TRPV1 sensitization rather than aiming at the blockade of TRPV1 itself. So far, two mechanisms to achieve pain reduction via TRPV1 modulation have been investigated: First, repeated or continuous TRPV1 activation leading to nociceptor defunctionalization, and second, direct blockade of TRPV1 via newly synthesized TRPV1 antagonists (Szallasi and Sheta, 2012). TRPV1-agonist-induced defunctionalization is a result of several events.

Neuronal excitability is decreased via the inactivation of voltage-gated Na+ channels and direct desensitization of TRPV1 expressed in the plasma membrane. Additionally, high calcium concentrations enter the cell via TRPV1, leading to the activation of calcium-dependent proteases and microtubule depolymarization, which interrupts fast axonal transport (reviewed in (Anand and Bley, 2011)). At ultra high concentrations, the TRPV1 agonist capsaicin can directly inhibit electron chain transport in neuronal mitochondria resulting in neurotoxicity. Finally, increased intracellular chloride accumulation (accompanied by an influx of positively charged ions) leads to osmotic swelling. All these processes are responsible for impaired nociceptor functionality (Anand and Bley, 2011). Preclinical studies investigating repeated subcutaneous capsaicin injections demonstrated a significant reduction of pain thresholds in animals. However, this procedure can not be translated into clinical settings (Szallasi and Sheta, 2012). Creams containing low concentrations of capsaicin did not show convincing results and were accompanied by local skin irritation (Derry and Moore, 2012; Szallasi and Sheta, 2012). In osteoarthritic pain, topical capsaicin application showed good efficacy and a positive safety profile. However, these studies have to be interpreted with caution due to their problems with blinding (Guedes et al., 2018). More recently, capsaicin patches were investigated in postherpetic neuralgia, HIV-associated neuropathy and peripheral diabetic neuropathy. However, systematic literature analysis revealed only *"moderate quality evidence that high-concentration (8%) capsaicin patches can give moderate pain relief,…"* (Derry et al., 2017). The benefits for patients with HIV-neuropathy and peripheral diabetic neuropathy were minor (Derry et al., 2017). On the other hand, local or perineuronal injections of the TRV1 ligand resiniferatoxin showed promising results in animal models of inflammatory, incisional, neuropathic and cancer pain (Szallasi and Sheta, 2012). However, a literature search in the Cochrane Library indicated that resiniferatoxin treatment in painful bladder syndrome/interstitial cystitis was without sufficient pain relief and yielded only one ongoing clinical trial in patients with knee pain (Ford et al., 2016; Klincewicz, 2018).

The other possibility is direct antagonism of TRPV1 with the intention to block the transduction of painful stimuli. TRPV1 antagonists showed pain-relieving actions in models of osteoarthritic pain, dental pain, gastroesophageal reflux-induced pain and a reduction of the severity of inflammatory bowel disease, mechanical bladder hyperactivity, atopic dermatitis and epileptic seizures (Aghazadeh Tabrizi et al., 2017). These promising preclinical (and some clinical) data resulted in an intense search for TRPV1-antagonists and in numerous clinical trials (reviewed in (Aghazadeh Tabrizi et al., 2017). However, most of these antagonists failed to induce pain relief or they induced adverse effects like burns or hyperthermia. The latter was possibly due

to the blockade of tonically active visceral TRPV1, which suppress the autonomic cold defense by thermogenesis and vasoconstriction (Romanovsky et al., 2009; Garami et al., 2018)) or burns, which precluded them from entering clinical phase III studies. A recent strategy is the development of antagonists that act via distinct molecular domains responsible for TRPV1 activation by heat, capsaicin and protons and therefore should lack hyperthermia induced by full TRPV1 blockade (NEO6860). Also the development of locally restricted TRPV1 modulators and molecules that only treat TRPV1 sensitization become more popular (Aghazadeh Tabrizi et al., 2017). The results of the present studies could be used for the development of molecules reducing TRPV1 sensitization under pathological conditions. Our findings that the residues S116, T144, and S774 of TRPV1 are phosphorylated by PKA or that TRPV1 interacts with other pain-sensitizing interaction partners (e.g. ARMS) in a cAMP/PKA dependent manner leading to TRPV1 sensitization, could be used for the generation of compounds directly shielding theses residues and therefore reversing the sensitization, but retaining normal channel functionality.

3.2. Recent strategies for the development of opioids without side effects

In the opioid field, two major concepts for the development of pain therapeutics without side effects are currently pursued, namely peripherally restricted opioids and “biased” agonists. Peripheral restriction of opioids can be achieved for example by covalent attachment of an opioid agonist to nanocarriers that are unable to permeate the blood brain barrier. We studied morphine attached to hyperbranched polyglycerol via a cleavable linker (polyglycerol-morphine) (Gonzalez-Rodriguez et al., 2017). Exploiting its high molecular weight and hydrophilicity, we assumed that polyglycerol-morphine should not cross the blood brain barrier and that morphine should be selectively released in injured tissue. In a rat model of inflammatory pain, we indeed found that intravenous polyglycerol-morphine exclusively activated peripheral opioid receptors and induced analgesia only in the inflamed paw without evoking central or intestinal side effects (Gonzalez-Rodriguez et al., 2017). Another example is the conjugation of opioid-loaded liposomes with an antibody to intercellular adhesion molecule-1 to mimic the properties of immune cells invading injured tissue. Intravenous injection of these liposomes reduced mechanical hypersensitivity in the rat inflamed paw via the local activation of opioid receptors ((Hua and Cabot, 2013) reviewed in (Machelska and Celik, 2018).

In contrast to these pharmacokinetic approaches, a pharmacodynamic concept is the peripheral restriction of opioid receptor activation based on the opioid ligand’s pK_a. This strategy is based

on the finding that most painful conditions like arthritis, cancer, trauma, neuropathy and surgery are associated with injury-induced inflammation and tissue acidification. Under such conditions, the expression and functionality of opioid receptors is upregulated at the site of injury, and their activation results in potent inhibition of neuronal excitability (reviewed in (Stein, 2016)). Activation of peripheral opioid receptors mediates a considerable proportion of opioid-induced analgesia in animals and humans, and the augmented signaling indicates conformational alterations of opioid receptors and/or ligands in the inflamed environment (Gaveriaux-Ruff et al., 2011; Weibel et al., 2013; Jagla et al., 2014; Stein, 2016). In cooperation with the ZUSE-Institute Berlin, we developed an innovative artificial intelligence-based design for peripherally acting opioids. This strategy used computational simulations of pathological receptor conformations and the finding that the protonation state of a ligand is crucial for its activity at opioid receptors. Our data indicated that the ligand's pK_a should be close to the acidic pH of injured tissue, which could be achieved by fluorination of the piperidine ring of the standard agonist fentanyl, leading to the novel compound NFEPP. We found that NFEPP selectively binds and activates opioid receptors at low pH (as in peripheral injured tissue) but not at normal pH (as in the CNS and myenteric plexus). In animal models of inflammatory, incisional and neuropathic pain, NFEPP exerted analgesic effects by selective activation of peripheral opioid receptors in injured tissue, but did not act in healthy tissue. Consistently, NFEPP was devoid of typical opioid side effects such as addiction potential, sedation, motor impairment, respiratory depression, and constipation (Spahn et al., 2017; Rodriguez-Gaztelumendi et al., 2018). In another study, we further developed our concept in that we exchanged hydrogen/fluorine in fentanyl′s ethylidene bridge leading to the compound FF3. FF3 displayed a pK_a of 7.2 and induced selective analgesia in injured tissue. However, high concentrations of FF3 induced sedation, motor disturbance, reward, constipation, and respiratory depression, leading us to the conclusion that a pK_a of 7.2 is too close to physiological pH (Spahn et al., 2018). Overall, our results indicate that the pK_a of an opioid receptor ligand can be used as a defining factor to predict the side effect profile of an opioid analgesic.

The second strategy to obtain opioids without side effects under intense current debate is the concept of "biased" opioid agonists. This concept is based on the finding that GPCR activation leads to the activation of both inhibitory G-proteins and β-arrestins. Investigations of β-arrestin 2 knockout mice revealed that morphine-induced antinociception was prolonged and respiratory depression was reduced, leading to the conclusion that opioid side effects are mediated via the β-arrestin 2 signaling pathway (Bohn et al., 1999; Raehal and Bohn, 2005; Schmid et al., 2017). Since this discovery, numerous studies were conducted to develop opioid

receptor agonists that selectively activate the G-protein, but not the β-arrestin pathway ("biased" agonists). One of the most promising candidates was TRV130 (oliceridin) (DeWire et al., 2013; Kingwell, 2015). However, a detailed analysis of the data uncovered similar side effect profiles of TRV130 compared to morphine (DeWire et al., 2013; Soergel et al., 2014; Viscusi et al., 2016; Singla et al., 2017; Negus and Freeman, 2018) (reviewed in (Machelska and Celik, 2018). Two subsequent phase 3 clinical studies in patients undergoing surgery showed effective analgesia but the respiratory safety endpoints were not achieved (FDA, 2018a). Finally, in October 2018, the US Food and Drug Administration (FDA) voted against the approval of TRV130 based on an advisory committee briefing document concluding that TRV130 is not *"safer than traditional opioids"* (FDA, 2018b; a). Recently, another G_i -biased agonist (PZM21) was discovered (Manglik et al., 2016). However, a subsequent study trying to reproduce the data measured concentration-dependent G_i-activation, β-arrestin recruitment, respiratory depression and tolerance to antinociceptive effects, again questioning this concept (Hill et al., 2018). Considering the cellular signaling mechanisms underlying opioid-induced side effects, the results of the above studies are not surprising, since most opioid-induced side effects are induced by the activation of G-proteins (reviewed in (Machelska and Celik, 2018). Additionally, a recent study has exploited MOR tolerance and desensitization to examine biased signaling: Both events are associated with phosphorylation of intracellular domains of MOR and subsequent β-arrestin signaling (Kliewer et al., 2019). The authors created a mouse line where MOR is unable to recruit β-arrestin due to a series of mutations of the receptor's C-terminus, and therefore served as the perfect biased agonist control (Kliewer et al., 2019). In these animals, opioid-induced analgesia was increased, tolerance was reduced, but opioid-induced adverse side effects were either unchanged or enhanced, leading to the conclusion that β-arrestin recruitment was not responsible for these side effects and questioning the concept of biased agonists (Kliewer et al., 2019).

Thus, the concept of biased ligands did not result in useful novel opioid analgesics so far. On the other hand, additional animal and human studies on peripherally restricted opioids like NFEPP have to be performed to examine their potential for clinical utility. Furthermore, long-term use and withdrawal of NFEPP have to be investigated, since opioid withdrawal can increase TRPV1 activity and induce hyperalgesia (see chapter 2.1.3. and (Spahn et al., 2013).

3.3. Concluding remarks

In summary, pain may be treated by influencing different components of the pain pathway: inhibition of pain generation, inhibition of pain transmission and/or inhibition of pain perception. Here, we investigated two approaches that address the peripheral origin of the pain pathway under pathological conditions. Figure 5 summarizes both appoaches.

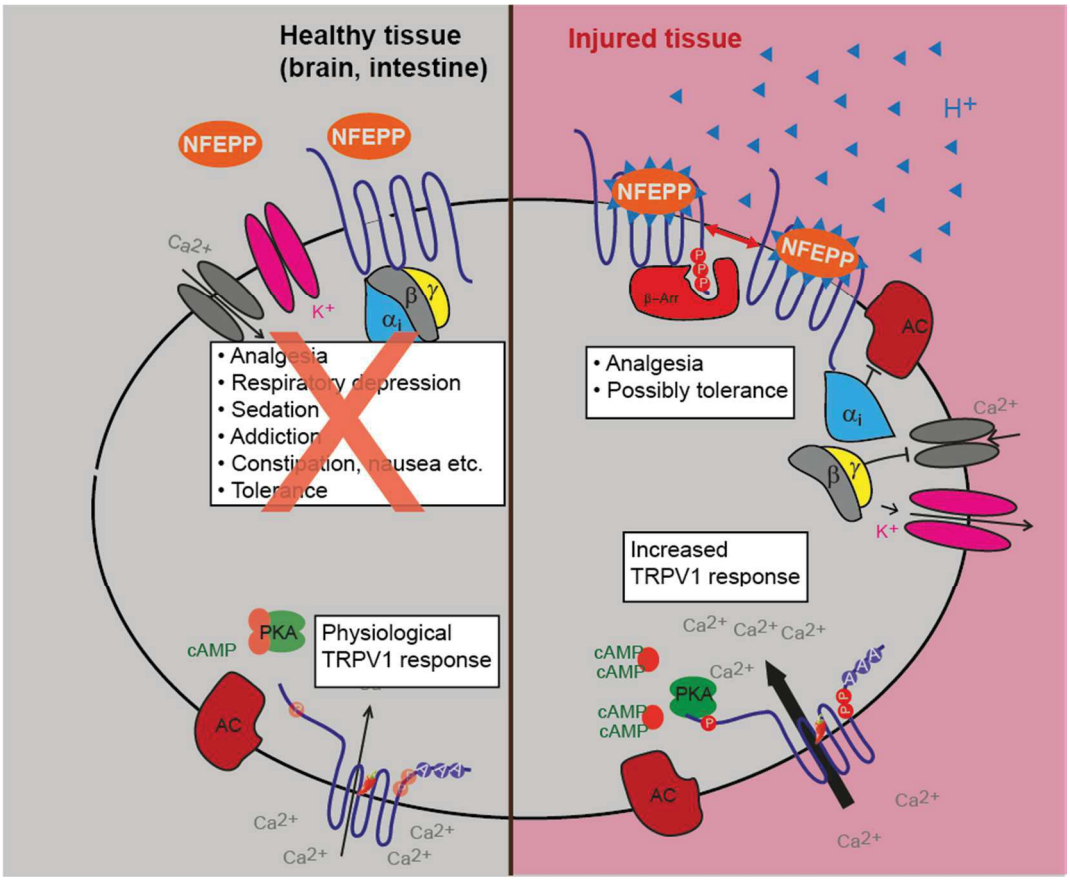


Figure 5: Summary of the investigated mechanisms. During inflammation, the tissue is acidified, NFEPP is protonated and able to bind and activate opioid receptors leading to analgesia (upper right). In healthy tissue, NFEPP is deprotonated and does not activate central or intestinal opioid receptors (upper left). During pathological conditions, PKA is highly active leading to sensitized TRPV1 and hyperalgesia (lower right). Under physiological conditions, PKA is mostly inactive resulting in a physiological TRPV1 response to painful stimuli (lower left). Reprinted adapted with permission from G. Del Vecchio, V. Spahn, C. Stein, Novel Opioid Analgesics and Side Effects, ACS Chem Neurosci, 2017, 8(8):1638-1640. Copyright (2017) American Chemical Society (Del Vecchio et al., 2017).

We found that prevention of TRPV1 sensitization could be a useful strategy to eliminate TRPV1-induced hyperalgesia under pathological conditions. However, the translation of these findings into the clinical setting is still in the distant future. Further steps would be the identification and detailed *in vitro*/*in vivo* investigation of targets at TRPV1, which cause inflammation-induced TRPV1 sensitization, followed by the development and preclinical characterization of compounds that interfere with these targets. On the other hand, our results regarding the development of safer opioids already demonstrate a powerful artificial

intelligence-based strategy for the design of peripherally restricted opioid receptor activation with the lead candidate NFEPP. NFEPP was extensively and successfully tested in *in vitro* and *in vivo* systems, which is the basis for further exploitation and regulatory pre-clinical and clinical testing.

3.4. Outlook

Treating pain at its peripheral source is a promising strategy to induce sufficient pain relief. The development of new TRPV1 targets with indication for inflammatory or neuropathic pain is an active research field in the pharmaceutical industry. However, the clinical development of TRPV1 antagonists is still ongoing and examines different strategies like the route of administration or "partial" TRPV1 blockade. The idea of preventing inflammation-induced TRPV1 sensitization is relatively new and still in its infancy (Aghazadeh Tabrizi et al., 2017). One potential strategy based on our findings could be the development of molecules that disrupt the TRPV1/ARMS/PKA interaction and therefore PKA-induced TRPV1 sensitization. On the other hand, there were no commercially successful innovations since the late 80ies in the opioid analgesic field (Faria et al., 2018). This might change if novel compounds like NFEPP would be further developed on a commercial basis. This might be highly beneficial for many patients with injury-induced painful syndromes. Furthermore, the development of other peripherally restricted opioids with different pK_a-values due to other electronegative moietie scould be of interest. Finally, future studies should investigate whether our concept is applicable to other classes of GPCRs and other types of pathological conditions.

4. Summary

Currently available pain medications are limited by adverse side effects leading to enormous individual and socioeconomic costs. Therefore, the investigation of pathological receptor conformations and mechanisms involved in pain sensitization is urgently needed. I investigated the pain pathway from two perspectives. In the first part, I focused on mechanisms underlying the initiation of pain and sensitization. I concentrated on the identification of mechanisms that sensitize TRPV1. TRPV1 is an excitatory ion channel that plays a fundamental role in neuronal sensitization during tissue injury and inflammation. I found that the interaction of TRPV1 with other proteins like TRPA1 or ARMS leads to a cAMP and PKA dependent channel sensitization. The same signaling pathway is responsible for TRPV1-induced hyperalgesia during opioid withdrawal, leading to the conclusion that targeting PKA-induced TRPV1 sensitization could be a strategy to circumvent TRPV1 sensitization without direct TRPV1 blockade.

In the second part, I concentrated on the investigation of inhibitory components of the pain pathway, particularly the opioid receptor system under pathological conditions. Results showed that decreased pH – a hallmark of tissue inflammation – can be used to design opioids that selectively activate opioid receptors under pathological conditions. Opioid ligands have to be protonated to bind and active their respective receptors. Classical opioids are protonated under both physiological and pathological conditions and therefore activate opioid receptors in both healthy and injured tissues. The reduction of the pK_a of an opioid ligand close to the pH of inflamed tissue resulted in the selective activation of opioid receptors in injured, but not healthy environments. Our lead candidate NFEPP produced efficient injury-restricted analgesia in animal models of inflammatory, visceral, and neuropathic pain without inducing side effects like respiratory depression, sedation, constipation, or addiction.

5. References

Aghazadeh Tabrizi, M., Baraldi, P.G., Baraldi, S., Gessi, S., Merighi, S., and Borea, P.A. (2017). Medicinal Chemistry, Pharmacology, and Clinical Implications of TRPV1 Receptor Antagonists. *Med Res Rev* 37**:**936-983.

Anand, P., and Bley, K. (2011). Topical capsaicin for pain management: therapeutic potential and mechanisms of action of the new high-concentration capsaicin 8% patch. *Br J Anaesth* 107**:**490-502.

Antonijevic, I., Mousa, S.A., Schafer, M., and Stein, C. (1995). Perineurial defect and peripheral opioid analgesia in inflammation. *J Neurosci* 15**:**165-172.

Basbaum, A.I., Bautista, D.M., Scherrer, G., and Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell* 139**:**267-284.

Benyamin, R., Trescot, A.M., Datta, S., Buenaventura, R., Adlaka, R., Sehgal, N., Glaser, S.E., and Vallejo, R. (2008). Opioid complications and side effects. *Pain Physician* 11**:**S105-120.

Bohn, L.M., Gainetdinov, R.R., Lin, F.T., Lefkowitz, R.J., and Caron, M.G. (2000). Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. *Nature* 408**:**720-723.

Bohn, L.M., Lefkowitz, R.J., Gainetdinov, R.R., Peppel, K., Caron, M.G., and Lin, F.T. (1999). Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286**:**2495-2498.

Brito, R., Sheth, S., Mukherjea, D., Rybak, L.P., and Ramkumar, V. (2014). TRPV1: A Potential Drug Target for Treating Various Diseases. *Cells* 3**:**517-545.

Califf, R.M., Woodcock, J., and Ostroff, S. (2016). A Proactive Response to Prescription Opioid Abuse. *N Engl J Med* 374**:**1480-1485.

Cao, E.H., Liao, M.F., Cheng, Y.F., and Julius, D. (2013). TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* 504**:**113-+.

Carcoba, L.M., Contreras, A.E., Cepeda-Benito, A., and Meagher, M.W. (2011). Negative affect heightens opiate withdrawal-induced hyperalgesia in heroin dependent individuals. *J Addict Dis* 30**:**258-270.

Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., and Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 288**:**306-313.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., and Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389**:**816-824.

Che, T., Majumdar, S., Zaidi, S.A., Ondachi, P., Mccorvy, J.D., Wang, S., Mosier, P.D., Uprety, R., Vardy, E., Krumm, B.E., Han, G.W., Lee, M.Y., Pardon, E., Steyaert, J., Huang, X.P., Strachan, R.T., Tribo, A.R., Pasternak, G.W., Carroll, F.I., Stevens, R.C., Cherezov, V., Katritch, V., Wacker, D., and Roth, B.L. (2018). Structure of the Nanobody-Stabilized Active State of the Kappa Opioid Receptor. *Cell* 172**:**55-67 e15.

Clapham, D.E., Runnels, L.W., and Strubing, C. (2001). The TRP ion channel family. *Nat Rev Neurosci* 2**:**387-396.

Corder, G., Castro, D.C., Bruchas, M.R., and Scherrer, G. (2018). Endogenous and Exogenous Opioids in Pain. *Annu Rev Neurosci* 41**:**453-473.

Coxib, Traditional, N.T.C., Bhala, N., Emberson, J., Merhi, A., Abramson, S., Arber, N., Baron, J.A., Bombardier, C., Cannon, C., Farkouh, M.E., Fitzgerald, G.A., Goss, P., Halls, H., Hawk, E., Hawkey, C., Hennekens, C., Hochberg, M., Holland, L.E., Kearney, P.M., Laine, L., Lanas, A., Lance, P., Laupacis, A., Oates, J., Patrono, C., Schnitzer, T.J., Solomon, S., Tugwell, P., Wilson, K., Wittes, J., and Baigent, C. (2013). Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. *Lancet* 382**:**769-779.

Darre, L., and Domene, C. (2015). Binding of Capsaicin to the TRPV1 Ion Channel. *Mol Pharm* 12**:**4454-4465.

Davis, J.B., Gray, J., Gunthorpe, M.J., Hatcher, J.P., Davey, P.T., Overend, P., Harries, M.H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S.A., Rance, K., Grau, E., Harper, A.J., Pugh, P.L., Rogers, D.C., Bingham, S., Randall, A., and Sheardown, S.A. (2000). Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 405**:**183-187.

Del Vecchio, G., Spahn, V., and Stein, C. (2017). Novel Opioid Analgesics and Side Effects. *ACS Chem Neurosci* 8**:**1638-1640.

Derry, S., and Moore, R.A. (2012). Topical capsaicin (low concentration) for chronic neuropathic pain in adults. *Cochrane Database Syst Rev***:**CD010111.

Derry, S., Rice, A.S., Cole, P., Tan, T., and Moore, R.A. (2017). Topical capsaicin (high concentration) for chronic neuropathic pain in adults. *Cochrane Database Syst Rev* 1**:**CD007393.

Dewire, S.M., Yamashita, D.S., Rominger, D.H., Liu, G., Cowan, C.L., Graczyk, T.M., Chen, X.T., Pitis, P.M., Gotchev, D., Yuan, C., Koblish, M., Lark, M.W., and Violin, J.D. (2013). A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J Pharmacol Exp Ther* 344**:**708-717.

Dray, A. (2009). "Pharmacological Modulation of Pain," in *Science of Pain,* eds. A.I. Basbaum & C. Bushnell. (Oxford, UK and San Diego, USA: Elsevier):796.

Endres-Becker, J., Heppenstall, P.A., Mousa, S.A., Labuz, D., Oksche, A., Schafer, M., Stein, C., and Zollner, C. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol Pharmacol* 71**:**12-18.

Evans, C.J., Keith, D.E., Jr., Morrison, H., Magendzo, K., and Edwards, R.H. (1992). Cloning of a delta opioid receptor by functional expression. *Science* 258**:**1952-1955.

Faria, J., Barbosa, J., Moreira, R., Queiros, O., Carvalho, F., and Dinis-Oliveira, R.J. (2018). Comparative pharmacology and toxicology of tramadol and tapentadol. *Eur J Pain* 22**:**827-844.

Fda (2018a). *FDA ADVISORY COMMITTEE BRIEFING DOCUMENT Oliceridine* [Online]. Available: https://www.fda.gov/media/121230/download [Accessed 08.05.2019 2019].

Fda (2018b). *Final Summary Minutes of the Anesthetic and Analgesic Drug Products Advisory Committee Meeting* [Online]. Available: https://www.fda.gov/media/119281/download [Accessed 08.05.2019 2019].

Ford, A., Ballard, P., Ramage, C., and Ogah, J. (2016). "2016 cochrane review: intravesical treatments for bladder pain syndrome/interstitial cystitis", in: *46th Annual Meeting of the International Continence Society.* (Tokyo, Japan).

Gangadharan, V., and Kuner, R. (2013). Pain hypersensitivity mechanisms at a glance. *Dis Model Mech* 6**:**889-895.

Garami, A., Pakai, E., Mcdonald, H.A., Reilly, R.M., Gomtsyan, A., Corrigan, J.J., Pinter, E., Zhu, D.X.D., Lehto, S.G., Gavva, N.R., Kym, P.R., and Romanovsky, A.A. (2018). TRPV1 antagonists that cause hypothermia, instead of hyperthermia, in rodents: Compounds' pharmacological profiles, in vivo targets, thermoeffectors recruited and implications for drug development. *Acta Physiol (Oxf)* 223**:**e13038.

Gaveriaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X.C., Weibel, R., Matifas, A., Reiss, D., Filliol, D., Nassar, M.A., Wood, J.N., Maldonado, R., and Kieffer, B.L. (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 152**:**1238-1248.

Gonzalez-Rodriguez, S., Quadir, M.A., Gupta, S., Walker, K.A., Zhang, X., Spahn, V., Labuz, D., Rodriguez-Gaztelumendi, A., Schmelz, M., Joseph, J., Parr, M.K., Machelska, H.,

Haag, R., and Stein, C. (2017). Polyglycerol-opioid conjugate produces analgesia devoid of side effects. *Elife* 6.

Gouin, O., L'herondelle, K., Lebonvallet, N., Le Gall-Ianotto, C., Sakka, M., Buhe, V., Plee-Gautier, E., Carre, J.L., Lefeuvre, L., Misery, L., and Le Garrec, R. (2017). TRPV1 and TRPA1 in cutaneous neurogenic and chronic inflammation: pro-inflammatory response induced by their activation and their sensitization. *Protein Cell* 8**:**644-661.

Granier, S., Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Weis, W.I., and Kobilka, B.K. (2012). Structure of the delta-opioid receptor bound to naltrindole. *Nature* 485**:**400-404.

Guedes, V., Castro, J.P., and Brito, I. (2018). Topical capsaicin for pain in osteoarthritis: A literature review. *Reumatol Clin* 14**:**40-45.

Hassan, A.H., Ableitner, A., Stein, C., and Herz, A. (1993). Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. *Neuroscience* 55**:**185-195.

Hill, R., Disney, A., Conibear, A., Sutcliffe, K., Dewey, W., Husbands, S., Bailey, C., Kelly, E., and Henderson, G. (2018). The novel mu-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. *Br J Pharmacol* 175**:**2653-2661.

Hua, S., and Cabot, P.J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16**:**E199-216.

Huang, W., Manglik, A., Venkatakrishnan, A.J., Laeremans, T., Feinberg, E.N., Sanborn, A.L., Kato, H.E., Livingston, K.E., Thorsen, T.S., Kling, R.C., Granier, S., Gmeiner, P., Husbands, S.M., Traynor, J.R., Weis, W.I., Steyaert, J., Dror, R.O., and Kobilka, B.K. (2015). Structural insights into micro-opioid receptor activation. *Nature* 524**:**315-321.

Iasp (2011). *IASP PainTerminology* [Online]. Available: https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698&navItemNumber=576 [Accessed 22.02.2019 2019].

Isensee, J., Wenzel, C., Buschow, R., Weissmann, R., Kuss, A.W., and Hucho, T. (2014). Subgroup-elimination transcriptomics identifies signaling proteins that define subclasses of TRPV1-positive neurons and a novel paracrine circuit. *PLoS One* 9**:**e115731.

Jagla, C., Martus, P., and Stein, C. (2014). Peripheral opioid receptor blockade increases postoperative morphine demands--a randomized, double-blind, placebo-controlled trial. *Pain* 155**:**2056-2062.

Jeanjean, A.P., Moussaoui, S.M., Maloteaux, J.M., and Laduron, P.M. (1995). Interleukin-1 beta induces long-term increase of axonally transported opiate receptors and substance P. *Neuroscience* 68**:**151-157.

Jeske, N.A., Patwardhan, A.M., Ruparel, N.B., Akopian, A.N., Shapiro, M.S., and Henry, M.A. (2009). A-kinase anchoring protein 150 controls protein kinase C-mediated phosphorylation and sensitization of TRPV1. *Pain* 146**:**301-307.

Kieffer, B.L., Befort, K., Gaveriaux-Ruff, C., and Hirth, C.G. (1992). The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* 89**:**12048-12052.

Kingwell, K. (2015). Pioneering biased ligand offers efficacy with reduced on-target toxicity. *Nat Rev Drug Discov* 14**:**809-810.

Kliewer, A., Schmiedel, F., Sianati, S., Bailey, A., Bateman, J.T., Levitt, E.S., Williams, J.T., Christie, M.J., and Schulz, S. (2019). Phosphorylation-deficient G-protein-biased mu-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* 10**:**367.

Klincewicz, S. (2018). *Study of Resiniferatoxin for Knee Pain in Moderate to Severe Osteoarthritis* [Online]. Available: https://clinicaltrials.gov/ct2/show/NCT03542838 [Accessed 08.05.2019 2019].

Latremoliere, A., and Woolf, C.J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain* 10**:**895-926.
Liao, M.F., Cao, E.H., Julius, D., and Cheng, Y.F. (2013). Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504**:**107-+.
Lynch, M.E., and Watson, C.P. (2006). The pharmacotherapy of chronic pain: a review. *Pain Res Manag* 11**:**11-38.
Machelska, H., and Celik, M.O. (2018). Advances in Achieving Opioid Analgesia Without Side Effects. *Front Pharmacol* 9**:**1388.
Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Mathiesen, J.M., Sunahara, R.K., Pardo, L., Weis, W.I., Kobilka, B.K., and Granier, S. (2012). Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* 485**:**321-326.
Manglik, A., Lin, H., Aryal, D.K., Mccorvy, J.D., Dengler, D., Corder, G., Levit, A., Kling, R.C., Bernat, V., Hubner, H., Huang, X.P., Sassano, M.F., Giguere, P.M., Lober, S., Da, D., Scherrer, G., Kobilka, B.K., Gmeiner, P., Roth, B.L., and Shoichet, B.K. (2016). Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537**:**185-190.
Meng, F., Xie, G.X., Thompson, R.C., Mansour, A., Goldstein, A., Watson, S.J., and Akil, H. (1993). Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc Natl Acad Sci U S A* 90**:**9954-9958.
Mickle, A.D., Shepherd, A.J., and Mohapatra, D.P. (2015). Sensory TRP channels: the key transducers of nociception and pain. *Prog Mol Biol Transl Sci* 131**:**73-118.
Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G., and Meunier, J.C. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. *FEBS Lett* 341**:**33-38.
Montell, C. (2005). The TRP superfamily of cation channels. *Sci STKE* 2005**:**re3.
Montell, C., and Rubin, G.M. (1989). Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron* 2**:**1313-1323.
Mousa, S.A., Cheppudira, B.P., Shaqura, M., Fischer, O., Hofmann, J., Hellweg, R., and Schafer, M. (2007). Nerve growth factor governs the enhanced ability of opioids to suppress inflammatory pain. *Brain* 130**:**502-513.
Negus, S.S., and Freeman, K.B. (2018). Abuse Potential of Biased Mu Opioid Receptor Agonists. *Trends Pharmacol Sci* 39**:**916-919.
Nestler, E.J. (1992). Molecular mechanisms of drug addiction. *J Neurosci* 12**:**2439-2450.
Neubrand, V.E., Cesca, F., Benfenati, F., and Schiavo, G. (2012). Kidins220/ARMS as a functional mediator of multiple receptor signalling pathways. *J Cell Sci* 125**:**1845-1854.
Patapoutian, A., Tate, S., and Woolf, C.J. (2009). Transient receptor potential channels: targeting pain at the source. *Nat Rev Drug Discov* 8**:**55-68.
Peter, J., Kasper, C., Kaufholz, M., Buschow, R., Isensee, J., Hucho, T., Herberg, F.W., Schwede, F., Stein, C., Jordt, S.E., Brackmann, M., and Spahn, V. (2017). Ankyrin-rich membrane spanning protein as a novel modulator of transient receptor potential vanilloid 1-function in nociceptive neurons. *Eur J Pain* 21**:**1072-1086.
Raehal, K.M., and Bohn, L.M. (2005). Mu opioid receptor regulation and opiate responsiveness. *AAPS J* 7**:**E587-591.
Ranade, S.S., Syeda, R., and Patapoutian, A. (2015). Mechanically Activated Ion Channels. *Neuron* 87**:**1162-1179.
Rittner, H.L., Amasheh, S., Moshourab, R., Hackel, D., Yamdeu, R.S., Mousa, S.A., Fromm, M., Stein, C., and Brack, A. (2012). Modulation of tight junction proteins in the perineurium to facilitate peripheral opioid analgesia. *Anesthesiology* 116**:**1323-1334.
Rittner, H.L., Brack, A., and Stein, C. (2008). Pain and the immune system. *Br J Anaesth* 101**:**40-44.

Rittner, H.L., Hackel, D., Voigt, P., Mousa, S., Stolz, A., Labuz, D., Schafer, M., Schaefer, M., Stein, C., and Brack, A. (2009). Mycobacteria attenuate nociceptive responses by formyl peptide receptor triggered opioid peptide release from neutrophils. *PLoS Pathog* 5**:**e1000362.

Rodriguez-Gaztelumendi, A., Spahn, V., Labuz, D., Machelska, H., and Stein, C. (2018). Analgesic effects of a novel pH-dependent mu-opioid receptor agonist in models of neuropathic and abdominal pain. *Pain* 159**:**2277-2284.

Romanovsky, A.A., Almeida, M.C., Garami, A., Steiner, A.A., Norman, M.H., Morrison, S.F., Nakamura, K., Burmeister, J.J., and Nucci, T.B. (2009). The transient receptor potential vanilloid-1 channel in thermoregulation: a thermosensor it is not. *Pharmacol Rev* 61**:**228-261.

Sandkuhler, J. (2009). Models and mechanisms of hyperalgesia and allodynia. *Physiol Rev* 89**:**707-758.

Schmid, C.L., Kennedy, N.M., Ross, N.C., Lovell, K.M., Yue, Z., Morgenweck, J., Cameron, M.D., Bannister, T.D., and Bohn, L.M. (2017). Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. *Cell* 171**:**1165-1175 e1113.

Shah, M., and Huecker, M.R. (2019). "Opioid Withdrawal," in *StatPearls*. (Treasure Island (FL).

Sharma, S.K., Klee, W.A., and Nirenberg, M. (1975). Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* 72**:**3092-3096.

Singla, N., Minkowitz, H.S., Soergel, D.G., Burt, D.A., Subach, R.A., Salamea, M.Y., Fossler, M.J., and Skobieranda, F. (2017). A randomized, Phase IIb study investigating oliceridine (TRV130), a novel micro-receptor G-protein pathway selective (mu-GPS) modulator, for the management of moderate to severe acute pain following abdominoplasty. *J Pain Res* 10**:**2413-2424.

Sip. 2018. Thematic Network on the Societal Impact of Pain - Framing Paper. Available: https://ec.europa.eu/health/sites/health/files/policies/docs/ev_20181112_co07_en.pdf [Accessed 22.02.2019].

Soergel, D.G., Subach, R.A., Burnham, N., Lark, M.W., James, I.E., Sadler, B.M., Skobieranda, F., Violin, J.D., and Webster, L.R. (2014). Biased agonism of the mu-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects versus morphine: A randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. *Pain* 155**:**1829-1835.

Spahn, V., Del Vecchio, G., Labuz, D., Rodriguez-Gaztelumendi, A., Massaly, N., Temp, J., Durmaz, V., Sabri, P., Reidelbach, M., Machelska, H., Weber, M., and Stein, C. (2017). A nontoxic pain killer designed by modeling of pathological receptor conformations. *Science* 355**:**966-969.

Spahn, V., Del Vecchio, G., Rodriguez-Gaztelumendi, A., Temp, J., Labuz, D., Kloner, M., Reidelbach, M., Machelska, H., Weber, M., and Stein, C. (2018). Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. *Scientific Reports* 8**:**8965.

Spahn, V., Fischer, O., Endres-Becker, J., Schafer, M., Stein, C., and Zollner, C. (2013). Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. *Pain* 154**:**598-608.

Spahn, V., Stein, C., and Zollner, C. (2014). Modulation of transient receptor vanilloid 1 activity by transient receptor potential ankyrin 1. *Mol Pharmacol* 85**:**335-344.

Stander, S., Moormann, C., Schumacher, M., Buddenkotte, J., Artuc, M., Shpacovitch, V., Brzoska, T., Lippert, U., Henz, B.M., Luger, T.A., Metze, D., and Steinhoff, M. (2004). Expression of vanilloid receptor subtype 1 in cutaneous sensory nerve fibers, mast cells, and epithelial cells of appendage structures. *Exp Dermatol* 13**:**129-139.

Stein, C. (2016). Opioid Receptors. *Annu Rev Med* 67**:**433-451.
Stein, C. (2018). New concepts in opioid analgesia. *Expert Opin Investig Drugs* 27**:**765-775.
Stein, C., Hassan, A.H., Przewlocki, R., Gramsch, C., Peter, K., and Herz, A. (1990). Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. *Proc Natl Acad Sci U S A* 87**:**5935-5939.
Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. *Pharmacol Rev* 63**:**860-881.
Stein, C., Pfluger, M., Yassouridis, A., Hoelzl, J., Lehrberger, K., Welte, C., and Hassan, A.H. (1996). No tolerance to peripheral morphine analgesia in presence of opioid expression in inflamed synovia. *J Clin Invest* 98**:**793-799.
Szallasi, A., and Sheta, M. (2012). Targeting TRPV1 for pain relief: limits, losers and laurels. *Expert Opin Investig Drugs* 21**:**1351-1369.
Thompson, A.A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., Huang, X.P., Trapella, C., Guerrini, R., Calo, G., Roth, B.L., Cherezov, V., and Stevens, R.C. (2012). Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* 485**:**395-399.
Toll, L., Bruchas, M.R., Calo, G., Cox, B.M., and Zaveri, N.T. (2016). Nociceptin/Orphanin FQ Receptor Structure, Signaling, Ligands, Functions, and Interactions with Opioid Systems. *Pharmacol Rev* 68**:**419-457.
Tominaga, M. (2006). "Gating, Sensitization, and Desensitization of TRPV1," in *The Nociceptive Membrane,* ed. U. Oh. Elsevier Inc.):181-197.
Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I., and Julius, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21**:**531-543.
Treede, R.-D. (2015a). "Definition von Schmerzen," in *Basisbuch Q 14 - Lehrbuch für das Querschnittfach Schmerzmedizin nach der neuen AO,* eds. A. Kopf, H. Rittner, M. Karst & R. Sabatowski. (Berlin, Germany: Deutsche Schmerzgesellschaft e. V.):163.
Treede, R.-D. (2015b). "Ethische und rechtliche Grundlagen der Schmerzmedizin," in *Basisbuch Q 14 - Lehrbuch für das Querschnittfach Schmerzmedizin nach der neuen AO,* eds. A. Kopf, H. Rittner, M. Karst & R. Sabatowski. (Berlin, Germany: Deutsche Schmerzgesellschaft e. V.):163.
Vay, L., Gu, C., and Mcnaughton, P.A. (2012). The thermo-TRP ion channel family: properties and therapeutic implications. *Br J Pharmacol* 165**:**787-801.
Vetter, I., Cheng, W., Peiris, M., Wyse, B.D., Roberts-Thomson, S.J., Zheng, J., Monteith, G.R., and Cabot, P.J. (2008). Rapid, opioid-sensitive mechanisms involved in transient receptor potential vanilloid 1 sensitization. *J Biol Chem* 283**:**19540-19550.
Viscusi, E.R., Webster, L., Kuss, M., Daniels, S., Bolognese, J.A., Zuckerman, S., Soergel, D.G., Subach, R.A., Cook, E., and Skobieranda, F. (2016). A randomized, phase 2 study investigating TRV130, a biased ligand of the mu-opioid receptor, for the intravenous treatment of acute pain. *Pain* 157**:**264-272.
Wang, J.B., Imai, Y., Eppler, C.M., Gregor, P., Spivak, C.E., and Uhl, G.R. (1993). mu opiate receptor: cDNA cloning and expression. *Proc Natl Acad Sci U S A* 90**:**10230-10234.
Weibel, R., Reiss, D., Karchewski, L., Gardon, O., Matifas, A., Filliol, D., Becker, J.A., Wood, J.N., Kieffer, B.L., and Gaveriaux-Ruff, C. (2013). Mu opioid receptors on primary afferent nav1.8 neurons contribute to opiate-induced analgesia: insight from conditional knockout mice. *PLoS One* 8**:**e74706.
Williams, J.T., Ingram, S.L., Henderson, G., Chavkin, C., Von Zastrow, M., Schulz, S., Koch, T., Evans, C.J., and Christie, M.J. (2013). Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* 65**:**223-254.

Woolf, C.J. (2010). Overcoming obstacles to developing new analgesics. *Nat Med* 16**:**1241-1247.

Woolf, C.J., and Ma, Q. (2007). Nociceptors--noxious stimulus detectors. *Neuron* 55**:**353-364.

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G.W., Vardy, E., Liu, W., Thompson, A.A., Huang, X.P., Carroll, F.I., Mascarella, S.W., Westkaemper, R.B., Mosier, P.D., Roth, B.L., Cherezov, V., and Stevens, R.C. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* 485**:**327-332.

Zhang, X., Huang, J., and Mcnaughton, P.A. (2005). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *EMBO J* 24**:**4211-4223.

Zhang, X., Li, L., and Mcnaughton, P.A. (2008). Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. *Neuron* 59**:**450-461.

Zhou, L., Zhang, Q., Stein, C., and Schafer, M. (1998). Contribution of opioid receptors on primary afferent versus sympathetic neurons to peripheral opioid analgesia. *J Pharmacol Exp Ther* 286**:**1000-1006.

Zollner, C., Mousa, S.A., Fischer, O., Rittner, H.L., Shaqura, M., Brack, A., Shakibaei, M., Binder, W., Urban, F., Stein, C., and Schafer, M. (2008). Chronic morphine use does not induce peripheral tolerance in a rat model of inflammatory pain. *J Clin Invest* 118**:**1065-1073.

Zollner, C., Shaqura, M.A., Bopaiah, C.P., Mousa, S., Stein, C., and Schafer, M. (2003). Painful inflammation-induced increase in mu-opioid receptor binding and G-protein coupling in primary afferent neurons. *Mol Pharmacol* 64**:**202-210.

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Erklärung § 4 Abs. 3 (k) der HabOMed der Charité

Hiermit erkläre ich, dass

- weder früher noch gleichzeitig ein Habilitationsverfahren durchgeführt oder angemeldet wurde,
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- mir die geltende Habilitationsordnung bekannt ist.

Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

11.07.2019 .....

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