# Investigation of SAFit2 as a potential treatment option for nerve injury- and chemotherapy-induced neuropathic pain

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## Zusammenfassung

Neuropathische Schmerzen beschreiben einen pathologischen und oft chronischen Schmerzzustand, der bis zu 10% der Weltbevölkerung betrifft. Hervorgerufen wird der neuropathische Schmerz durch Nervenschäden, die durch verschiedene Auslöser wie Krankheiten, medizinische Eingriffe und Vorfälle verursacht werden. Jedoch weisen derzeitig empfohlenen Behandlungsstrategien eine geringe Wirksamkeit sowie ein hohes Risiko für Nebenwirkungen auf. Folglich gibt es einen großen Bedarf an neuen Zielstrukturen sowie neuen Medikamenten für die Therapie von neuropathischen Schmerzen. Basierend darauf wurde in dieser Arbeit der selektive FKBP51 Inhibitor und potentielle Arzneimittelvorläufer SAFit2 im Kontext neuropathischer Schmerzen untersucht, die durch Nervenverletzung oder Chemotherapie verursacht werden.

Im ersten Teil dieser Arbeit wurde der Einfluss von SAFit2 auf neuropathische Schmerzen nach Nervenverletzungen und die zugrundeliegenden Mechanismen in einem Mausmodell untersucht. Hierbei linderte SAFit2 die mechanische Hypersensitivität von Mäusen durch Verminderung der exzessiven Nervenentzündung. Mechanistisch betrachtet, wirkte SAFit2 einer verstärkten NF-κB Aktivierung entgegen und verringerte dadurch die Infiltration von Immunzellen sowie die Konzentration von proinflammatorischen Zytokinen und Chemokinen in den Spinalganglien und im Rückenmark. SAFit2 beeinträchtigte jedoch nicht die Auflösung der Entzündung an der Verletzungsstelle, dem Ischiasnerv. Außerdem desensibilisierte SAFit2 den schmerzvermittelnden Ionenkanal TRPV1 in primären sensorischen Neuronen, indem SAFit2 die Aktivität der Phosphatase Calcineurin verstärkte, was zu einer Verringerung des TRPV1 vermittelten Calciuminfluxes führte. Infolgedessen verringerte SAFit2 die Freisetzung des proinflammatorischen Neuropeptids *calcitonin gene-related peptide* (CGRP), wodurch die Schmerzübertragung nach Nervenverletzung verringert und eine neurogene Entzündung gemindert wurde.

Im zweiten Teil dieser Arbeit wurde die Wirkung von SAFit2 auf das Lipidprofil in neuronalen Geweben untersucht, da Lipide wichtige Schmerzmediatoren darstellen. Interessanterweise stellte eine SAFit2 Behandlung basale Lipidkonzentrationen nach Nervenverletzung wieder her, insbesondere die des C16 Dihydroceramids in den Spinalganglien. Die Charakterisierung des C16 Dihydroceramids zeigte in primären sensorischen Neuronen, dass das Lipid den

schmerzvermittelnden TRPV1 Kanal ebenfalls desensibilisieren und die CGRP-Freisetzung von sensorischen Neuronen verringern kann. Weiterhin linderte das C16 Dihydroceramid die akute thermische Hypersensitivität in einem Capsaicin Mausmodell, was auf dessen anti-nozizeptive Eigenschaften hinweist.

Im dritten Teil dieser Arbeit wurde die Wirkung von SAFit2 auf neuropathische Schmerzen nach Chemotherapie, insbesondere durch Paclitaxel induzierte neuropathische Schmerzen, in einem Mausmodell untersucht. Hierbei konnte gezeigt werden, dass SAFit2 die durch Paclitaxel verursachte mechanische Hypersensitivität wirksam lindern konnte. Darüber hinaus wirkte SAFit2 der Paclitaxel induzierten Spinalgliose entgegen, da es die Aktivierung von Astrozyten und Mikroglia sowie die Konzentration schmerzvermittelnder Chemokine im dorsalen Rückenmark reduzieren konnte. Neben der immunologischen Komponente ergab die Analyse des Lipidprofils, dass SAFit2 die Spiegel von entzündungshemmenden und - auflösenden Oxylipinen nach Paclitaxel Behandlung in den Spinalganglien und im Rückenmark erhöhen konnte. Diese Ergebnisse erweitern das mechanistische Verständnis entscheidend und identifizieren, inwiefern SAFit2 Paclitaxel induzierte neuropathische Schmerzen lindern und schmerzverursachende Prozesse verringern kann.

Zusammenfassend zeigen diese Erkenntnisse vielversprechende analgetische Eigenschaften von SAFit2 im Kontext von Nervenverletzung und durch Paclitaxel hervorgerufener neuropathischer Schmerzen. SAFit2 linderte neuropathische Schmerzen, indem es durch Nervenverletzung veränderte Lipidspiegel wiederherstellte und mehrere zugrunde liegende neuroinflammatorische Mechanismen regulierte. Hingegen ist eine Verbesserung der Bioverfügbarkeit und Selektivität des Wirkstoffs SAFit2 für eine präklinische und klinische Anwendung notwendig. Auf dieser Grundlage stellt SAFit2 eine neue und vielversprechende Leitstruktur sowie einen möglichen Arzneimittelvorläufer für die Behandlung von Nervenverletzungen und Paclitaxel induzierten neuropathischen Schmerzen dar.

## Summary

Neuropathic pain describes a pathological and often chronic pain state that affects around 10% of the world population. The pathological pain arises after nerve lesions which are caused by diverse etiologies, encompassing diseases, medical conditions, and interventions. However, current and recommended treatment strategies carry a high risk of adverse effects and are inefficient in many cases. Consequently, the demand of novel targets and drug candidates for neuropathic pain emerges. In line with this, the focus of this thesis is set on the investigation of SAFit2, a selective FKBP51 inhibitor, as novel treatment option for nerve injury- and chemotherapy-induced neuropathic pain.

In the first part of this thesis, the influence of SAFit2 on nerve injury-induced neuropathic pain and the underlying mechanisms was assessed in a spared nerve injury mouse model. The results showed that SAFit2 ameliorates mechanical hypersensitivity as it diminishes excessive neuroinflammation after nerve injury. More specifically, SAFit2 counteracted an enhanced NF-κB activation and thereby reduced immune cell infiltration as well as pro-inflammatory cytokine and chemokine levels in dorsal root ganglia and spinal cord of nerve injured mice. However, SAFit2 did not impair the immune cell distribution and resolution of inflammation at the site of injury, the sciatic nerve. Besides this, SAFit2 desensitized the pain-mediating ion channel TRPV1 in primary sensory neurons by enhancing the activity of calcineurin, resulting in a reduction of TRPV1-mediated calcium transients. In consequence, SAFit2 decreased the release of the pro-inflammatory neuropeptide calcitonin gene-related peptide (CGRP) as downstream event, leading to a reduction of exacerbated pain transmission and neuroinflammation after nerve injury.

In the second part of this thesis, the impact of SAFit2 on lipid distribution was assessed in a spared nerve injury mouse model, since lipids represent crucial mediators in pain. Interestingly, SAFit2 restored lipid levels after nerve injury, especially levels of the C16 dihydroceramide in dorsal root ganglia. Like SAFit2, C16 dihydroceramide desensitized the pain-mediating ion channel TRPV1 in primary sensory neurons as well as reduced the CGRP release from primary sensory neurons. In line with this, C16 dihydroceramide alleviated acute thermal hypersensitivity in a capsaicin mouse model, indicating its anti-nociceptive properties.

In the third part of this thesis, the effect of SAFit2 on chemotherapy-induced neuropathic pain, especially paclitaxel-induced neuropathic pain, was evaluated in a multiple low dose mouse model. The data demonstrated that SAFit2 efficiently ameliorates paclitaxel-induced mechanical hypersensitivity. Moreover, SAFit2 counteracted paclitaxel-mediated spinal cord gliosis since it reduced the activation of astrocytes and microglia, as well as the levels of pain-mediating chemokines in the spinal cord. Beside the immunological component, the analysis of lipids revealed that SAFit2 increases anti-inflammatory and pro-resolving oxylipin levels in dorsal root ganglia and spinal cord after paclitaxel treatment. These results crucially expand our mechanistic understandings as well as identify how SAFit2 alleviates paclitaxel-induced neuropathic pain and decreases pain contributing processes.

In summary, these insights reveal promising analgesic properties of SAFit2 in the context of nerve injury- and paclitaxel-induced neuropathic pain. Furthermore, SAFit2 mediated a neuropathic pain relief by restoring injury-altered lipid levels and addressing several underlying neuroinflammatory mechanisms. However, the bioavailability and selectivity of SAFit2 has to be improved for preclinical and clinical usage. Based on this, SAFit2 constitutes as novel and promising lead compound and drug precursor for the treatment of nerve injury- and paclitaxel-induced neuropathic pain.

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# 1 General Introduction

## 1.1 Nociception and physiological pain

Pain is classified by the international association for the study of pain (IASP) as an unpleasant sensory perception of the nervous system that is influenced by many biological, psychological, and social factors (1). Even though it is experienced as an unpleasant sensation, pain in its physiological form serves as warning system in which nociception protects the body from potential tissue damage. Furthermore, it enables a well-working interaction of the body with its surrounding environment (2).

Nociception is caused by noxious thermal (hot or cold), mechanical or chemical stimuli, which are detected by a population of terminal nerve fibers, called nociceptors (3). To transduce such noxious stimuli, nociceptors express a variety of ion channels such as transient receptor potential (TRP) channels at their membrane to convert noxious stimuli into electric activity (4). To activate such ligand-gated and voltage-gated ion channels under physiological conditions, a stimulus has to reach a high-threshold in a noxious range (5). Once those ion channels are activated, intracellular calcium levels increase which subsequently alter the membrane potential and in turn open voltage-gated ion channels. This finally results in the generation of an action potential which is transmitted to the dorsal root ganglia (DRGs), the cell bodies of nociceptors. From the DRGs, the electrical signal is subsequently transmitted to the laminae I and II of the spinal cord, where the signal is firstly processed and conveyed in a synaptic connection. After this first synaptic transduction, the signal is further transmitted through the spinothalamic tract and the thalamus to the somatosensory cortex. In the brain, the noxious stimulus signal is processed, and a withdrawal information is sent through descending pathways into the periphery (Figure 1) (2, 3, 6, 7).

Nociceptors are pseudounipolar nerve fibers which means that they have axonal branches into both ascending and descending directions which enables them to additionally innervate the surrounding organs such as muscles and skin but also internal organs (8). Nociceptors in general describe a highly heterogeneous group which was loosely classified and categorized upon its conducting and molecular properties (9). Based on the conducting properties, nociceptors can be mainly classified into two classes. The first class consists of

myelinated medium diameter nociceptors that are called Aδ fibers and transduce stimuli rapidly to the central nervous system, resulting in a fast and well-localized pain sensation. In contrast, the second class of nociceptors comprises unmyelinated nerve fibers with small diameter that conduct pain slower than Aδ fibers. Accordingly, these nerve fibers transmit the "second slow" or more diffuse pain and are termed C fibers. In addition, C fibers can be further subdivided into two classes: the "peptidergic" and "non-peptidergic" sensory neurons. Peptidergic nerve fibers release neuropeptides such as neuropeptide P and calcitonin gene-related peptide (CGRP) upon activation, whereas non-peptidergic nerve fibers do not (2).
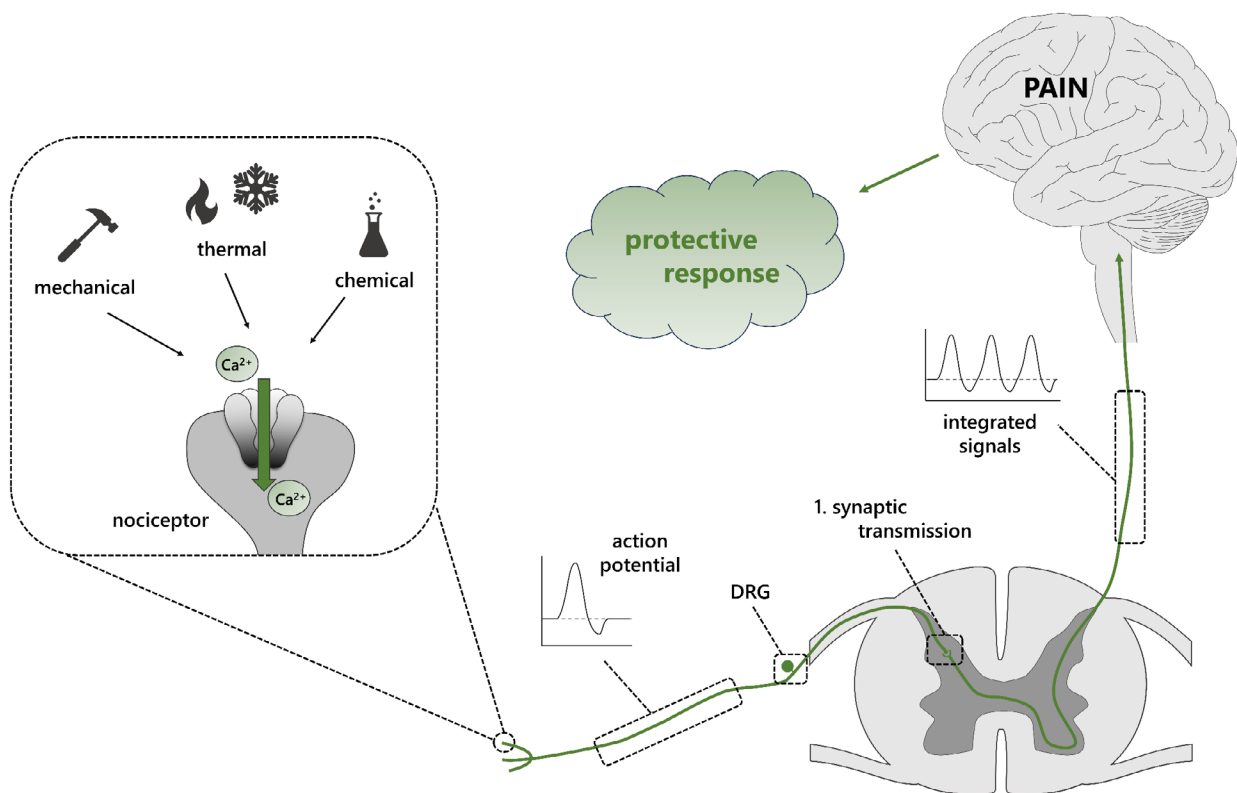


**Figure 1: Nociception in physiological pain**. Noxious stimuli (thermal, mechanical, or chemical) are recognized by nociceptors at terminal endings of sensory neurons. The respective nociceptors transduce high-threshold stimuli into electric signals via the influx of cationic ions such as calcium. Next, the electric signal is conveyed via the cell bodies of sensory neurons, located in the dorsal root ganglia (DRGs), to the dorsal horn of the spinal cord. In the spinal cord, the signal reaches the first synaptic transmission and is processed. Afterwards, the signal is further transduced via ascending fibers into the brain to the somatosensory cortex. In the somatosensory cortex, the signal is finally processed and converted into a protective response which is then send via descending pathways to the executing organ. The illustration was created with images from motifolio.
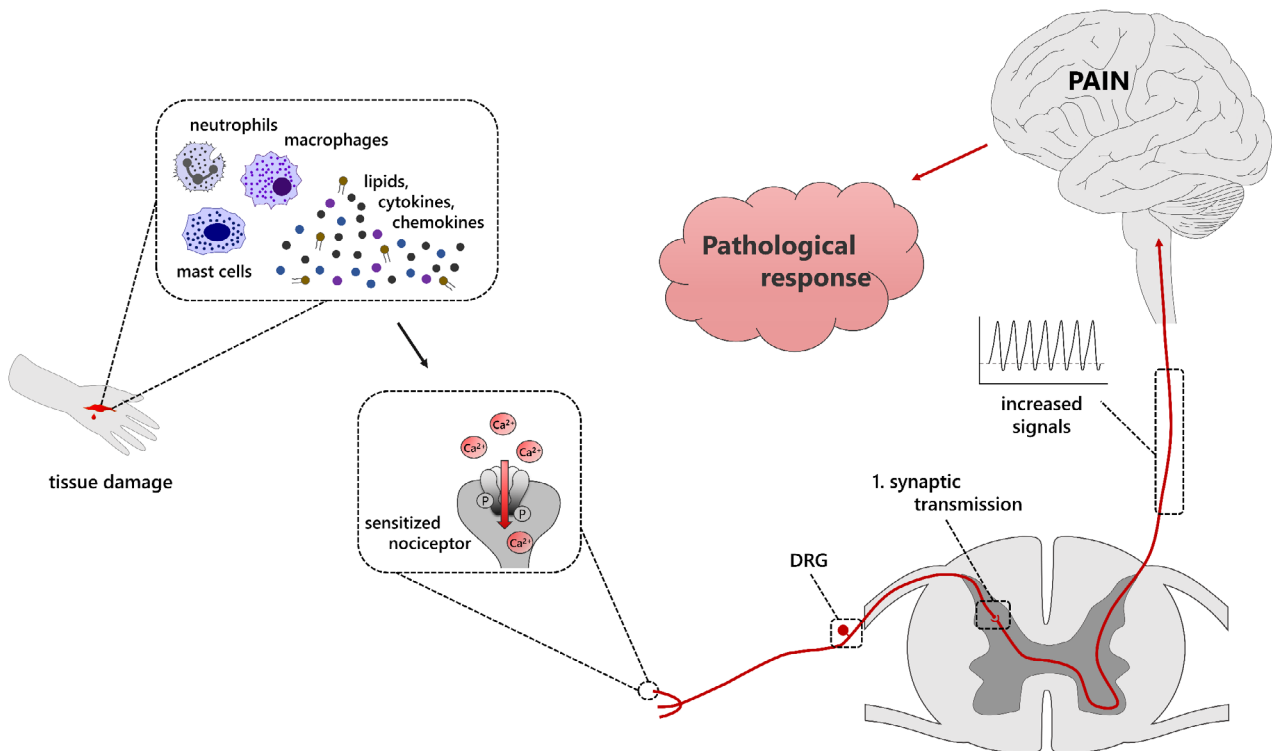
## 1.2 Pathological nociceptive pain

The second type of pain is termed pathological nociceptive or inflammatory pain and serves as protective function of the body like physiological pain. However, inflammatory pain is defined as a pathophysiological type of pain since it occurs after tissue damage and the subsequent resulting inflammation (10). Nevertheless, the primary function of inflammatory pain is to prevent further tissue damage and to enable a successful tissue healing. Therefore, the sensory system undergoes changes regarding its responsiveness towards external stimuli (11). In line with this, an increased responsiveness leads to an enhanced and prolonged perception of noxious stimuli and to the sensation of pain after innocuous stimuli. These changes in pain perception are termed hyperalgesia and allodynia respectively and describe typical symptoms of inflammatory pain (12). More specifically, hyperalgesia is described as signal amplification of painful stimuli, leading to an increased sensation of pain after a painful stimulus. Whereas allodynia is described as a more severe form of hypersensitivity, since it is defined as the sensation of pain after non-nociceptive stimuli like light touches (12).

Usually, symptoms of inflammatory pain such as an altered pain perception are locally restricted to the site of inflammation and do not affect the non-inflamed surrounding tissue. Furthermore, the altered pain perception is mainly caused by pro-inflammatory mediators which are secreted from the inflamed tissue (13). In turn, further immune cells are recruited to the site of inflammation and pro-inflammatory mediators are released, generating an "inflammatory soup" (3). This inflammatory soup can directly affect and increase the responsiveness of nociceptors which is called peripheral sensitization (Figure 2) (14).

Under physiological circumstances, the altered pain perception typically resolves after tissue healing and resolution of inflammation. Thereby, sensitizing mechanisms are reverted and mechanisms of physiological pain restored (15). However, inflammatory pain can become chronic in disorders such as rheumatic arthritis. In these cases, the inflammatory pain persists as long as the inflammation is active and mechanisms of central sensitization become more relevant (16, 17). Central sensitization is defined as changes in the ascending pathways like the dorsal horn of the spinal cord, resulting in a prolonged and spatially non-restricted perception of pain (18). The mechanisms of both peripheral and central sensitization are described in detail in a separate following chapter.

**Figure 2: Pain transmission in pathological nociceptive pain**. Upon tissue damage, sensitization processes such as peripheral sensitization are established to prevent the body from further tissue damage and to enable a successful wound healing. Therefore, resident immune cells like mast cells and macrophages release pro-inflammatory mediators. These in turn recruit immune cells which release further mediators such as cytokines, chemokines, and nerve growth factors. Beside immune cells, sensory neurons additionally release mediators such as lipids and neuropeptides that contribute to the inflammatory soup and mediate a crosstalk between neurons and immune cells. The inflammatory soup leads to the sensitization of nociceptors and an increased calcium response upon stimuli, resulting in an enhanced action potential firing and a pathologically increased pain response. The illustration was created with images from motifolio.

## 1.3 The role of TRP channels in the context of pain

TRP channels represent a major class of nociceptive signal transducers and are polymodally expressed in nociceptors throughout the body (19). Furthermore, the large ion channel superfamily encompasses the capability to detect a broad variety of physical and chemical noxious stimuli (20). Although the family comprises many functional differences, TRP channels have been characterized as non-selective cation channels, which are permeable even to large calcium ions (21). In total, the TRP channel superfamily consists of 28 members. The superfamily can be subdivided into six subfamilies that are classified as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastin (TRPM), polycistin (TRPP) and mucolipin (TRPML) (22).

Structurally, TRP channels are composed of four subunits forming a tetramer, with each subunit consisting of six transmembrane domains (23). Furthermore, the transmembrane domains five and six of each subunit build up together a hydrophilic loop that functions as ion conducting pore (24). The amino acid residues within this pore determine the cation selectivity of the respective ion channel (25). The most structural variation between TRP families occurs at the cytosolic amino and carboxy terminal end. However, the TRPC, TRPV, and TRPM family share a motif at the carboxy terminal which consists of a six amino acid sequence and is termed as TRP box (24). In addition, the TRPC and TRPV family have a conserved ankyrin repeat sequence at their amino terminal end which mostly consists of three to six repeats. In contrast, the amino terminal of the TRPA family consists of 14 repeats (26). Beside those conserved structures, many other structural elements as the calcium binding EF-hand domain, PDZ domain, coiled-coil domain, kinase domain and other domains characterize different TRP channels (27).

In the context of pain, especially members of the TRPV, TRPA and TRPM family like the TRPV1, TRA1 and TRPM8 have been shown to be key players in the transmission of pain (28). The latter one is primarily activated by thermal changes, transducing noxious cold stimuli (29). Nevertheless, it can also be activated by its ligands icilin, menthol, several lysophosphatidylcholines and poly unsaturated fatty acids (PUFAs) (24, 30). However, the TRPM8 channel is only expressed in a small subpopulation of DRG neurons which corresponds to 5-10% of all DRG neurons. Based on that, TRPM8 is expressed to a lesser extent than TRPV1 and TRPA1 in sensory neurons (31). Hence, only the two other key nociceptive TRP channels, TRPA1 and TRPV1, are described in the following.

## 1.3.1 TRPV1 as member of the TRPV family

The TRPV1 channel is the most well-characterized TRP channel of the TRPV family (26). It is predominantly and widely expressed in the peripheral nervous system (PNS), albeit its expression has additionally been observed in the central nervous system (CNS) but to a lesser extend (32). In the PNS, TRPV1 is mainly expressed by small and medium sized nociceptive nerve fibers such as peptidergic and non-peptidergic C fibers as well as in a lower amount by Aδ fibers. Based on that, the expression of TRPV1 was confirmed in the associated ganglia such as the dorsal root ganglia, the trigeminal ganglion, the nodal ganglion, and the sympathetic ganglion (33, 34). Furthermore, some studies confirmed the expression of

TRPV1 in other nerve fibers of the PNS, in which TRPV1 covers other functions than the sensation of noxious stimuli such as the sensing of irritants in the lung and airways, innervating the bladder or contributing to hearing processes in the cochlea (35). In the CNS, TRPV1 is primarily expressed pre-synaptically in dorsal horn neurons of laminae I and II, where it regulates the synaptic transmission of nociceptive signals (24, 27, 36). However, some groups also suggested the involvement of TRPV1 in the hippocampal synaptic transmission and described its expression in the caudal hypothalamus (37).

Structurally, TRPV1 is set up like all TRP channels with six transmembrane domains and a conducting pore between domain five and six. Furthermore, TRPV1 comprises a TRP box at the carboxy terminal end and six ankyrin repeats at the amino terminal end (Figure 3) (38, 39). While TRPV1 is a non-selective cation channel such as the other TRP channels, it has a preference for calcium ions upon activation (40). It gets polymodally activated by heat (> 43 °C), chemical ligands such as vanilloid capsaicin, and protons (pH < 5.9) (Figure 3) (41). Moreover, several other TRPV1 agonists have already been identified such as resiniferatoxin, eicosanoids, endocannabinoid lipids like anandamide or oxidized lipid mediators like 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE (41-44).

Since TRPV1 can be activated polymodally, activation of TRPV1 by different agonists can affect its responsiveness, respectively. For instance, an extracellular acidification to a pH around 6.5 lowers the thermal activation threshold of TRPV1 to a range of 35 to 37 °C, reflecting a temperature range already below body temperature (27, 45). This cross-regulation of TRPV1 becomes particularly relevant in pain states that cover acidosis such as cancer or injury-induced inflammation (33, 46). Beside those various direct activating mechanisms, TRPV1 can also be sensitized by endogenous mechanisms mediated by pro-inflammatory and proalgesic mediators. These mediators in turn initiate intracellular signaling cascades via their receptors, leading to the activation of kinases and phosphatases. These activated kinases, on the one hand, phosphorylate TRPV1 at serine and threonine residues, lowering the activation threshold, or, on the other hand, increase the trafficking and incorporation of TRPV1 into the plasma membrane (27).

However, the particular phosphorylation site determines the affected functional properties of TRPV1. The phosphorylation of TRPV1 by protein kinase A (PKA) and PKC increases the proton sensitivity of TRPV1, shifting its activation threshold into a physiological pH range. In

contrast, the phosphorylation by the calcium calmodulin-dependent kinase II (CaMKII) affects the TRPV1 vanilloid binding site, influencing the activation of TRPV1 by its ligands (31, 47-49). Nevertheless, the activation of all the respective kinases as well as the activation of the mitogen-activated protein kinase (MAPK) lead to an increased expression of TRPV1, which enables a maintenance of sensitization (50). Furthermore, previous studies showed that the activation of those kinases can contribute to the trafficking of TRPV1 to the plasma membrane (51). In addition, its activation triggers the release of neuropeptides like CGRP and substance P that are capable to contribute to the development of neurogenic inflammation (52). The counterpart of TRPV1 sensitization displays TRPV1 desensitization. Thereby, either TRPV1 channels are internalized or the phosphorylation state of TRPV1 is reduced by the phosphatase calcineurin (PP2B) (53). This phosphatase is primarily expressed in neurons and is activated among other mechanisms by the calcium calmodulin complex, which is formed upon increasing intracellular calcium concentrations (54, 55).
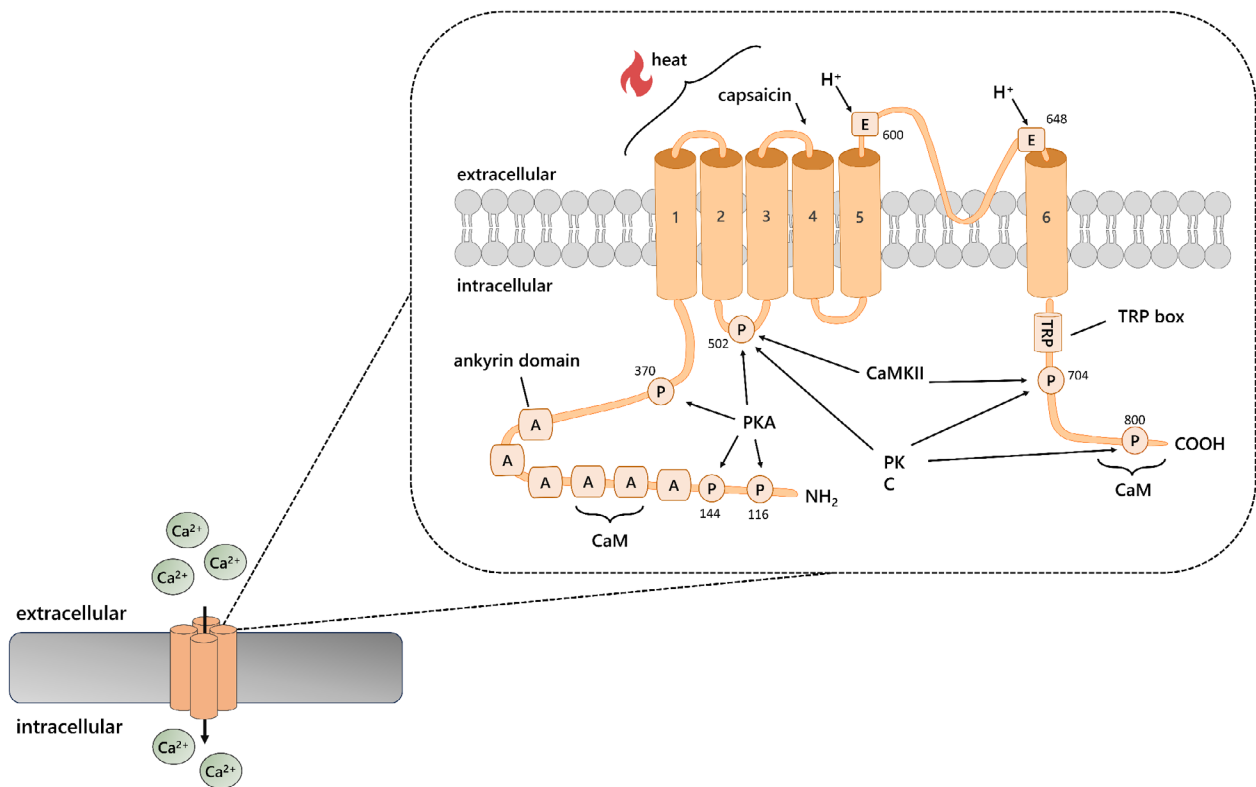


**Figure 3: Structure of the TRPV1 channel.** The TRPV1 channel is composed of four subunits building a tetramer as all members of the TRP channel family (bottom left). One subunit of the homotetramer consists of six transmembrane domains from which domains five and six build up the conducting pore with the respective domains of the other subunits (top right). Both the amino and carboxy terminal of the channel are located intracellularly and comprise six ankyrin repeat domains and a TRP box domain, respectively. Furthermore,

several phosphorylation sites, consisting of serine and threonine, are located along both terminal ends that can in turn be phosphorylated by protein kinase A and C or CaMKII. In addition, each terminal end provides a calmodulin (CaM) binding site. The TRPV1 channel can be activated by noxious heat stimuli, protons that act on glutamic acid residues (indicated at the extracellular loops) and capsaicin that was proposed to bind close to the fourth transmembrane domain. The illustration was created with images from motifolio.

Based on the involvement of TRPV1 in the development and maintenance of various pain states, it was highlighted as a potential target for the treatment of pain (56-60). Furthermore, it has been suggested that targeting TRPV1 has a low probability of central side effects, since it is predominantly expressed in sensory neurons. However, targeting TRPV1 with first generation antagonists resulted in hyperthermia in clinical trial patients. This severe side effect was induced since the first generation of antagonists interfered in the TRPV1-mediated body temperature regulation (61). Nevertheless, the second generation of antagonists, which is currently tested in clinical trials, seems to comprise analgesic properties without impairing the body temperature regulation (62).

## 1.3.2 TRPA1 as member of the TRPA family

The TRPA1 channel is the only member of the TRPA family, and it is named ankyrin TRP channel as its amino terminal domain comprises 14 (mouse) or 16 (human) ankyrin repeats (Figure 4). Furthermore, the TRPA1 amino terminal domain displays the largest part of the channel as it represents 64% of the entire protein and contains a cysteine rich domain (63). However, TRPA1 lacks the typical TRP box at the carboxy terminal (26). Furthermore, TRPA1 is mainly expressed in small diameter neurons of the dorsal root ganglia, trigeminal ganglia and nodose ganglia (63, 64). Beside the PNS, TRPA1 was also found to be expressed in various other tissues like the brain, hair, skin, and in different cell types such as myocytes and renal tubular cells (26). Nevertheless, the function of TRPA1 in those tissues is poorly understood until now.

Comparable to TRPV1, TRPA1 can be polymodally activated by a wide range of chemicals. Hence, TRPA1 is mainly known as a chemosensor, although its involvement in cold and mechanical sensation has been broadly discussed (65, 66). The respective chemical agonists can be loosely subdivided into electrophilic agonists which covalently modify TRPA1 and non-electrophilic agonists that activate TRPA1 non-covalently (67). Based on the variety of chemical agonists, studies show that electrophilic agonists such as isothiocyanate and allyl

isothiocyanate (AITC) which are found in wasabi, mustard, and mustard oil, in common interact with the cysteine rich domain at the amino terminal (Figure 4) (31, 68). Beside isothiocyanate and AITC, lipids such as prostaglandins, mediators of oxidative stress, cytokines, and chemokines are also identified as endogenous electrophilic agonists (69, 70). In addition, there are also non-electrophilic agonists known such as menthol, cannabinoids, carvacrol, thymol, and gingerol that activate the TRPA1 channel (71, 72). However, the binding mechanism of these agonists is largely unknown, except for the suggestion that some of them might bind to some kind of binding pocket located in close proximity to the fifth transmembrane domain (Figure 4). Nevertheless, this was only indicated for menthol and is assumed for other non-electrophilic agonists (63).



**Figure 4: Structure of the murine TRPA1 channel.** The TRPA1 channel is composed of four subunits that build a tetramer (bottom left). One subunit comprises six transmembrane domains from which domains five and six build up the conducting pore with the respective domains of the other subunits (top right). The amino terminal of the murine TRPA1 channel consists of 14 ankyrin repeat domains and several phosphorylation sites. Furthermore, the amino terminal comprises a suggested binding region for electrophilic agonists. In contrast menthol was predicted to bind near the fifth transmembrane domain. The carboxy terminal comprises predominantly a large voltage sensitive domain and provides a calmodulin (CaM) binding site. The illustration was created with images from motifolio.

In the context of pain, TRPA1 also displays an interesting target, since it is known as the "gatekeeper of inflammation" (11). Several studies have already shown that the pharmacological inhibition or deletion of TRPA1 led to a reduction of cold and mechanical hypersensitivity in inflammatory pain models (70). Furthermore, TRPA1 was shown to be involved in the mediation of persistent pain states such as neuropathic pain after nerve injuries or chemotherapy (70). Based on the emerging evidence of TRPA1 being involved in pain development and maintenance, its role as potential target in pain treatment increases more and more (70, 73, 74).

## 1.4 Peripheral and central sensitization

Peripheral sensitization describes a mechanism which occurs after any kind of lesion and increases the excitability of nociceptors. Thereby peripheral sensitization establishes a pain state in which innocuous stimuli are experienced as pain and noxious stimuli are sensed exaggerated, as described previously. These symptoms can either be caused by a direct activation of nociceptors or a reduction of the nociceptor threshold. In the case of a direct activation, ion channels such as TRP channels are activated by mediators secreted from injured cells or mediators from the inflammatory soup (3, 75). Mediators such as adenosine triphosphate (ATP) and protons are immediately released from damaged cells after injury (76). Both ATP and protons can directly activate ion channels which are expressed on the peripheral membrane of nociceptors. ATP activates the purinic receptor P2X3 and protons activate TRPV1, leading to a calcium influx, alterations in the membrane potential, depolarization as well as subsequently to the generation of an action potential (38, 77).

In the case of a nociceptor threshold reduction, mediators from the pro-inflammatory soup like cytokines, growth factors as nerve growth factor (NGF), kinins as bradykinin, purines, amines as histamine, lipids as prostaglandins, and other mediators indirectly affect the activity of nociceptors (46, 78). Those mediators bind to G protein coupled receptors (GPCRs) and tyrosine kinase receptors which are subsequently activated and in turn internally activate protein kinases of the family A and C (79). Upon their activation, protein kinases phosphorylate TRP channels at serine and threonine residues which is termed as sensitization (80). Based on the sensitization, the threshold of those ion channels is reduced and the open probability increased, leading to a pathologically increased pain perception. Furthermore, the persistent activation of nociceptors can lead to transcriptional changes in

terminal nerve fibers like the enhanced expression of ion channels and neuropeptides such as substance P and CGRP. Afterwards, these neuropeptides are released upon the activation of nociceptors and act on immune cells as well as on other sensory neurons, contributing to the maintenance of peripheral sensitization (6, 76).

In contrast to peripheral sensitization, central sensitization describes a state which involves the first synaptic connection at the dorsal horn of the spinal cord (18). Furthermore, central sensitization contributes to an enhanced and prolonged pain perception as well as a spatially extended sensation of peripheral stimuli, leading to increased pain perception in non-inflamed surrounding tissue (81). The amplification of peripheral signals occurs due to structural and functional changes in synaptic connections which are induced by various mechanisms (82). Firstly, an increased excitability of primary afferents leads to an enhanced excitement of postsynaptic neurons (83). Furthermore, an enhanced primary afferent activity leads to the release of neurotransmitters such as bone derived neurotrophic factor (BDNF), glutamate, and neuropeptides such as substance P and CGRP, which bind to postsynaptic dorsal horn neurons (84). Upon the binding of those mediators, various mechanisms are initiated in dorsal horn neurons: (1) phosphorylation and subsequent sensitization of ion channels, (2) activation of amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, and (3) translocation of ionotropic AMDA receptors to the post-synaptic membrane (85, 86). Moreover, under physiological conditions, the activation of NMDA receptors is blocked by a magnesium ion located in the pore of the receptor. However, due to an increased excitability and a constantly depolarized membrane potential, the magnesium blockage is removed and NMDA receptors get activated by glutamate, which further depolarizes post-synaptic neurons in the dorsal horn (87). In addition, functional modulation such as sensitization of NMDA receptors through second messengers like kinases would even intensify the external signal amplification (88). Thereby, innocuous stimuli like light touches would be experienced as unpleasant pain.

In later phases of central sensitization, transcriptional changes arise due to the activation of transcription factors such as nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) (79). Thereby, inflammatory genes are induced and repressors such as downstream regulatory element antagonist modulator (DREAM) are removed, initiating further intracellular cascades that contribute to the maintenance of sensitization (3, 89). Beside those transcriptional changes, alterations additionally occur at gap junctions as well as in the

interaction between neurons, astrocytes, microglia or even invading immune cells (90, 91). These interactions play an essential part in central sensitization mechanisms and neuroinflammation, which occurs especially in neuropathic pain states and is described in the neuropathic pain chapter.

Furthermore, lipid mediators and lipid oxidizing enzymes have been proposed to contribute to sensitized states. More specifically, oxidized lipid mediators can modulate the sensitivity of pain-mediating ion channels and thereby influence pain transmission (92). Especially the enhanced activity of the cyclooxygenase 2 leads to the production of prostaglandin E2 which represses the inhibitory function of glycine receptors and enhances the activity of terminal afferents (93). Accordingly, the function of further lipid mediators is described in the following chapter. In summary, peripheral sensitization is described as accumulation of mechanisms that amplify external stimuli in the periphery and central sensitization is characterized by mechanisms that lead to central signal amplification and induce long lasting changes in neuronal plasticity (82).

## 1.5 Lipid mediators in pain

Lipids in general describe a loosely defined group that covers a broad variety of hydrophobic molecules (94). Based on the high group diversity, there are also several subgroups existing such as fatty acids, phospholipids, sterol lipids, sphingolipids, and glycerolipids among others that affect various processes in the body and properties of compartments (94-97). In the context of pain, lipid mediators function as important signaling mediators in paracrine signaling and interaction of neurons and glial cells (98, 99). More specific, mainly mediators from the endocannabinoid system, the group of fatty acids and the group of sphingolipids have been identified as mediators in the context of pain (98, 100, 101). However, the relevance of lipids emerges in the context of pain with the increase of described endogenous lipid mediators and characterized pain-mediating TRP channel modulators (92, 102, 103).

### 1.5.1 Sphingolipids in the context of pain

From the group of sphingolipids predominantly ceramides and the ceramide/sphingosine pathway were shown to contribute to pain transmission (104). Ceramides are active metabolites of sphingosines that are known to regulate and modulate various cellular mechanisms such as cell cycle arrest, apoptosis, and inflammation (105, 106). In detail,

ceramides can act as second messengers themselves, activating transcription factors like NF-κB or kinases like MAPKs. These in turn induce or enhance the expression of pro-inflammatory cytokines among other pro-inflammatory mediators (107). In addition, ceramides serve as precursors for other mediators in pain such as the sphingosine-1-phosphate (S1P). Especially S1P emerged as an important mediator in pain as it was shown to contribute to peripheral and central sensitization (107-109). However, targeting the ceramide/sphingosine pathway with therapeutics led to exacerbating pain states as well (110). Based on this, the ceramide/sphingosine pathway contributes to pathological pain states but cannot be targeted easily because of the pleiotropic effects of sphingolipids.
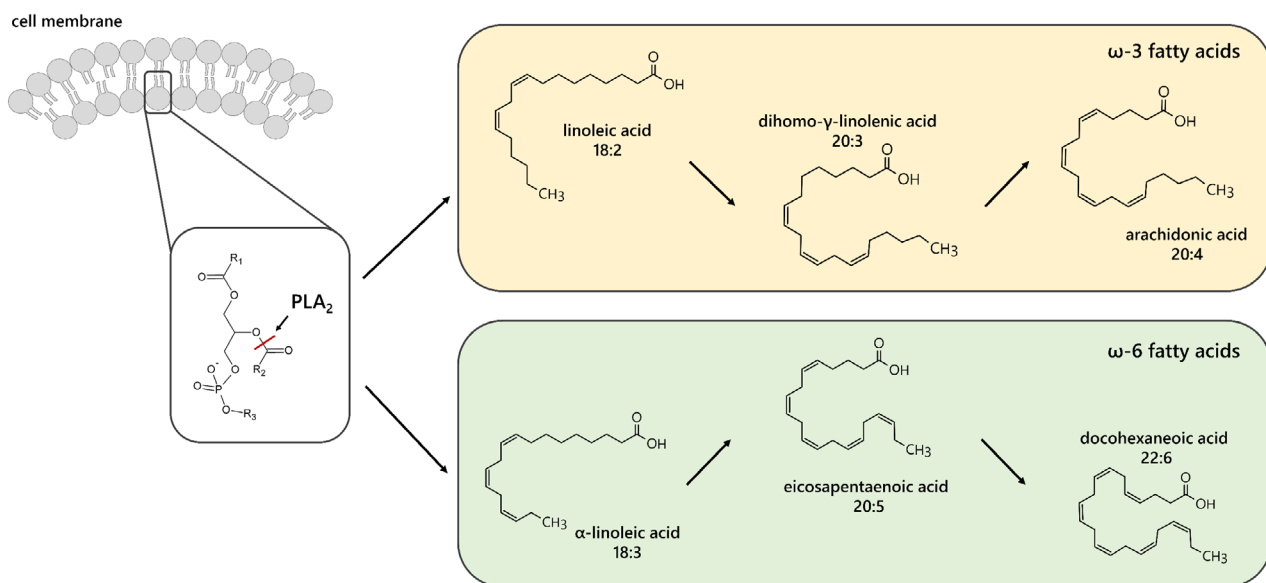
## 1.5.2 PUFAs in the context of pain

In contrast to sphingolipids in pain, the influence of polyunsaturated fatty acids (PUFAs) and their metabolites on pain transmission has been described in several studies (111, 112). PUFAs in general can be further categorized upon their degree and position of unsaturation (113). Thereby, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) belong to the group of omega-3 fatty acids, whereas linoleic acid (LA) and arachidonic acid (AA) belong to the group of omega-6 fatty acids (Figure 5). Both PUFA groups are also known to comprise essential fatty acids that cannot be synthesized by the body itself and have to be taken up by nutrition (111, 114). Once taken up, these PUFAs are either directly metabolized and oxidized, or processed and esterified into phospholipids which get incorporated into cell membranes. In turn, PUFAs can be released from the cell membrane through the activation of the phospholipase A II ($PLA_2$) pathway (Figure 5) (115).

In mammals the $PLA_2$ superfamily comprises more than 50 enzymes. Despite the variety of $PLA_2$ enzymes, only $cPLA_2$, $iPLA_2$, and $sPLA_2$ were shown to play an important role in the context of inflammation and pain (116-118). Although the expression of the respective phospholipases is confirmed in CNS and PNS, their activation is not fully understood yet (116). There is evidence in literature that the activation of $cPLA_2$ and $sPLA_2$ requires increased intracellular calcium conditions, whereas the activation of $iPLA_2$ does not (119, 120). In addition, it was shown that $cPLA_2$ can be activated by extracellular stimuli like ATP and colony-stimulating factor 1 (CSF1) or under neuroinflammatory conditions by the activated CaMKII pathway (121, 122). In contrast, the $iPLA_2$ activity has been shown to be regulated by ATP binding, calmodulin, or caspase cleavage (123). Nevertheless, the respective $PLA_2$

enzymes have in common that they hydrolyze the second ester bond of phospholipids upon their activation and thereby release a lysophospholipid and a PUFA (123).

From the cell membrane released PUFAs consist of different chain lengths and unsaturated double bonds. Usually, they are rapidly processed into their metabolites after their release, but they can also serve as antioxidants, scavenging reactive oxygen species (124). In general, fatty acids such as AA and LA are associated with pro-inflammatory properties and fatty acids such as EPA and DHA with anti-inflammatory properties, although the respective groups can also comprise metabolites with opposing properties (113, 125). Nevertheless, previous studies showed that long chain PUFAs such as DHA are capable to directly activate the chemosensor TRPA1, leading to a desensitization of TRPA1 in the long term. Based on such results, analgesic properties are ascribed to long chain PUFAs such as DHA (126).



**Figure 5: PUFA release by the phospholipase A₂ from cellular membrane.** Polyunsaturated fatty acids (PUFAs) can be released by the phospholipase A2 from the cellular membrane. Thereby, phospholipase A2 hydrolyses the second esther bond of glycerophospholipids, liberating a lysophospholipid and a PUFA. Furthermore, PUFAs can be converted into other PUFAs upon elongation and desaturation processes. However, the depicted direction of PUFA conversion serves only for explanatory purposes and does not implicate that e.g. arachidonic and docosahexaenoic acid cannot be incorporated into cell membranes. The molecular structures were drawn with ChemSketch and the lipid bilayer was modified and originates from motifolio.

### 1.5.3 Oxidized PUFA mediators in pain

In general, oxidized PUFA mediators are produced by cyclooxygenases, lipoxygenases and cytochrome-$P_{450}$-epoxygenases and -hydroxylases that rapidly metabolize PUFAs into their oxidized metabolites (127-129). In detail, cyclooxygenases predominantly generate under incorporation of oxygen molecules bicyclic hydroperoxides that get subsequently peroxidized (128, 130, 131). Lipoxygenases catalyze the incorporation of one oxygen molecule in dependency of iron ions, creating a hydroperoxyl fatty acid, and cytochrome-$P_{450}$-epoxygenases attach either an epoxide group or a terminal hydroxyl to the molecule also in dependency of iron ions (Figure 6) (127, 132). Thereby, many different lipid mediators can be generated, which are named oxylipins and comprise various properties and functions. In the following, the resulting fatty acid metabolites are described and sorted by the originating fatty acid, starting with the metabolites of arachidonic acid which belong to the group of eicosanoids.

The PUFA arachidonic acid gets processed by cyclooxygenases (COX) into prostaglandins and thromboxanes, by lipoxygenases (LOX) into leukotrienes and predominantly hydroxyeicosatetraenoic acids (HETEs), and by cytochrome-$P_{450}$-epoxygenases (CYP) into various epoxy, hydroxy and dihydroxy metabolites (133, 134). The first group of metabolites, prostaglandins and thromboxanes, consists of pro-inflammatory mediators that contribute to inflammation and enhance inflammatory pain (135, 136). Furthermore, various non-steroidal anti-inflammatory drugs (NSAIDs) target the synthesis of prostaglandins and thromboxanes by inhibiting non-selectively COX enzymes, indicating the relevance of those mediators in inflammation (137-139). Beside COX metabolites, the group of leukotrienes is also involved in the mediation of inflammatory processes, however their major function encompasses the mediation of allergic reactions (140). In contrast, other LOX enzyme mediators such as HETEs have already been described in the context of pain (92, 141). However, HETEs are not directly generated by lipoxygenases. For the oxidation of AA into HETEs, arachidonic acid firstly gets processed via LOX enzymes into hydroperoxyeicosatetraenoic acids (HPETEs) and then subsequently reduced into HETEs by the glutathione peroxidase system (142). Nevertheless, both mediator classes were previously shown to comprise pro-inflammatory and proalgesic properties. In particular, the

mediators 5-,12- and 15-HETE were shown to function as direct agonists of the pain-mediating TRPV1 channel or at least to increase pain sensation (101, 143-145).

The latter group of AA oxylipins describes a heterogenous group of mediators since CYP enzymes can either generate HETEs or expoxyeicosatrienoic acids (EETs) (100). Although they generate other HETEs like 19-HETE and 20-HETE than LOX enzymes, their properties such as activating the pain-mediating TRPV1 channel are similar (146-148). In contrast to HETEs, EETs, also termed as EpETrEs, have been shown to comprise anti-inflammatory properties and to ameliorate neurodegenerative diseases as they reduce microglial activation and immune cell infiltration (125, 129). However, 5,6-EET has also been described as TRPA1 agonist (149). In summary, the PUFA arachidonic acid is predominantly metabolized into pro-inflammatory mediators except for some epoxy metabolites, which possess more anti-inflammatory properties.

The PUFA linoleic acid can be metabolized into hydroxyotadecadienoic acids (HODEs) and epoxyoctadecenoid acids (EpOMEs) which can be further processed into dihydroxy-octadecenoic acid (DiHOMEs) (150, 151). However, the generation of the respective metabolites and the required enzymes are not fully identified. Nevertheless, emerging evidence indicates that HPODE, the HODE precursors, can be generated by LOX enzymes (92, 152). Afterwards, HPODEs get subsequently processed into the stable metabolites like 9-HODE and 13-HODE by the glutathione peroxidase (92, 153). In addition, both metabolites were shown to sensitize the TRPV1 channel in primary sensory neurons and to induce spontaneous pain and thermal hypersensitivity after administration *in vivo* (154, 155). Furthermore, both mediators can activate the TRPA1 channel in a heterologous expression system, revealing proalgesic properties of both (156). In line with this, chemotherapeutically treated mice that developed neuropathic pain symptoms, showed elevated 9-HODE levels in neuronal tissues such as sciatic nerve and DRGs (157).

Beside HODEs, EpOMEs such as 9,10- and 12,13-EpOME represent another group of LA metabolites. These are mainly generated by CYP enzymes and can be further metabolized by the soluble epoxide hydrolase (sEH) into their corresponding diols 9,10- and 12,13- DiHOME (158). In addition, EpOMEs and DiHOMEs, are predominantly attributed with pro-inflammatory and proalgesic properties (151). In particular, the mediators 9,10-EpOME and 12,13-DiHOME sensitized the pain-mediating TRPV1 channel in primary sensory

neurons and 12,13-DiHOME induced a thermal hyperalgesia *in vivo* (141, 159). In line with this, another study postulated that all four oxidative LA metabolites activate TRPV1 and TRPA1 and contributed to thermal hyperalgesia *in vivo* (160). In addition to the described proalgesic properties, EpOMEs are known to be especially synthesized by leucocytes in inflammatory diseases in which they contribute to inflammation (92). Furthermore, DiHOMEs directly activate neutrophils, indicating that oxidated LA metabolites contribute to inflammation and increase pain perception in inflammatory and chronic pain states (92, 161).

Beside these oxidized linoleic acid metabolites, LA can be metabolized into dihomo-gamma-linolenic acid (DGLA) which itself gives rise to either hydroxyeicosatrienoic acids (HETrEs) via LOX enzymes or prostaglandins via COX enzymes (162). HETrEs and especially 15-HETrE was shown to comprise a more anti-inflammatory profile as it inhibits the production of leukotrienes in immune cells via inhibiting the lipoxygenase 5 (163). Beside the omega-6 fatty acid DGLA, there is another linolenic acid called alpha-linolenic acid (α-LA). However, α-LA is classified as an omega-3 fatty acid and is known to comprise anti-inflammatory properties (164, 165). The α-LA in turn can be metabolized by LOX enzymes into hydroxyoctadecatrienoic acids (HOTrEs) which also encompass anti-inflammatory properties (166, 167). Although less is known about HOTrEs in the context of pain, their anti-inflammatory properties indicate that they might be beneficial for the reduction or resolution of neurogenic inflammation in pathological pain states.

Other omega-3 PUFAs like EPA or DHA can be generated by the desaturation and elongation of α-LA (168). Both comprise anti-inflammatory properties as α-LA and give rise to anti-inflammatory metabolites (15, 165, 169-172). Moreover, both PUFAs can be metabolized into less potent prostaglandins and thromboxanes, thereby preventing the prostaglandin synthesis from arachidonic acid as they compete directly as substrate with AA (173). Beside repressing the prostaglandins synthesis, EPA and DHA can be metabolized into resolvins and DHA additionally into maresins and protectins. These metabolites belong to the group of specialized pro-resolving mediators (SPMs) (95, 174-176). Moreover, these SPMs were shown to encompass essential regulatory functions in the context of pain and to be involved in the active resolution of inflammation (169, 177, 178). However, the synthesis of SPMs is not elucidated in detail and their occurrence controversially discussed in literature as SPMs are difficult to detect even with sensitive methods such as tandem mass spectrometry. Nevertheless, the generation of their precursors such as hydroxyeicosapentaenoic acids

(HEPEs) and hydroxydocosahexaenoic acids (HDHAs) is characterized and their detection well established (179-184). HDHAs originate from DHA and are produced in the same way as HEPEs via LOX enzymes and the glutathione peroxidase. Furthermore, DHA can be processed into epoxydocosapentaenoic acids (EDPs) and dihydroxydocosapentaenoic acids (DiHDPAs) by CYP enzymes as well as into hydroxydocosahexaenoic acids (HDoHEs) by LOX enzymes (179, 185). In addition, CYP enzymes give rise to epoxyeicosatetraenoic acids (EEQs) by metabolizing EPA (179).
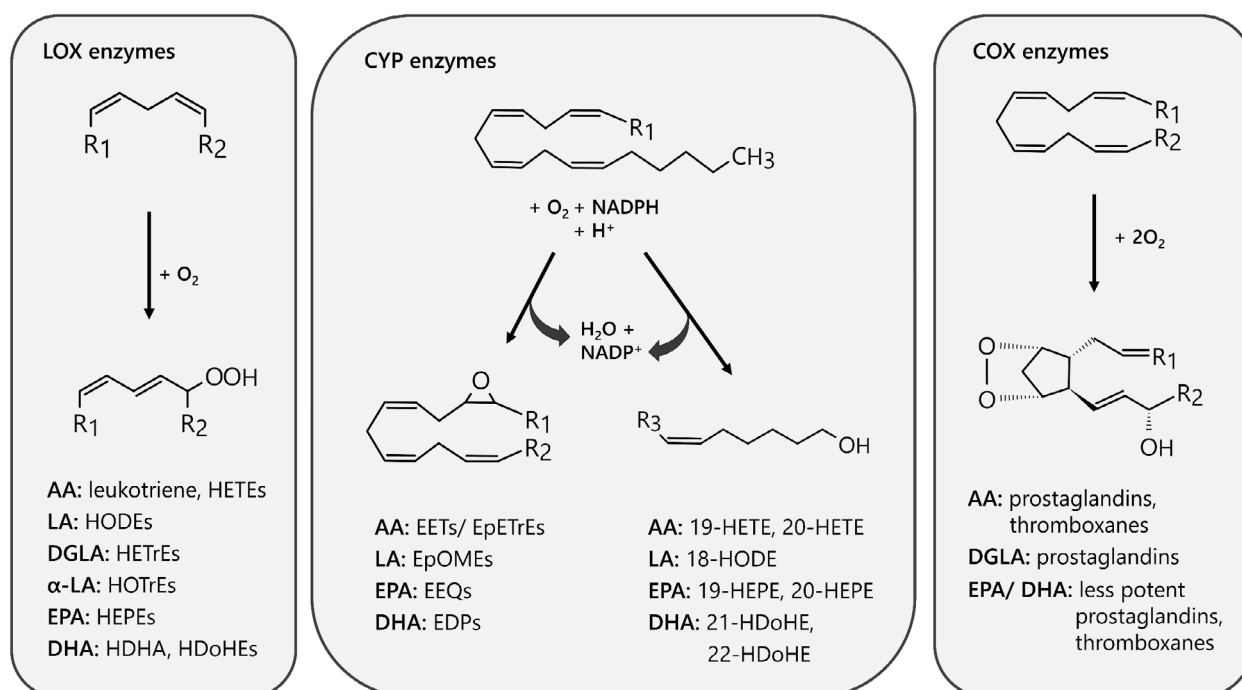


**Figure 6: Enzymatic reactions catalyzed by LOX, CYP and COX enzymes.** The iron containing lipoxygenases (LOX enzymes) generate hydroperoxyl fatty acids under incorporation of one oxygen molecule. Cytochrome-$P_{450}$-epoxygenases (CYP enzymes) catalyze epoxygenase and hydroxylase activities in dependency of iron ions, attaching either an epoxide group, preferentially to the double bond closest to the amino group, or a terminal hydroxyl group, respectively. Cyclooxygenases (COX enzymes) catalyze the generation of predominantly bicyclic hydroperoxides under the incorporation of two oxygen molecules. The oxidized metabolites generated from the respective PUFA are depicted per enzyme. The molecular structures were drawn with ChemSketch.

In summary, oxidized PUFA mediators, which are also termed as oxylipins, play an essential and very divers role in the regulation of various inflammatory diseases and pain states. Nevertheless, many underlying mechanisms are not elucidated yet and have to be further investigated. However, the extremely low endogenous level of lipids as well as their short half times make the investigation of lipids challenging.

## 1.6 Neuropathic pain

The third type of pain, beside physiological and inflammatory pain, is termed neuropathic pain. Neuropathic pain is characterized as a pain state which arises after a lesion or disease of the somatosensory nervous system (186). Although pain serves as protective function of the body, neuropathic pain is commonly defined as maladaptive pain response and persistent pain state (187). Furthermore, this maladaptive pain state is not only provoked by the nerve damage itself but also by an imbalanced inflammatory response, the subsequent initiated mechanisms, and resulting disorders (188, 189).

The initial nerve damage can be induced by a variety of causes like metabolic diseases such as diabetes mellitus, nervous diseases such as herpes zoster and multiple sclerosis, infectious diseases like HIV, surgical interventions like amputations, medical conditions like stroke, nerve traumas and nerve injuries or even by neurotoxic substances like chemotherapeutics (190). Based on the high variety of elicitors, neuropathic pain is defined by a broad symptom scope which includes several phenotypes of abnormal pain perception. In detail, neuropathic pain patients experience hypersensitivities, allodynia, and hyperalgesia but also paresthesia and spontaneous pain which feels like an electroshock or burning pain (191, 192). Unfortunately, the latter described symptoms occur stimuli-independent, demanding neuropathic pain patients even more (193). Based on the severity and persistency of symptoms, neuropathic pain is classified as one of the most difficult pain forms to treat, which leads to a high burden for pain patients. Furthermore, neuropathic pain can even become incurable and life long lasting (193).

Such harsh symptoms are mediated by a variety of underlying mechanisms, which are partially understood and differ depending on the type of neuropathic pain. However, mechanisms such as peripheral and central sensitization as well as events like ectopic discharges were shown to contribute to the most frequent neuropathic pain symptoms (194). In addition, independent from the initial elicitor, all nerve lesions lead at first to an inflammation at the site of injury. This specific type of inflammation is termed neuroinflammation since it describes a neuroimmune interaction (195). Furthermore, it is characterized by the activation of glial cells like astrocytes and microglia, the infiltration of immune cells into PNS and CNS compartments like DRGs or spinal cord, respectively, and the synthesis of inflammatory mediators (196). Nevertheless, the intensity of

neuroinflammation is divers in different types of neuropathic pain. Among the different neuropathic pain types, predominantly nerve injury-induced neuropathic pain is characterized by a strong ongoing neuroinflammation. In contrast, other forms of neuropathic pain such as the chemotherapy-induced neuropathic pain comprise a weaker neuroinflammation (197, 198). Both forms of neuropathic pain (nerve injury- and chemotherapy-induced) and their underlying mechanisms such as neuroinflammation are described in the following in detail since these two neuropathic pain models were used to firstly investigate the effects of SAFit2 on neuropathic pain.

## 1.6.1 Nerve injury-induced neuropathic pain

Nerve injury-induced neuropathic pain describes a specific type of neuropathic pain, which arises after nerve injuries like nerve transections or nerve stretches and crushes that can occur during events such as surgeries or even amputations (199-203). As previously described, this type of nerve lesion induces a strong neurogenic inflammation which characterizes this type of neuropathic pain. Immediately after lesion, resident glial and immune cells such as Schwann cells or macrophages and mast cells, respectively, get activated in the surrounding tissue (198). Upon their activation, Schwann cells dedifferentiate and start to degrade the myelin sheath at the site of nerve injury to prepare the site of injury for regeneration (204). In addition, resident immune cells such as mast cells release pro-inflammatory mediators such as cytokines, growth factors and histamine among others. These recruit further immune cells and firstly sensitize nociceptors as explained in the peripheral sensitization chapter. Beside peripheral sensitization, those mediators also recruit further immune cells to the site of injury (198, 205, 206). However, the resulting immune cell infiltration occurs in a distinct time response. Firstly, neutrophils are infiltrating within hours followed by macrophages within days (196). Furthermore, the differentiation of monocytes into macrophages is initiated as well as the polarization of macrophages into a pro-inflammatory phenotype is established (207). Lastly, T cells infiltrate in the inflamed region within days and weeks (196). In summary, all these infiltrating immune cells further release pro-inflammatory mediators, building up the so-called inflammatory soup which further mediates the development of inflammation as well as modulates nociceptor sensitization (208).

The complex interaction of immune cells and mediators is usually strictly regulated and balanced to facilitate the resolution of neuroinflammation and nerve injury (209). Unfortunately, in the case of arising neuropathic pain, this beneficial regulation is out of balance and the local neurogenic inflammation cannot be resolved. This in turn promotes the appearance of central mechanisms like central sensitization and enhanced glial activation (90, 210). Nevertheless, the respective central mechanisms can also arise even faster when nerve lesions occur directly in compartments of the CNS (211). Especially the activation of glial cells such as microglia and astrocytes displays a key mechanism of neuroinflammation and is described in the following (212, 213). The underlying neuronal mechanisms of central sensitization are not explained in detail in the following as they have been described in a previous chapter.

The activation of glial cells is induced by either mediators of the inflammatory soup or by mediators like ATP, chemokines, CSF1 or CGRP released from primary nerve terminals after spontaneous discharges (196, 214, 215). Furthermore, glial cells can respectively influence their activation by the secretion of distinct mediators (207). Upon glial activation, glial cells like microglia and astrocytes change their phenotype from a controlling resting state into an effector state (216, 217). Though, the activation of astrocytes and microglia takes places in a time dependent manner, in which microglia get activated immediately after nerve injuries and astrocytes within days (212).

Microglia start to rapidly proliferate, change their morphology, and start to express several markers like chemokine receptors and the ionized calcium-binding adaptor molecule-1 (IBA1) upon their activation (218-220). Furthermore, they facilitate other responses such as antigen presentation, migration, phagocytosis, as well as production of inflammatory mediators (221). Mechanistically, the generation of pro-inflammatory mediators is induced through external stimuli, which activate further signaling cascades including MAPK or initiate the NF-κB pathway (222). Thereby, the activated microglia release mediators like tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, C-C motif ligand 5 (CCL5) and BDNF, acting paracrine on neurons and astrocytes (223, 224). Thereby, effector microglia also actively contribute to the development and maintenance of sensitization and neuroinflammation.

Beside microglia, astrocytes are also activated upon noxious stimuli as well as subsequently undergo morphological changes, proliferate, and express several markers such as the glial fibrillary acidic protein (GFAP) (212, 225). Although astrocytes are activated later than microglia, their activation is more persistent and can last for months (226). In contrast to microglia, astrocytes are connected via gap junctions in a network and form contacts with synapses through which they have a direct influence on signal transduction (212, 227). This is of particular interest, since activated astrocytes release D-serine as gliotransmitter into synaptic gaps. D-serine itself is characterized as an endogenous co-agonist of NMDA receptors and can thereby directly modulate synaptic transmission as well as interfere in the regulation of synaptic plasticity (193, 228).

Beside the neuron-glial interaction, glial cells can also crosstalk with other glial cells through the secretion of glial mediators. These glial mediators can therefore, on the one hand amplify the activation of other glial cells, or on the other hand resolve it, depending on their properties (207). In the case of astrocytes, pro-inflammatory gliomediators are pre-dominantly chemokines such as CCL2, CCL5, CCL7 and chemokine C-X-C motif ligand 10 (CXCL10) among others that are secreted upon the activation of MAPK and the NF-κB signaling pathway (207, 229, 230). Furthermore, these chemokines play an important role in the transmission of pain as they enhance or sustain the activation of microglia (231).

Nevertheless, those secreted chemokines also contribute to the sensitization of neurons. In particular, CCL2 displays a prominent chemokine candidate in pain transmission as it directly binds to the C-C motif chemokine receptor 2 (CCR2) on neurons. Thereby, CCL2 induces an increase of AMPA and NMDA mediated currents, resulting in an amplification of amplitude and frequency of post synaptic currents (212). In summary, the orchestrated release of mediators by neurons and glial cells as well as the possible interactions between neurons and glial cells in neuroinflammatory conditions result in multi-combinatorial possibilities of effects. Nevertheless, the prevention of both astrogliosis and microgliosis as well as the neutralization of glial mediators was shown to attenuate chronic pain states after nerve injury, highlighting both as target in chronic and especially neuropathic pain states (232-235). However, due to the multitude of interactions and influences, the underlying mechanism of nerve injury-induced neuropathic pain are only superficially elucidated and have to be studied further in depth.

## 1.6.2 Chemotherapy-induced neuropathic pain

Another prominent type of neuropathic pain is described as chemotherapy-induced peripheral neuropathy (CIPN) and arises during or after chemotherapy. Unfortunately, CIPN affects up to 80% of all cancer patients and appears as a dose dependent and severe side effect of chemotherapy (197, 236). CIPN can already occur immediately after the first cytostatic treatment, multiple treatment cycles or even after the end of chemotherapy (197, 237). The latter described event is especially challenging for oncologists as they cannot respond anymore in order to modulate chemotherapy and to reduce CIPN. During chemotherapy, CIPN is firstly recognized by altered sensory perceptions of patients, starting in hands and feet. In the progression of CIPN, these altered sensory perceptions such as numbness, paresthesia, and hypersensitivities spread throughout the limbs to more central regions (236-238). Thereby, CIPN limits and impedes an effective cancer treatment in many cases as chemotherapy has to be interrupted or reduced in frequency and dose after the occurrence of CIPN (239, 240).

The commonly used cytostatics that can cause CIPN can be classified into taxanes such as paclitaxel, platinum compounds like cisplatin and oxaliplatin, and vinca alkaloids such as vincristine. In addition, newer cytostatic classes include protease inhibitors like bortezomib and immunomodulatory compounds like thalidomide (238, 241, 242). Although these cytostatics are used to preferentially target and kill proliferating malignant cells, they also affect non-malignant and non-proliferating cells. Thereby, cytostatics often affect the somatosensory system since it is well susceptible due to a low nerve-blood barrier (243). The most affected parts of the PNS are distal nerve fiber endings, cell bodies of sensory neurons and their axonal components such as myelin, mitochondria, and microtubules among others. Furthermore, cytostatics also affect glial cells like astrocytes and microglia (244). However, the underlying mechanisms vary between the different chemotherapeutics as they target different structures, leading to different lesions and symptoms.

In this work, the focus is further set on the cytostatic paclitaxel and the underlying mechanisms of paclitaxel-mediated neuropathic pain. Paclitaxel was chosen for a chemotherapeutic model since it is efficiently and widely used for the treatment of solid tumors such as lung, prostate, or ovarian cancer (245, 246). Moreover, paclitaxel is used as a first-line chemotherapy for breast cancer which affects one out of eight women in their life

time based on the current Robert Koch incidence rates (247, 248). In addition, paclitaxel is used for the treatment of malignancies which are previously refractory for other cytostatics such as small cell lung cancer or bladder cancer (249-251). However, although paclitaxel is known as one of the most successful natural cytostatics, unfortunately up to 87% of paclitaxel patients develop neuropathic pain after their treatment (244, 252). Furthermore, some patients even experience an acute pain syndrome immediately after paclitaxel treatment, reaching its peak 3-4 days after treatment (197, 253). In summary, the high efficiency of paclitaxel and frequency of side effects display a high need of therapeutics for paclitaxel-mediated neuropathic pain.

Paclitaxel itself belongs to the group of taxanes and is described as a microtubule stabilizing agent (254). Under physiological conditions, microtubules are characterized as dynamic cytoskeletal polymers that polymerize and depolymerize. However, upon the binding of paclitaxel to β-tubulin, the microtubule lattice is stabilized and the depolymerization repressed. Thereby, paclitaxel efficiently arrests cell cycle between metaphase and anaphase and disrupts cell proliferation. Moreover, those arrested cells, predominantly rapid proliferating cancer cells, undergo apoptosis over time (255). Unfortunately, the stabilizing of microtubule affects also other cytoskeleton-related mechanisms beside proliferation. In particular, paclitaxel impairs the axonal transport in neurons, leading to axonal degeneration and a phenomenon termed as axonal dying back pattern. This in turn can lead to demyelination processes and a complete loss of sensory nerve action potentials (236). Furthermore, the disruptions of microtubule-related functions can even lead to the complete loss of intraepidermal nerve fibers, which are predominantly pain-mediating C-fibers and Aδ-fibers, resulting in a loss-of-function symptoms like numbness (256, 257).

The impaired axonal transport further affects mitochondria, since they are primary localized in axons and rely on the rapid trafficking of mRNA and nutrients (258, 259). In addition, they play a crucial role in the excitability of neurons as they provide a lot of energy, which is required for the regeneration of ion gradients and membrane potentials (260). In addition to the impaired supply of mitochondria, paclitaxel altered the morphology of mitochondria. After paclitaxel treatment, mitochondria were shown to be swollen up, start to vacuolize and show fragmentation of cristae (236, 261). This effect is presumably caused by an increased permeability of the mitochondrial permeability transition pore (mPTP), leading to alterations in the calcium flux in mitochondria (262). Furthermore, a sustained opening of mPTP leads

to the disruption of the electrochemical gradient and the respiratory chain which finally results in ATP deficiencies and the leakage of pro-apoptotic mediators (263).

Beside the ATP deficiency, the paclitaxel-mediated disruption of mitochondria causes the generation of reactive oxygen species (ROS) and oxidative stress (197). However, ROS and oxidative stress themselves were shown to affect mitochondria as they induce mitochondrial membrane potential loss, revealing two closely dependent and regulating systems (264). Furthermore, accumulating ROS induce the expression of matrix-metalloproteinases, which enables the infiltration of immune cells into neuronal tissue (236). Nevertheless, paclitaxel-mediated oxidative stress and mitochondrial dysfunction were shown to be reduced by antioxidants and other compounds that counteract an enhanced ROS production or promote an endogenous antioxidant cascade (265, 266). Taken together, the diverse effects of paclitaxel on axonal transport and mitochondria are closely related, dependent on each other and lead to the experience of neuropathic pain in variable intensities.

Beside those oxidative stress and axonal transport effects of paclitaxel, paclitaxel itself is described as ligand of toll-like receptor 4 (TLR4) (265). This receptor is expressed by sensory neurons, glial cells, and other immune cells such as macrophages (267-269). TLR4 is a transmembrane receptor which is coupled to two adaptor proteins: myeloid differentiation primary response 88 (MyD88), and TIR domain-containing adaptor inducing IFN-$\beta$ (TRIF) (270). Upon its activation, the respective adaptor proteins promote signal transduction and activate intracellular signaling cascades. These downstream signaling cascades then result in the activation of transcription factors like NF-$\kappa$B, activator protein 1 (AP-1) and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (271, 272). Thereby, paclitaxel induces the expression of several pro-inflammatory cytokines and chemokines that contribute to sensitization processes and recruit further immune cells (265). Moreover, paclitaxel directly activates resident immune cells as well as initiates the degranulation of mast cells through the TLR4 mediated signaling (265). Based on these downstream effects, paclitaxel itself generates a pro-inflammatory environment and contributes to the development of neuroinflammation.

Despite to the paclitaxel-mediated expression of cytokines and activation of immune cells, paclitaxel was additionally shown to enhance the expression of several factors, contributing to the development of neuropathic pain. For instance, paclitaxel increased the expression of

chemokine receptors (273). This expressional upregulation plays an important role in the maintenance of paclitaxel-induced neuropathic pain as chemokines are strongly involved in the mediation of neuroinflammation and pain transmission (274-277). Furthermore, the blockage of chemokines such as CCL2 was shown to prevent paclitaxel-induced hypersensitivity (278). Beside the inflammation-related expression, paclitaxel increased the expression of TRPV1 in sensory neurons (279, 280). In addition, it enhanced the expression of neuropeptides like substance P which play an essential role in sensitization processes (265, 281, 282). Moreover, paclitaxel was shown to induce a glutamate release from sensory neurons and to disrupt glutamate homeostasis in the dorsal horn which further contributes to paclitaxel-induced neuropathic pain (274, 283). In summary, paclitaxel is used as a potent antineoplastic agent for the treatment of various cancer types. However, it additionally affects multiple other processes than proliferation, resulting in many cases to the development of neuropathic pain.

## 1.6.3 Therapeutic options for neuropathic pain

Although neuropathic pain affects up to 10% of the world population, the treatment strategies for neuropathic pain are limited due to either severe side effects or a low efficacy of the available therapeutics. Nevertheless, the pharmacological guidelines for neuropathic pain recommend gabapentinoids like gabapentin or pregabalin, tricyclic antidepressants such as amitriptyline as well as serotonin-noradrenaline reuptake inhibitors (SNRIs) such as duloxetine as first-line treatments (284). Gabapentin and pregabalin bind both to subunits of voltage-dependent calcium channels and thereby reduce calcium influxes and the calcium-mediated release of excitatory neurotransmitters (285, 286). Although this mechanism of action seems to be beneficial for many neuropathic pain types, the treatment with gabapentinoids showed no amelioration in a clinical phase III study with CIPN patients (284, 287, 288). Furthermore, such channel blockers lead to adverse effects like vertigo, lethargy, obesity and peripheral swelling (288, 289). Tricyclic antidepressants were found to be efficient in nerve injury-induced neuropathic pain patients among others (284). However, they are contraindicated for patients with cardiovascular diseases as they could lead to a reduced blood flow and have an inhibitory influence on choline, adrenalin, and histamine receptors (290, 291). Based on this, tricyclic antidepressants can induce arrhythmia, walking difficulties, and cognitive impairment, which could lead to an accidental overdosing and an

increased suicide risk (288). SNRIs were shown to be beneficial in diverging types of pain like nerve injury-induced and chemotherapy-induced neuropathic pain (197, 284). Those SNRIs such as duloxetine are beneficial for the treatment of neuropathic pain as they balance neurotransmitter concentrations by inhibiting the reuptake of serotonin and noradrenaline (292). Nevertheless, the treatment with SNRIs should be closely surveilled as those agents increase the blood pressure which can lead to vertigo, nausea, hypertension, and ataxia (284).

As second-line treatments, topical agents like lidocaine and capsaicin as TRPV1 agonist were used to locally reduce and prevent spontaneous discharges (293). These treatments were shown to be quite analgesic in cases of post-operative pain but failed to mediate an efficient pain relief in more systemic neuropathic pain forms (284, 294). Beside the topical treatments, opioids such as tramadol and tapentadol are recommended as second-line treatment for more systemic forms of neuropathic pain such as diabetic neuropathy (295). These weak μ-opioid receptor agonists also act on neurotransmitter balances as they additionally function as SNRIs (197, 296). Although these opioids are recommended as second-line treatments, since they comprise a low potential of abuse, the risk of addiction and other adverse effects such as confusion should not be underestimated. Third-line treatments include more potent opioids like morphine and oxycodone as well as neurotoxins like botulinum toxin (284, 288). Nevertheless, strong opioids were used less in the treatment of neuropathic pain as they carry a high risk of misuse and are not adequate for long-term treatments (297). Botulinum toxin is recommended as last choice for the treatment of refractory cases. It inhibits neural transmission and therefore can efficiently reduce peripheral neuropathic pain states, however it inhibits also the transmission of any other neuronal signal (298).

In addition to the limited choice of unsuitable drugs, several of which are used off-label to treat neuropathic pain, many patients develop resistance or tolerance to their therapeutics. In these cases, alternative therapies such as spinal cord stimulation, transcranial and epidural stimulation, or deep brain stimulation can be considered (288, 299). These therapy options try to address pathological pain transmission with electrical or magnetic signals. However, these techniques are characterized as invasive treatment options and carry potential risks (300-302). In summary, neuropathic pain patients have to deal with a limited and often inadequate choice of treatments, highlighting an emerging need for novel therapeutics to cope with different types of neuropathic pain.

## 1.7 FKBP51 as potential target for neuropathic pain

### 1.7.1 FKBP51 structure and its main function as co-chaperone

The FK506 binding protein 51 (FKBP51) is encoded by the gene *FKBP5* and belongs to the family of immunophilins (303). Immunophilins are characterized by the ability to bind immunosuppressing agents such as FK506 and rapamycin (304). Within the family of immunophilins FKBP51 is homologous to FKBP52, sharing 60% sequence identity and a similarity of 75% (305). Structurally, FKBP51 consists of three domains. The amino terminal constitutes the FK1 domain, followed by the FK2 domain in the center and the tetratricopeptide repeat (TRP) domain at the carboxy end (Figure 7) (306). The best characterized domain of FKBP51 is the FK1 domain which comprises five antiparallel beta strands, surrounding a central alpha helix (306). Furthermore, it possesses a catalytic pocket that facilitates a prolyl-peptidyl-isomerase (PPIase) activity and binds ligands such as FK506 (306, 307). The function and structure of the FK2 domain is less characterized compared to FK1. However, FK2 is assumed to comprise scaffold functions since it displays neither an enzymatic function nor the ability to bind immunosuppressing agents, although it shares almost the same structure with FK1 except for some residues (308). The TRP domain at the carboxy terminal comprises three TRP repeats that are folded into seven alpha helices (309). In addition, it serves as further binding site for protein-protein interactions especially for the chaperone heat shock protein 90 (HSP90) (310, 311).

Based on the binding affinity of the TRP domain to HSP90, FKBP51 and other immunophilins are described as co-chaperones and are involved in the mediation of protein folding and stabilization (306, 312, 313). Although the regulatory function of FKBP51 as co-chaperone is barely understood in many pathways, it was shown to be involved in the regulation of steroid hormone receptors (SHR) (314-317). The family of SHRs includes the androgen receptor (AR), the estrogen receptor (ER), the mineralcorticoid receptor (MR), the progesterone receptor (PR), as well as the glucocorticoid receptor (GR) (308, 318). However, FKBP51 was shown to be most sensitive to the glucocorticoid receptor and to negatively regulates its activity (308). In contrast, FKBP52 potentiates the signal transduction of GR, although FKBP51 and FKBP52 share almost the same structure (319, 320). These contrary effects are suggested to be mediated by conformational differences since the relative orientation of FK1 and FK2 was described to be twisted in FKBP52 compared to FKBP51 (305).
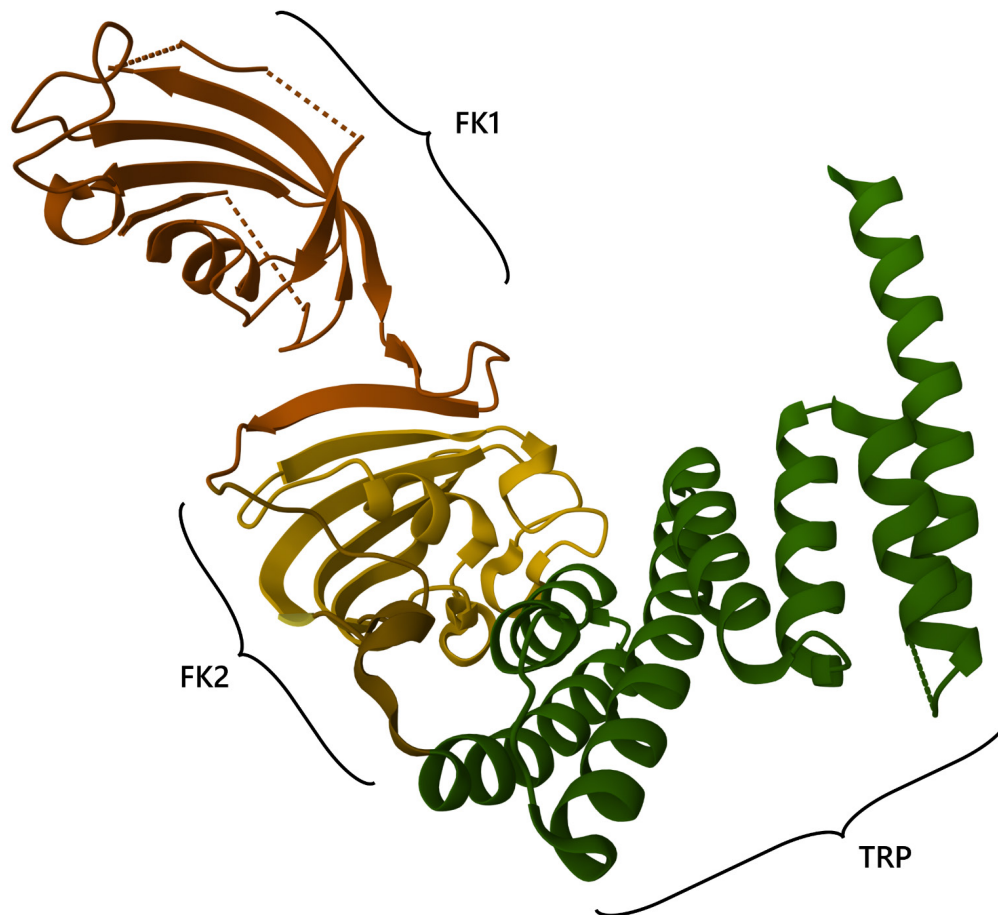
**Figure 7: Structure of FKBP51.** FKBP51 comprises a FK1 domain at the amino terminal (brown), an FK2 domain at the center (yellow) and a TRP domain at the carboxy terminal (green). The crystallographic structure was taken from the RSCB database (PDB-ID: 1KT0) and colored as well as labeled individually (306).

The GR activity is closely regulated by the binding of FKBP51 or FKBP52 to the heterocomplex including the GR itself, HSP90 and other co-chaperones (321). While glucocorticoid concentrations are low in the cytosol, predominantly FKBP51 was shown to occupy the binding site of the heterocomplex. However, upon glucocorticoid binding, the heterocomplex undergoes a conformational change and FKBP51 is exchanged to FKBP52 (322, 323). FKBP52 in turn recruits the dynein-dynactin motor complex which allows the translocation of the heterocomplex into the nucleus (324). Next, the expression of GR targets such as FKBP51 is enhanced (325). This subsequently leads to increased FKBP51 levels in the cytosol that counterbalance the FKBP51/FKBP52 homeostasis and reduce the transcriptional activity of GRs. Based on that, the GR-FKBP51 regulation functions as an ultrashort negative feedback loop and can lead to a glucocorticoid resistance and a lack of negative feedback signaling towards the hypothalamic–pituitary–adrenal (HPA) axis (326, 327). Thereby, the diminished negative feedback towards the HPA axis results in a prolonged stress response

and the development of stress-related diseases and mood disorders such as depression (321, 323).

In line with the development of stress-related diseases, human genetic studies revealed a correlation of *FKBP5* gene variants with a higher risk of psychiatric disorders. In particular, *FKBP5* single nucleotide polymorphisms (SNPs) are associated with either a dysregulation of the FKBP51-GR system, enhanced levels of FKBP51 or both, leading to various types of disorders (328-333). Thereby, patients, carrying such *FKBP5* SNPs, have been found to be prone to develop depressive disorders, a post-traumatic stress disorder, bipolar disorders, a psychosis, or to show suicidality (334-338). Beside psychiatric disorders, FKBP51 has also been linked to metabolic diseases since studies identified increased FKBP51 levels in diabetes type II patients. Furthermore, several studies implicated a FKBP51-mediated glucose intolerance in obesity, and an insulin resistance in type II diabetes (339, 340). In summary, various animal and human studies showed that increased FKBP51 levels are related to many disorders that can be improved by the deficiency or reduction of FKBP51.

## 1.7.2 FKBP51 in the context of pain

While FKBP51 is quite well known as target for stress disorders, the understanding of its role is rather limited and is currently increasing in the context of pain. Based on the role of *FKBP5* in neuropsychiatric disorders, it was firstly associated with being involved in the development of post-traumatic chronic musculoskeletal pain (MSP) (341, 342). Although the development of MSP is poorly understood, genetic studies identified *FKBP5* gene variants as predictor for the severity of MSP (341). Besides MSP, FKBP51 deficiency was shown to reduce mechanical hypersensitivity in an inflammatory joint mouse model (343). However, a FKBP51 deficiency did not impair acute nociceptive pain, which is quite important for the protective function of nociception (343). Based on this data, FKBP51 was firstly revealed to be involved in the mediation of pathological pain. Furthermore, the deficiency of FKBP51 ameliorated inflammatory pain in both male and female mice (344). This is of particular interest, as other studies suggested sex specifies in inflammatory pain mechanisms. Based on this, targets postulated from earlier studies were less efficient in either male or female mice in the inflammatory context (345-351), highlighting FKBP51 as potential target for both sexes.

Previous studies also investigated the involvement of FKBP51 in the establishment of chronic pain states such as neuropathic pain (344). Thereby, FKBP51 was identified as central mediator of chronic and especially neuropathic pain, and as pharmacological target for neuropathic pain. This conclusion was based on data which shows that FKBP51 deficient mice experienced less nerve injury-induced neuropathic pain than wild type animals (344). Furthermore, the treatment of nerve injured wild type mice with an FKBP51 silencing RNA resulted in comparably low pain behavior (344). Beside the nerve injury-induced neuropathic pain, FKBP51 was also shown to play an essential role in chemotherapy-induced neuropathic pain states, since FKBP51 deficient mice showed less hypersensitivity after cytostatic treatment (344). In addition, Maiaru et al. confirmed elevated FKBP51 levels in the dorsal horn of wild type mice after nerve injury. The increase of FKBP51 was suggested to induce a disbalance in the FKBP51-GR regulation and to lead to chronic pain states. However, these chronic pain states could be counteracted by pharmacologically targeting FKBP51 (344).

Beside the *in vivo* data, FKBP51 has additionally been described as regulator of the NF-κB signaling pathway (308). Although the influence of FKBP51 on the NF-κB signaling pathway is controversially discussed in literature, there is emerging evidence that FKBP51 seems to promote the activation of the NF-κB signaling pathway (352-357). This is of particular relevance in the context of pain as NF-κB induces the expression of various cytokines and chemokines, which play an essential role in the development of neuroinflammation (189, 222, 358-360). In addition, the NF-κB signaling pathway was associated with FKBP51-mediated cell migration, indicating its involvement in metastasis (361, 362). However, the underlying mechanism of FKBP51-mediated NF-κB regulation are poorly elucidated. Nevertheless, a reduction of FKBP51 levels was shown to be beneficial in many chronic inflammatory disorders (342-344, 363, 364).

### 1.7.3 SAFit2 as selective FKBP51 inhibitor

Since FKBP51 was revealed as potential target for neuropsychiatric disorders, metabolic diseases and chronic pain states, the development of an FKBP51 specific inhibitor gained interest. However, the structural resemblance of other FKBPs, especially FKBP52 to FKBP51, hampered the development of a FKBP51 specific inhibitor for a long time (365, 366). Nevertheless, Gaali et al. firstly developed two FKBP51 selective ligands by an induced fit mechanism (367). Based on this, the FKBP51 inhibitors were termed SAFit (selective

antagonist of FKBP51 by an induced fit mechanism) (367). From these two FKBP51 ligands, SAFit1 showed the highest affinity with 4 nM, however SAFit2 was shown to be more CNS permeable, while comprising an affinity of 6 nM towards FKBP51 (367, 368). Based on these results, the selective FKBP51 inhibitor SAFit2 was used for further animal studies (369-371).

The selectivity of SAFit2 to FKBP51 is mainly facilitated by a conformational change, which is induced at the FK1 domain of FKBP51 upon its binding (367). Mechanistically, the phenylalanine (Phe) 67 of FK1 is displaced towards lysine (Lys) 58 and Lys60, due to the Cα of SAFit2, and gets stabilized by Phe129 (Figure 8) (367). In contrast, FKBP52 is unsusceptible to undergo such conformational changes as the respective residues (threonine 58, tryptophan 60 and valine 129) are too bulky therefore (367). Nevertheless, FKBP52 could undergo such conformational changes but the resulting conformation is very strained and unlikely. Based on that, SAFit2 comprises a 10,000 fold selectivity for FKBP51 over FKBP52 (367).
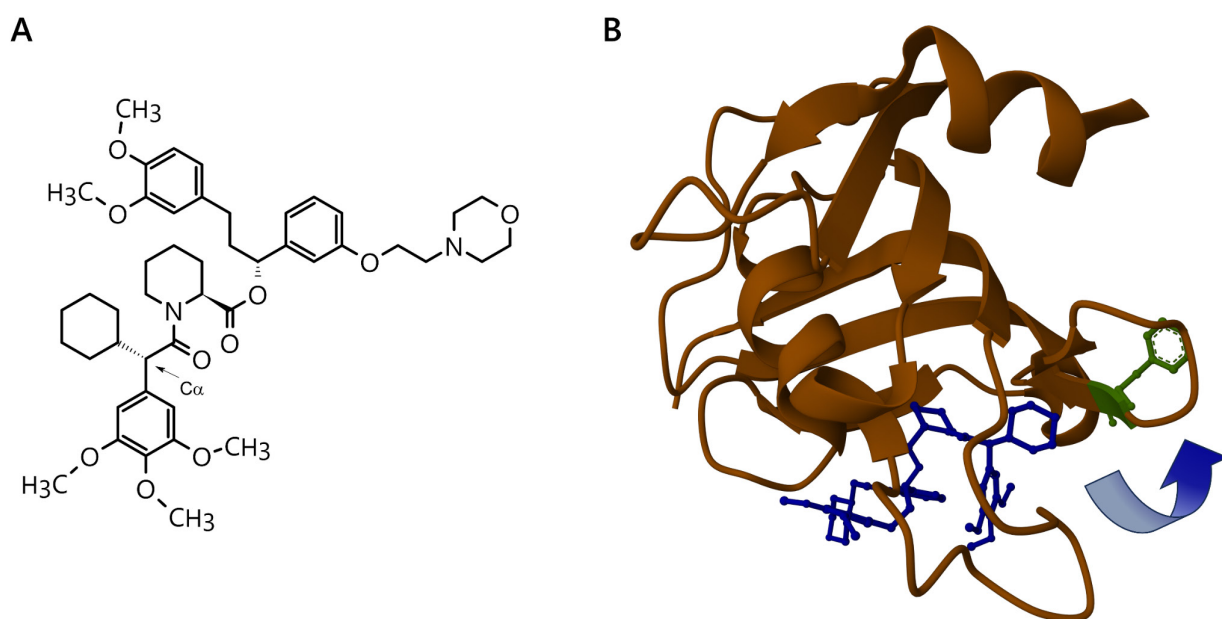


**Figure 8: Structure of SAFit2 and SAFit2 bound to FK1 of FKBP51. (A)** Molecular structure of SAFit2 with indicated Cα which facilitates the conformational change in FKBP51. **(B)** Crystallographic structure of the FK1 domain of FKBP51 bound to SAFit2. The FK1 domain is colored in brown and SAFit2 in blue. The arrow indicates the displacement of phenylalanine 67 (green) due to the binding of SAFit2. The molecular structure was drawn with ChemSketch and the crystallographic structure was taken from the RSCB database (PDB-ID: 6txx) (372).

## 1.8 Aim of this thesis

Based on the high demand of neuropathic pain therapeutics and the limited and inadequate options for neuropathic pain treatment, the need of novel therapeutical drug candidates emerges. Interestingly, previous studies revealed a significant pain relieve of nerve injury- and chemotherapy-induced neuropathic pain in FKBP51 deficient mice, highlighting FKBP51 as potential target for these types of neuropathic pain. However, the pharmacological targeting of FKBP51 was hampered for years due to the lack of a selective FKBP51 inhibitor. Nevertheless, the development of SAFit2 as specific and highly potent FKBP51 inhibitor enabled the opportunity to target FKBP51 pharmacologically *in vitro* and *in vivo* (Figure 9).
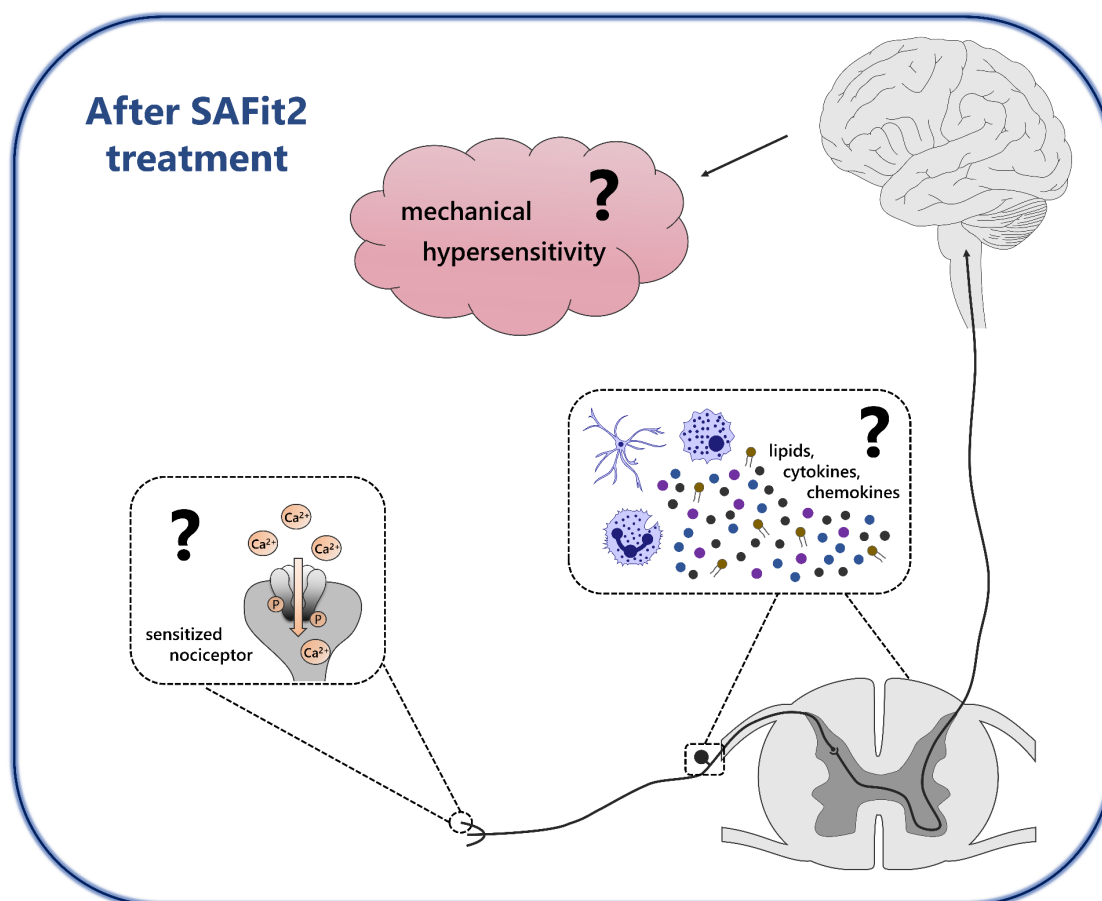


**Figure 9: Illustration of aims scheduled within this thesis**. To address the central question whether SAFit2 constitutes as potential novel treatment option for nerve injury- and paclitaxel-induced neuropathic pain, we firstly measured the mechanical hypersensitivity of mice as neuropathic pain behavior in both neuropathic pain models after SAFit2 treatment. Furthermore, lipid levels, cytokine and chemokine levels, the number of invading immune cells and the activation of glial cells was analyzed in dorsal root ganglia and spinal cord samples. In addition, we investigated the sensitization state of different pain-mediating TRP channels after SAFit2

treatment via calcium transients in primary sensory neurons and assessed related underlying mechanisms. The illustration was created with images from motifolio.

Hence, the overall objective of this thesis was to address and assess the hypothesis that SAFit2 constitutes as novel treatment option for nerve injury- and chemotherapy-induced neuropathic pain. In line with this, the **first aim** of this thesis was to investigate whether inhibiting FKBP51 with SAFit2 ameliorates nerve injury-induced and chemotherapy-induced neuropathic pain *in vivo* like the deficiency of FKBP51 does. The **second aim** encompassed the description of endogenous mediators such as lipids, chemokines, and cytokines in spinal cord and dorsal root ganglia of nerve injured and paclitaxel treated mice. Furthermore, the direct influence of SAFit2 on the excitability of primary sensory neurons and migration of primary macrophages was investigated. The **third aim** was to identify which impact changes of mediator profiles, sensory neuron excitability and macrophage migration might have on mechanisms occurring in neuropathic pain. In addition, first approaches should be made to understand how these changes are mediated after SAFit2 treatment.

## 2 Publication 1

# SAFit2 reduces neuroinflammation and ameliorates nerve injury-induced neuropathic pain

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## 2.1 Author contributions

Conceptualization: FH, MS and GG conceptualized the project.

Funding acquisition: MS, FH, OR, NdB and MF acquired the funding.

Experimental design: **SW**, OR, AW, NdB, and MS designed the experiments.

Data acquisition: **SW** performed the multiplex assays, qPCRs, Western Blots, transwell assays, calcium imaging experiments, CGRP assay, calcineurin assay, WST assay and prepared all primary cells and tissue samples for measurements. PM performed the FACS measurements. OR performed the electrophysiological experiments. TH synthesized ddSAFit2. CC performed the SAFit2 screening in HEK cells. DF performed the seahorse run.

Data analysis: **SW** analyzed all the data. AW helped with the FACS analysis and OR performed the analysis of the electrophysiological measurements.

Data curation: **SW** performed the data curation.

Original draft preparation: **SW** and MS wrote and revised the manuscript.

All authors approved the final manuscript.

## 2.2 Manuscript

# SAFit2 reduces neuroinflammation and ameliorates nerve injury-induced neuropathic pain

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## Abstract

**Background:** Neuropathic pain is experienced worldwide by patients suffering from nerve injuries, infectious or metabolic diseases or chemotherapy. However, the treatment options are still limited because of low efficacy and sometimes severe side effects. Recently, the deficiency of FKBP51 was shown to relieve chronic pain, revealing FKBP51 as a potential therapeutic target. However, a specific and potent FKBP51 inhibitor was not available until recently which hampered targeting of FKBP51.

**Methods:** In this study, we used the well-established and robust spared nerve injury model to analyze the effect of SAFit2 on nerve injury-induced neuropathic pain and to elucidate its pharmacodynamics profile. Therefore, the mice were treated with 10 mg/kg SAFit2 after surgery, the mice behavior was assessed over 21 days and biochemical analysis were performed after 14 and 21 days. Furthermore, the impact of SAFit2 on sensory neurons and macrophages was investigated in vitro.

**Results:** Here, we show that the FKBP51 inhibitor SAFit2 ameliorates nerve injury-induced neuropathic pain in vivo by reducing neuroinflammation. SAFit2 reduces the infiltration of immune cells into neuronal tissue and counteracts the increased NF-κB pathway activation which leads to reduced cytokine and chemokine levels in the DRGs and spinal cord. In addition, SAFit2 desensitizes the pain-relevant TRPV1 channel and subsequently reduces the release of pro-inflammatory neuropeptides from sensory neurons.

**Conclusions:** SAFit2 ameliorates neuroinflammation and counteracts enhanced neuronal activity after nerve injury leading to an amelioration of nerve injury-induced neuropathic pain. Based on these findings, SAFit2 constitutes as a novel and promising drug candidate for the treatment of nerve injury-induced neuropathic pain.

**Keywords:** SAFit2, FKBP51, Neuropathic pain, Neuroinflammation, Sensory neurons

## Background

Neuropathic pain is defined as lesion or disease of the somatosensory system [8]. It is a pathological pain state experienced worldwide by patients suffering from nerve injuries, diseases such as diabetes or chemotherapy [50]. However, the therapeutic options for neuropathic pain are very limited due to low treatment responses and severe side effects of available drugs [12]. Nerve

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injury-induced neuropathic pain comprises several types of symptoms such as mechanical hypersensitivity which is defined as the reduction of the mechanical pain threshold, and painful sensations that can be mediated by innocuous stimuli [60].

The pain state itself arises by an increased activity of damaged nerve fibers, often in combination with an inflammatory response of the organism, which results in a pathological neuroinflammation [11]. Neuroinflammation is characterized as the infiltration of immune cells into neuronal tissue and the secretion of pro-inflammatory and proalgesic mediators such as cytokines, chemokines and neuropeptides that directly affect sensory neurons [22]. Moreover, the activation and sensitization of pain-mediating transient receptor potential (TRP) ion channels lead to the release of neuroinflammation-promoting neuropeptides [23].

During nerve injury-induced neuropathic pain, the release of pro-inflammatory and proalgesic mediators changes the microenvironment of sensory neurons which enhances neuronal activity. This can lead to peripheral and central sensitization and can subsequently to an increased pain perception [22]. The multitude of responsible mediators and the variety of different immune cell types, involved in their orchestrated synthesis and release, makes it difficult to target neuroinflammation at a large scale. Unfortunately, inhibiting individual cytokines, such as the tumor necrosis factor alpha, interleukin 6 or blocking their respective receptors does not significantly ameliorate neuropathic pain in patients [27, 58].

However, neuroinflammation can also be initiated by neurons themselves. More specifically, enhanced neuronal activity caused, for example, by increased activity of ion channels and depolarization can lead to an augmented downstream release of pro-inflammatory neuropeptides, such as calcitonin gene-related peptide (CGRP). These neuropeptides can cause vasodilation and recruitment of T cells and initiate neuroinflammation via their G protein-coupled receptors [3, 15, 56]. These studies indicate that a broader approach is required to target neuroinflammation-mediated persistent pain in patients.

Interestingly, previous studies revealed the FK506 binding protein 51 (FKBP51, encoded by the FKBP5 gene) as a novel potential therapeutic target for relieving neuropathic pain since its deficiency led to a significant amelioration of inflammatory and neuropathic pain [30, 31]. Initially, FKBP51 was discovered as a potential target for treating stress endocrinology [52] and glucocorticoid signaling related diseases [17]. Nevertheless, its strong upregulation, exclusively in sensory neurons of the dorsal horn after an ankle joint inflammation, indicated its involvement in the pathophysiological progress leading to chronic pain [31]. However, the pharmacological targeting of FKBP51 inhibitor was not possible for a long time due to low specificity of synthesized inhibitors and cross-interactions with other related proteins.

Nevertheless, these challenges were overcome, exploiting an FKBP51-specific conformation [20], to generate an highly potent FKBP51 inhibitor called SAFit2 (selective antagonist of FKBP51 by induced fit) [2, 13]. In addition, a good pharmacokinetic availability and an improved blood–brain barrier permeability of SAFit2 compared to SAFit1 was already confirmed in previous pharmacokinetic studies for the dose of 10 mg/kg SAFit2 injected intraperitoneally [13, 14]. However, the underlying analgesic and anti-hyperalgesic mechanisms have not been identified yet. Here, we used the well-established and robust spared nerve injury (SNI) mouse model to analyze the effect of SAFit2 on nerve injury-induced neuropathic pain. Further, we investigated the neuronal and neuroinflammatory effects of a SAFit2 treatment in vivo and in vitro to elucidate its pharmacodynamics profile.

## Materials and methods
### Animals, spared nerve injury surgery and SAFit2 treatment
In all experiments, wild-type mice (male C57Bl/6N mice, age from 8 to 12 weeks at the start of the study) were purchased from commercial breeding companies (Janvier and Charles River). For inducing neuropathic pain in mice, a spared nerve injury (SNI) surgery was performed under anesthesia to establish neuroinflammation. During the surgery, the sciatic nerve was exposed by a blunt dissection on the level of the knee joint. Then, two of the three sciatic nerve branches, common peroneal and tibial branch, were ligated with 6/0 non-sterile silk thread and cut distally from the ligature, leaving the sural nerve branch intact. Contact with the sural nerve branch was avoided to prevent stretching or harming. Afterwards, muscle and skin were closed in two layers [9].

For assessing the impact of SAFit2 on neuropathic pain behavior and neuroinflammation, animals were treated intraperitoneally with either 10 mg/kg SAFit2 or vehicle (PBS supplemented with 5% PEG400, 5% Tween and 0.7% ethanol) two times daily on six consecutive days starting on day five after surgery.

### Behavioral experiments
During all behavioral experiments, the experimenter was blinded. The motoric function of all animals was verified via rotarod before treatment. All animals were transferred into respective test cages for at least one hour before the measurement to allow habituation. For the determination of the mechanical withdrawal threshold, a dynamic plantar test was performed, using a Dynamic Plantar Aesthesiometer (Ugo Basile) as described

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previously. Briefly, a steel rod was pushed against the mid-plantar area of the hind paw with linear ascending force up to 5 g over 10 s and a holding force of 5 g until a withdrawal response occurred. For behavioral experiments a cut-off time of 20 s was set [49].

### Tissue isolation

For tissue isolation, mice were euthanized by isoflurane, cardio puncture and cranial dislocation either on day 14 or on day 21 after surgery covering different time points in neuroinflammation. The sciatic nerve, lumbar (L4–L6) dorsal root ganglia (DRGs) and the respective segments of the spinal cord were dissected from injured (ipsilateral) and unimpaired (contralateral) sites, followed by freezing tissue samples in liquid nitrogen for either RNA isolation, Western blot or multiplex assay. For flow cytometry analysis, the tissue was dissected and stored in 500 µl ice-cooled PBS at 4 °C until further processing.

### Quantitative real-time PCR

Total RNA was isolated from L4–L6 DRGs and the respective segments of spinal cord using the mirVana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer´s instructions. Afterwards, RNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and a cDNA synthesis was performed with 200 ng RNA from DRGs and 400 ng RNA from spinal cord. The reverse transcription was performed with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. The quantitative real-time PCR was conducted with Quant-Studio™ Design & Analysis Software v 1.4.3 (Thermo Fisher Scientific) in a TaqMan® Gene Expression Assay System (Table 1, Thermo Fisher Scientific) according to the manufacturer´s instructions. The raw data were evaluated using the $\Delta\Delta C(T)$ method, as described previously [29, 47].

### Multiplex assay

For performing the ProcartaPlex multiplex immunoassay (Thermo Fisher), proteins were isolated with a manufacturer recommended cell lysis buffer, which was further supplemented with a phosphatase inhibitor cocktail (PhosSTOP, Roche) and a protease inhibitor cocktail (cOmplete, Roche). DRG samples were suspended in 100 µl and spinal cord samples in 200 µl of cell lysis buffer. The spinal cord samples were further processed by a cell grinder. Afterwards, the tissue was homogenized two times per sample using a Sonopuls Sonicator (Bandelin) with the setting $6 \times 10\%$. During sonication, the samples were cooled in an ice bath, preventing proteins from degradation. Finally, the samples were centrifuged with $16,000 \times g$ at 4 °C for 10 min, followed by collecting the supernatant for a protein concentration determination via Bradford.

The ProcartaPlex multiplex immunoassay was performed according to the manufacturer's recommendations. Briefly, a dark wall 96-well plate was prepared by several washing steps and coating steps with respective magnetic beads. Afterwards, standards were prepared in a serial dilution (1:4, (v/v)) and added to the plate, followed by further washing steps. Lastly, the samples were diluted (1:2, (v/v)) and added to the plate, which was sealed and incubated for 40 min with 500 rpm on an orbital shaker at room temperature, overnight at 4 °C and further 50 min with 500 rpm at room temperature. On the next day, the detection antibody mixture was added after a washing step and the plate was further incubated on an orbital shaker for 30 min at room temperature. Again, the plate was washed and streptavidin phycoerythrin was added to the plate and incubated for 30 min as described above. After the last washing step, the plate

**Table 1** List of used TaqMan® gene expression assays

| Target | Gene | Article number | Company |
| --- | --- | --- | --- |
| ATF3 | Activating transcription factor 3 | Mm00476033_m1 | Thermo Fisher |
| cFOS | FBJ osteosarcoma oncogene | Mm00487425_m1 | Thermo Fisher |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Mm99999915_g1 | Thermo Fisher |
| iNOS | Inducible nitric oxide synthase 2 | Mm00440502_m1 | Thermo Fisher |
| MMP9 | Matrix metallopeptidase 9 | Mm00442991_m1 | Thermo Fisher |
| NFATc3 | Nuclear factor of activated T cells, calcineurin dependent 3 | Mm01249200_m1 | Thermo Fisher |
| NFATc4 | Nuclear factor of activated T cells, calcineurin dependent 4 | Mm00452375_m1 | Thermo Fisher |
| NOX2 | NADPH oxidase 2 | Mm01287743_m1 | Thermo Fisher |
| NOX4 | NADPH oxidase 4 | Mm00479246_m1 | Thermo Fisher |
| XDH | Xanthine dehydrogenase | Mm00442110_m1 | Thermo Fisher |

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was prepared with reading buffer, incubated for 5 min with 500 rpm on an orbital shaker, and measured with the Luminex 200 system (Bio-Rad).

### Western blot

For Western blot purposes, the tissue of five animals was pooled and proteins isolated from the respective tissue samples. Therefore, 300 µl of cell lysis buffer, which was described in part 2.5, were added to spinal cord samples and 100 µl to DRG samples. The tissue samples were homogenized as previously described and the protein amount was determined by a Bradford assay.

Afterwards, 30 µg tissue lysate was loaded and separated by SDS-polyacrylamide gel electrophoresis (4% stacking gel, 12% running gel) and transferred on a nitrocellulose membrane with the Trans-Blot®Turbo™ Transfer System (BioRad). For total protein detection, the membrane was blocked with TBST buffer (20 mM Tris, 150 mM NaCl and 0.1% Tween20) containing 5% skimmed milk powder at room temperature for two hours, followed by an incubation overnight with primary antibodies at 4 °C: p65 1:500 (8242S, cell signaling technology), IKBα 1:500 (4812S, cell signaling technology) and IKKβ 1:250 (2370S, cell signaling technology). For phosphorylated protein detection, the membranes were blocked with TBST buffer containing 5% BSA at room temperature for 2 h, followed by an incubation for at least 48 h with primary antibodies at 4 °C: p-p65 1:500 (3033, cell signaling technology) and p-IKBα 1:250 (2859S, cell signaling technology). Beta-actin 1:1000 (ab8229, abcam) was used as a loading control. For labeling targets, the fluorescent-labeled secondary antibodies anti-rabbit labeled with IRDye680ED from donkey (Licor), and anti-goat labeled with IRDye800CW from donkey (Licor) were used in 1:5000 dilutions for one hour at room temperature. The antibody detection was performed with an Odyssey CLx device from Licor, followed by a quantification with Image Studio Software.

### Flow cytometry analysis

For creating single-cell suspensions, sciatic nerves were sliced with a tissue scissor and spinal cords were briefly pottered with a tissue grinder at first. Afterwards, all tissue samples (DRGs, sciatic nerve and spinal cord) were incubated in 500 µl Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 3 mg/mL collagenase and 1 µl/mL DNase for 30 min at 37 °C. Then, the enzymatic reaction was stopped by further adding 500 µl DMEM supplemented with 10% FCS. To obtain a single-cell suspension, the samples were filtered through a 70-µm cell strainer and centrifuged at 400 g for 5 min. The supernatant was discarded and the cell pellet washed with 500 µl PBS supplemented with 0.5% BSA, followed

by a further centrifugation step. Lastly, the cell pellet was suspended in 500 µl PBS supplemented with 0.5% BSA and 1 mM EDTA to prevent cells from aggregating until and during flow cytometry analysis. The flow cytometry analysis was performed essentially as described previously [37]. Single-cell suspensions were blocked with FcR blocking reagent (Miltenyi Biotec) in 0.5% PBS-BSA for 20 min, stained with fluorochrome-conjugated antibodies (Table 2) and analyzed on a FACSymphony A5 flow cytometer (BD Biosciences). The data were analyzed using FlowJo VX (TreeStar). All antibodies and secondary reagents were titrated to determine optimal concentrations. Comp-Beads (BD Biosciences) were used for single-color compensation to create multicolor compensation matrices. For gating, fluorescence minus one control were used. The instrument calibration was controlled daily using Cytometer Setup and Tracking beads (BD Biosciences). For characterization of immune cell subsets, the antibodies in Table 2 were used.

### Isolation and purification of dorsal root ganglia (DRGs)

For methods using sensory neurons in cell culture, DRGs were dissected and transferred into ice-cold HBSS with CaCl$_2$ and MgCl$_2$ (Gibco) directly after dissection. Afterwards, DRGs were treated with a collagenase/dispase solution, 500 U/mL collagenase and 2.5 U/mL dispase diluted in neurobasal medium (Gibco), at 37 °C for 75 min. The collagenase/dispase solution was removed by centrifuging and discarding the supernatant, the

**Table 2** List of antibodies used for flow cytometry analysis

| Target | Dye | Cell type | Identifier | Source |
|---|---|---|---|---|
| CD11b | BV605 | Monocytes, eosinophils, neutrophils | AB_2737951 | Biolegend |
| CD11c | BV711 | AMs | AB_2734778 | BD Biosciences |
| CD19 | APC-H7 | B cells | AB_1645234 | BD Biosciences |
| CD3 | APC-Cy7 | T cells | AB_1727461 | BD Biosciences |
| CD326 | BV711 | Epithelial cells | AB_2738022 | BD Biosciences |
| CD4 | BV711 | CD4+T cells | AB_2737973 | BD Biosciences |
| CD45 | VioBlue | All leukocytes | AB_2659925 | Miltenyi |
| CD8a | BV650 | CD8+T cells | AB_2563056 | Biolegend |
| CD90 | PE | Lymphocytes | AB_2659874 | Miltenyi |
| F4/80 | PE-Cy7 | Macrophages | AB_893478 | Biolegend |
| GITR | FITC | Tregs | AB_1089125 | Biolegend |
| Ly6C | PerCP-Cy5.5 | Monocytes | AB_1727558 | BD Biosciences |
| Ly6G | APC-Cy7 | Neutrophils | AB_10640819 | Biolegend |
| MHCII | APC | cDCs, IMs | AB_313329 | Miltenyi |
| NK1.1 | BV510 | NK cells | AB_2738002 | Biosciences |
| SiglecF | PE | Eosinophils | AB_394341 | Biolegend |
| γδTCR | APC | γδ T cells | AB_1731813 | Biolegend |

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Capsaicin, SAFit2 and RuR supplemented bath solutions were prepared directly before the measurements by adding the appropriate volume of stock solutions to the external solution. Bath solution exchange was performed using a gravity flow perfusion system. Whole-cell currents were elicited by repetitively applying 500 ms voltage pulses from $-120$ mV to $+80$ mV (20 mV increments) from a holding potential of $-40$ mV and 500 ms voltage ramps from $-60$ mV to $+60$ mV from a holding potential of $-60$ mV. Currents were recorded with a 10 kHz low-pass filter and sampled with a frequency of 50 kHz. Data were memorized with Patchmaster (HEKA Elektronik) and analyzed with Fitmaster (HEKA Elektronik). To account for the different sizes of the measured cells, whole-cell currents were normalized to the cell membrane capacity.

### Calcitonin gene-related peptide assay

For measuring the effect of SAFit2 on calcitonin gene-related peptide (CGRP) secretion, DRGs were isolated as previously described (part 2.8) and stimulated with the respective compound the next day. Therefore, the cells were washed with HBSS and incubated with 250 µl of the respective compound, diluted in HBSS to their final concentration, and 1 µM capsaicin for 15 min at 37 °C. The respective volume of the vehicle was used as a negative control, and 1 µM capsaicin alone as positive control. The CGRP quantification was performed with an ELISA kit from Bertin Bioreagent, which was used according to the manufacturer´s recommendations. The final detection was performed measuring the absorption at 405 nm. The raw data were evaluated using linear regression according to the manufacturer´s recommendation.

### Calcineurin assay

The calcineurin activation was measured with a cellular calcineurin phosphatase activity assay kit from abcam, which was used according to the manufacturer's instructions. The enzyme calcineurin was isolated from HEK-293 cells using 1 mL of TBS buffer containing 20 mM Tris and 150 mM NaCl (adjusted to pH 7.3) for a 90% confluent T75 flask. The cell lysate was further purified according to the kit protocol. To measure the impact of the respective compounds on the activity of calcineurin, the purified cellular extract with calcineurin was supplemented with the respective compound and added to the prepared assay plate. Afterwards, the plate was incubated for 30 min at 30 °C to stimulate the activity of the enzyme for a distinct period of time. Finally, the reaction was terminated by adding the assay reagent to the plate and the absorption was detected after 20 to 30 min at 620 nm. The raw data were evaluated using linear regression according to the manufacturer's instructions.

### Differentiation of bone marrow-derived macrophages (BMDM)

For bone marrow isolation, mice were killed and hind legs were dissected and transferred into ice-cold PBS. The bones were cleaned from muscle tissue, cut open and transferred into 0.5-mL Eppendorf tubes with holes at their peak end. To isolate the bone marrow via centrifugation (1300 rpm, 1 min, RT), the tubes were placed into 1.5-mL Eppendorf tubes, containing 50 µL of BMDM medium (RPMI + GlutaMAX (Thermo Fisher Scientific) supplemented with 10% FCS (Sigma-Aldrich), 100 U/mL penicillin + 100 µg/mL streptomycin (Thermo Fisher Scientific) and 20 ng/mL M-CSF (Peprotech). After centrifugation, the bone marrow pellet was resuspended in BMDM medium and distributed equally into a 6-well plate. For macrophage differentiation, cells were incubated overnight at 37 °C, followed by a change of BMDM medium the next day. At day four of differentiation, fresh growth factors were provided by adding the same amount of BMDM medium to the differentiating cells. After 7 days, the cells were fully differentiated and used for further experiments [57].

### Human macrophage isolation and purification

For isolating buffy coats from whole bloods samples, 50-mL Leucosep tubes were equilibrated with 15 mL lymphocyte separation medium, which was passed through the membrane by centrifuging at $1000 \times g$ for 5 min. Afterwards, 40 mL whole blood was transferred into a tube, which was then filled up to 50 mL with PBS containing 2 mM EDTA. The samples were centrifuged at $440 \times g$ for 35 min at room temperature without break to enable a clear separation of fractions comprising a plasma fraction at the top, the buffy coat in the middle and an erythrocyte and granulocyte fraction at the bottom. The plasma was discarded and the buffy coat transferred into a fresh 50-mL tube, which was then again filled up to 50 mL with PBS containing 2 mM EDTA for purification. The suspension was again centrifuged at $500 \times g$ at room temperature for 5 min and the purification step was repeated until the supernatant was almost clear. Afterwards, the cell pellet was resuspended in 50 mL RPMI medium supplemented with 1% P/S and the cell solution was plated with 1 mL/ well into 6-well plates. The cells were incubated for one hour at 37 °C, followed by a medium change with 2 mL RPMI supplemented with 1% P/S and 3.3% human plasma for differentiation purpose. The medium was changes every 2–3 days until day seven of differentiation. Monocytes were now fully differentiated into macrophages and can be used for further experiments [43].

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sensory neurons were washed twice with neurobasal medium containing 10% FCS, followed by an incubation with 0.05% trypsin (v/v) (Gibco) for another 10 min. The washing steps were repeated, and the cells were mechanically dissociated in neurobasal medium (Gibco) supplemented with L-glutamine (2 mM; Gibco), penicillin (100 U/mL; Gibco), streptomycin (100 µg/mL; Gibco), B-27 (Gibco) and gentamicin (50 µg/mL; Gibco). Afterwards, the cell solution was plated on poly-L-lysine-coated cover slips or 48-well plates. After two hours of incubation, 2 mL of neurobasal medium was added and the cells were incubated overnight at 37 °C. For further investigations, the cultured DRGs were further used and treated as described in part 2.9 and 2.13.

### Calcium imaging

For calcium imaging, the cultured sensory neurons were stained with fura-2-AM (Thermo Fisher) for at least 60 min at 37 °C and washed afterwards twice with Ringer´s solution. This was set up freshly with 145 mM NaCl, 1.25 mM $CaCl_2 \times 2H_2O$, 1 mM $MgCl_2 \times 6 H_2O$, 5 mM KCl, 10 mM D-glucose and 10 mM HEPES and adjusted to a pH of 7.3. During the experiments, Ringer's solution was also used for baseline measurements and for washing out agonists and compounds between stimulations. For investigating the effect of SAFit2 on different TRP channels, the sensory neurons were pre-incubated with the respective compound for 2 min and stimulated with respective agonists afterwards: 100 nM capsaicin for 30 s (TRPV1 agonist) and 100 mM allyl isothiocyanate for 45 s (TRPA1 agonist). Control experiments were performed with the respective volume of the corresponding vehicle. All stimulating compounds were dissolved in Ringer's solution to their final concentration. The measurements were performed using a DMI4000 B Microscope, the compact light source CTR550 HS (Leica) and the ValveBank II system (AutoMate Scientific).

### Flexstation method

Cells were grown in 96-well black-walled plates. Human TRPV1 was transiently expressed in HEK-293t cells using jetPEI transfection reagent (Polyplus Transfection). Cells were loaded with Calcium 6 for two hours (Calcium 6 Kit; Molecular devices, San Jose, CA), in an extracellular solution containing 145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 1.25 mM $CaCl_2$, and 1 mM $MgCl_2$, buffered to pH 7.4 with NaOH. According to the manufacturer's protocol, cells are not washed, but extracellular dye is chemically quenched. Calcium 6 fluorescence excited at 488 nm every 2.5 s served as an index of intracellular calcium. Assays were performed at 25 °C. A volume of 50 µL of each applied solution was pipetted automatically according to a preset protocol into 100 µL

of extracellular solution in the wells. Fluorescence change was measured by a Flexstation3 (Molecular devices) and is reported relative to baseline fluorescence.

### Transfection of HEK-293 cells

For functional expression of green fluorescent protein (GFP) flanked TRPV1, HEK-293 cells were transfected 16–24 h before the start of patch-clamp experiments using TransfeX™ Transfection Reagent (LGC Standards GmbH) according to the manufacturer's instructions. HEK-293 cells (German Collection of Microorganisms and cell cultures) were grown at 37 °C in a humidified 95% air/ 5% CO2 incubator in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% v/v heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate and 2 mM L-glutamine (all from Invitrogen). After reaching approximately 80% confluence HEK-293 cells were transfected in a 35-mm petri dish with 1 µg of hTRPV1_GFP (NM_080705, RG217713, OrgiGene) plasmid DNA.

### Electrophysiology

On the day of experiment, transfected HEK-293 cells were separated by trypsinization, seeded at low density on coverslips (15 mm in diameter), and then incubated for 2 to 4 h to allow adhering of cells on the glass surface. For patch-clamp experiments, coverslips were transferred to a perfusion chamber filled with bath solution and placed on the stage of an inverted microscope. Transfected cells were identified by the fluorescence of the GFP fused to the C-terminus of TRPV1. Patch-clamp experiments were performed in the whole-cell configuration with an EPC-10 patch-clamp amplifier (HEKA Elektronik) at room temperature (20–25 °C). Patch-pipettes were pulled from borosilicate capillaries (DWK Life Sciences) using a single stage glass microelectrode puller (PP-830) resulting in pipettes with 1.5–3 MOhm resistances. The capillaries were coated with Sigmacote® (Merck KGaA) and baked after pulling at 65 °C for 45 min. The pipette solution contained: 140 mM CsCl, 1.93 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), and 2 mM MgATP. pH and osmolarity were adjusted to 7.2 with 1 M CsOH and 320 mOsmol/kg with D-mannitol, respectively. The bath solution contained: 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM D-glucose. pH and osmolarity were adjusted to 7.4 with 1 M NaOH and 320 mOsmol/kg with D-mannitol, respectively. Capsaicin (M2028, Sigma Aldrich), SAFit2 and Ruthenium Red (RuR) (557,450, Sigma Aldrich) stock solutions were prepared by dissolving 10 mM, 2.5 mM and 10 mM in DMSO, respectively.

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### Transswell assay

For investigating the migration of macrophages, cultivated macrophages were starved overnight and harvested the next day by incubating them 10 min with 600 µl accutase/ well (Sigma-Aldrich) at 37 °C and using a cell scraper. The reaction was terminated using 1% (w/v) BSA solution for murine macrophages. Afterwards, the macrophages were centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended in 1 mL of the respective starving medium. The cell concentration was determined, using a Neubauer counting chamber and the cell concentration adjusted to $10^6$ cells/mL. Transwell inserts (Greiner) were placed into 24-well plates, which contain 500 µl medium/well with either 10% FCS as chemoattractant for murine cells or 3.3% human plasma for human cells and the respective compound. The cells were seeded by adding 300 µl cell solution per insert. After incubating the setup for two hours at 37 °C, the inserts were rinsed with PBS and cells were fixed with 2% PFA for two minutes, followed by further washing steps and a further fixation and permeabilization step with 100% ice-cooled methanol for 20 min. Lastly, the membranes were stained with DAPI in a final concentration of 0.2 ng/mL in the dark for two minutes and embedded on slides with mounting media for quantification purposes. Five images were taken per membrane with the fluorescence microscope Observer.Z1 (Carl Zeiss) and quantified with ImageJ software.

### Cytotoxicity assay with WST-1

Primary murine macrophages were isolated and differentiated as previously described (part 2.15). The cells were harvested the same way as described in part 2.17. Afterwards, the cell concentration was adjusted to $3.6 \times 10^5$ cells/ mL and cells were seeded with 1 mL/ well into 24-well plates, followed by an incubation for further 48 h at 37 °C. For analyzing the compound´s cytotoxicity, DMEM medium was supplemented with 10% (v/v) WST-1 and either the compound of interest or the according volume of the dissolvent. The cells were treated with 300 µl of prepared medium for two hours at 37 °C and the absorption wavelength was measured at 450 nm and 600 nm afterwards.

### Seahorse bioanalyzer

For measuring the metabolic parameters with the Seahorse bioanalyzer device, primary murine macrophages were isolated and differentiated as previously described (part 2.15). The macrophages were harvested in the same way as described in the part 2.17, seeded ($25 \times 10^3$ cells/well) into Seahorse 96-well cell culture plates and incubated overnight. On the day of measurement, the media was replaced by XF RPMI medium supplemented with 10 mM glucose and 2 mM glutamine (all from Agilent). The plate was equilibrated for 30 min in a non-$CO_2$ incubator at 37 °C. Metabolic parameters were measured on a Seahorse XFe 96 extracellular flux analyzer (Agilent). To analyze the ATP production rate, 2.5 µM oligomycin and 500 nM rotenone together with antimycin A were sequentially added to the cells. All chemicals were purchased from Cayman chemicals. Data were processed using Wave Desktop (Version 2.6.0.31) and ATP rates were calculated using the Seahorse Analytics online tool (Version from February 2022).

### Data analysis and statistics

All data are presented as mean $\pm$ SEM. Normal distribution was confirmed using the Shapiro–Wilk test. For in vitro experiments comparing only two groups, an unpaired and heteroskedastic Student's $t$ test was conducted with Welch's correction. When comparing more than two groups, a one-way analysis of variance (ANOVA) was used and for the comparison of more than three groups a two-way ANOVA was conducted. For statistical analysis of behavioral experiments, an ANOVA was performed, followed by Bonferroni's post hoc correction. For all statistical analysis the software GraphPad Prism 9 was used. A $p$ value of $< 0.05$ was considered statistically significant.
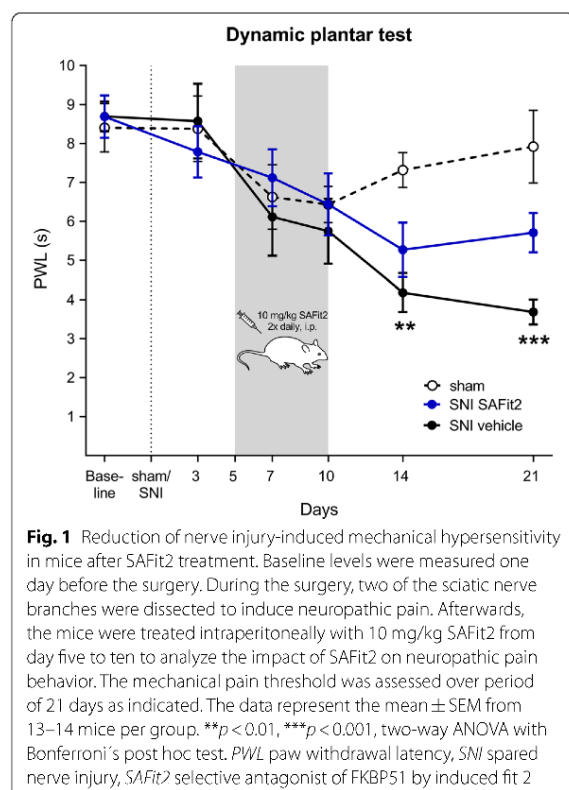
## Results

### SAFit2 ameliorates nerve injury-induced mechanical hypersensitivity

Our initial goal was to investigate whether SAFit2 has an influence on nerve injury-induced neuropathic pain. Therefore, we chose the robust and well described spared nerve injury (SNI) model to generate neuropathic pain in mice [9] and treated the mice intraperitoneally either with 10 mg/kg SAFit2 or vehicle from day five to ten after the surgery.

To analyze neuropathic pain, we determined the mechanical pain threshold of mice paws in a dynamic plantar test over 21 days (Fig. 1). After the surgery, the vehicle-treated SNI animals developed a significant mechanical hypersensitivity over time as compared to the sham-treated animals. In contrast, the SAFit2-treated SNI animals showed less mechanical hypersensitivity than the vehicle-treated animals (Fig. 1).

### SAFit2 reduces cytokine and chemokine levels in dorsal root ganglia and spinal cord after nerve injury

Since we detected that SAFit2 reduces the mechanical hypersensitivity in nerve-injured mice, we isolated lumbar dorsal root ganglia (DRGs) and spinal cord (SC) at day 21 after the surgery to investigate how amelioration of mechanical hypersensitivity is mediated. Therefore,

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**Fig. 1** Reduction of nerve injury-induced mechanical hypersensitivity in mice after SAFit2 treatment. Baseline levels were measured one day before the surgery. During the surgery, two of the sciatic nerve branches were dissected to induce neuropathic pain. Afterwards, the mice were treated intraperitoneally with 10 mg/kg SAFit2 from day five to ten to analyze the impact of SAFit2 on neuropathic pain behavior. The mechanical pain threshold was assessed over period of 21 days as indicated. The data represent the mean $\pm$ SEM from 13–14 mice per group. $**p < 0.01$, $***p < 0.001$, two-way ANOVA with Bonferroni´s post hoc test. *PWL* paw withdrawal latency, *SNI* spared nerve injury, *SAFit2* selective antagonist of FKBP51 by induced fit 2

we analyzed at first the expression of neuronal stress markers (activating transcription factor 3, matrix metallopeptidase 9, cFOS) and oxidative stress markers (xanthine dehydrogenase, NADPH oxidase 2 and 4, inducible nitric oxide synthase) but did not detect any significant differences between vehicle and SAFit2-treated animals (Additional file 1: Fig. S1). Since we did not observe any changes in the expression of common stress markers, we next analyzed the concentrations of inflammatory and proalgesic mediators in DRGs and spinal cord. We therefore performed a multiplex immunoassay to detect alterations in inflammatory and pain-mediating cytokines and chemokines after SAFit2 treatment (Figs. 2, 3, Additional file 1: Fig. S2).

All cytokines and chemokines were significantly upregulated in lumbar DRGs of vehicle-treated SNI animals (Fig. 2A–G, I–P) as compared to sham-treated animals, except for interleukin (IL)-18 (Fig. 2H). However, DRGs of SAFit2-treated SNI mice showed quite similar cytokine and chemokine levels as the DRGs of sham-treated mice (Fig. 2A–P). Moreover, we detected significant differences between vehicle and SAFit2-treated mice for both pro-inflammatory cytokines as IL-1β, IL-6, IL-18, IL-23 (Fig. 2 A, D, H, J) and anti-inflammatory

cytokines as IL-4, IL-5 IL-13, IL-22 (Fig. 2B, C, F, I). Also, significant differences were observed for pain-mediating chemokines as C-C motif ligand (CCL) 2, CCL3, CCL4 and C-X-C ligand 2 (Fig. 2K, L, M, P). In DRGs SAFit2 overall reduced cytokine and chemokine levels after nerve injury, which were thereby largely similar to levels in sham operated animals.
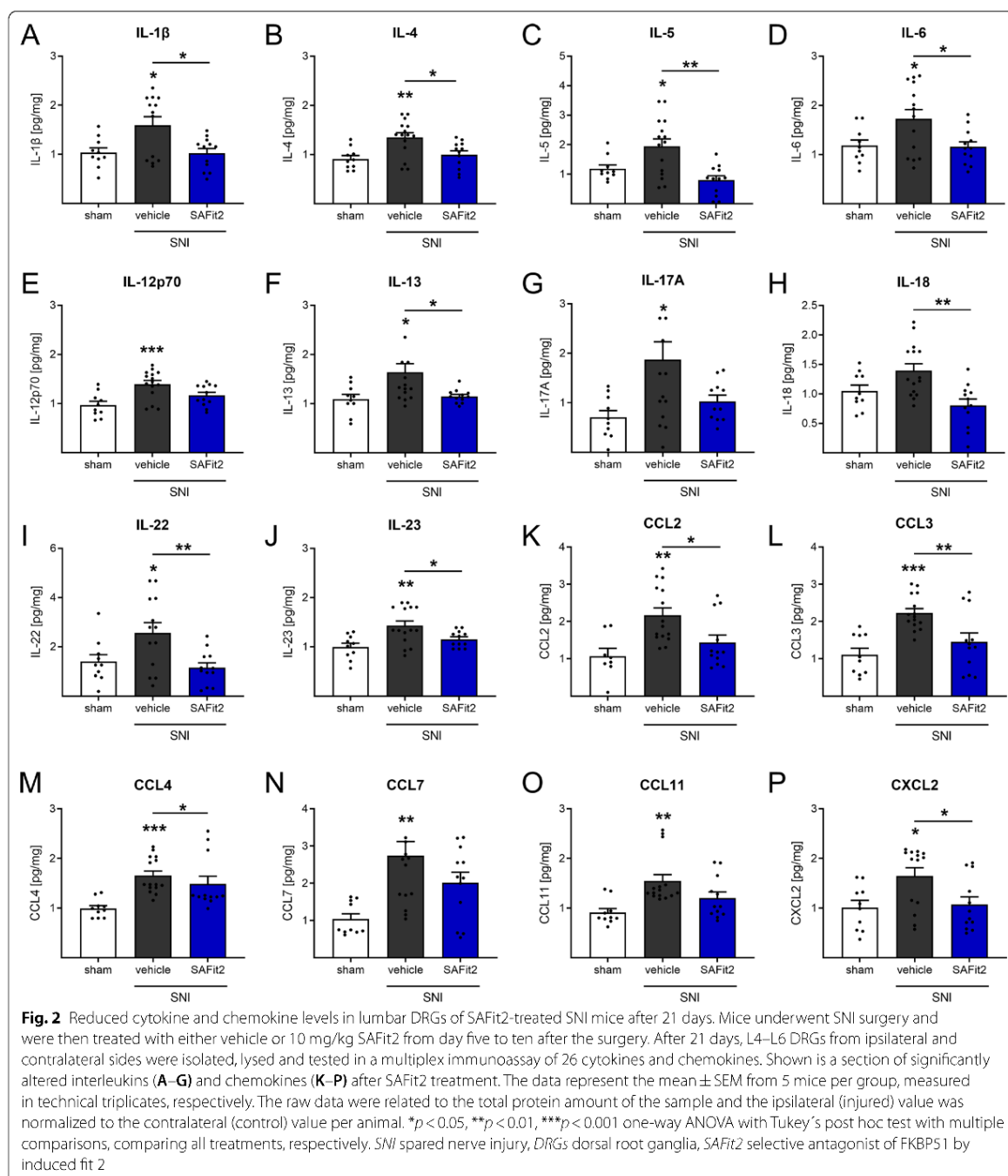
We next analyzed the concentrations of cytokines and chemokines in the dorsal spinal cord (Fig. 3). Interestingly, the analysis of spinal cord samples revealed similar trends for SAFit2 treatment as shown in the DRG measurements, including an increase of mediators after vehicle treatment which was blunted after SAFit2 treatment. However, SAFit2 reduced pain-mediating chemokines CCL2, CCL3, CCL4, CCL7, CCL11 and C-X-C ligand 2 (Fig. 3I–N) more substantially in the spinal cord than in DRG samples. In addition, we observed significant differences in the cytokines IL-2, IL-4, IL-10 and IL-18 (Fig. 3B, C, E, G) for vehicle and SAFit2-treated animals. Overall, SAFit2 treatment had a major effect on chemokine levels in the spinal cord and on cytokine levels in DRGs. In summary, these results indicate that SAFit2 significantly reduces inflammation and pain-mediating cytokine and chemokine secretion after nerve injury.

### SAFit2 in vivo treatment reduces NF-κB pathway activation after SNI

Based on the significant reduction of cytokines and chemokines in lumbar DRGs and spinal cord after SAFit2 treatment, we hypothesized that SAFit2 has an impact on the NF-κB signaling pathway and its activation, since NF-κB is one of the most relevant transcription factors being involved in the regulation of cytokine and chemokine expression. Moreover, FKBP51 has repeatedly been implicated in the NF-κB signaling pathway [17].

To investigate this hypothesis, we again performed an SNI mouse model and treated mice with either vehicle or 10 mg/kg SAFit2 (i.p.) over the same period of time as in the previous experiment. For examining the underlying mechanisms, we isolated lumbar DRGs and the respective parts of the spinal cord at day 14 after SNI surgery. This timepoint represents a phase of acute to chronic neuropathic pain with an established neuroinflammation [9, 44], being appropriate to analyze the ongoing inflammation on its peak.
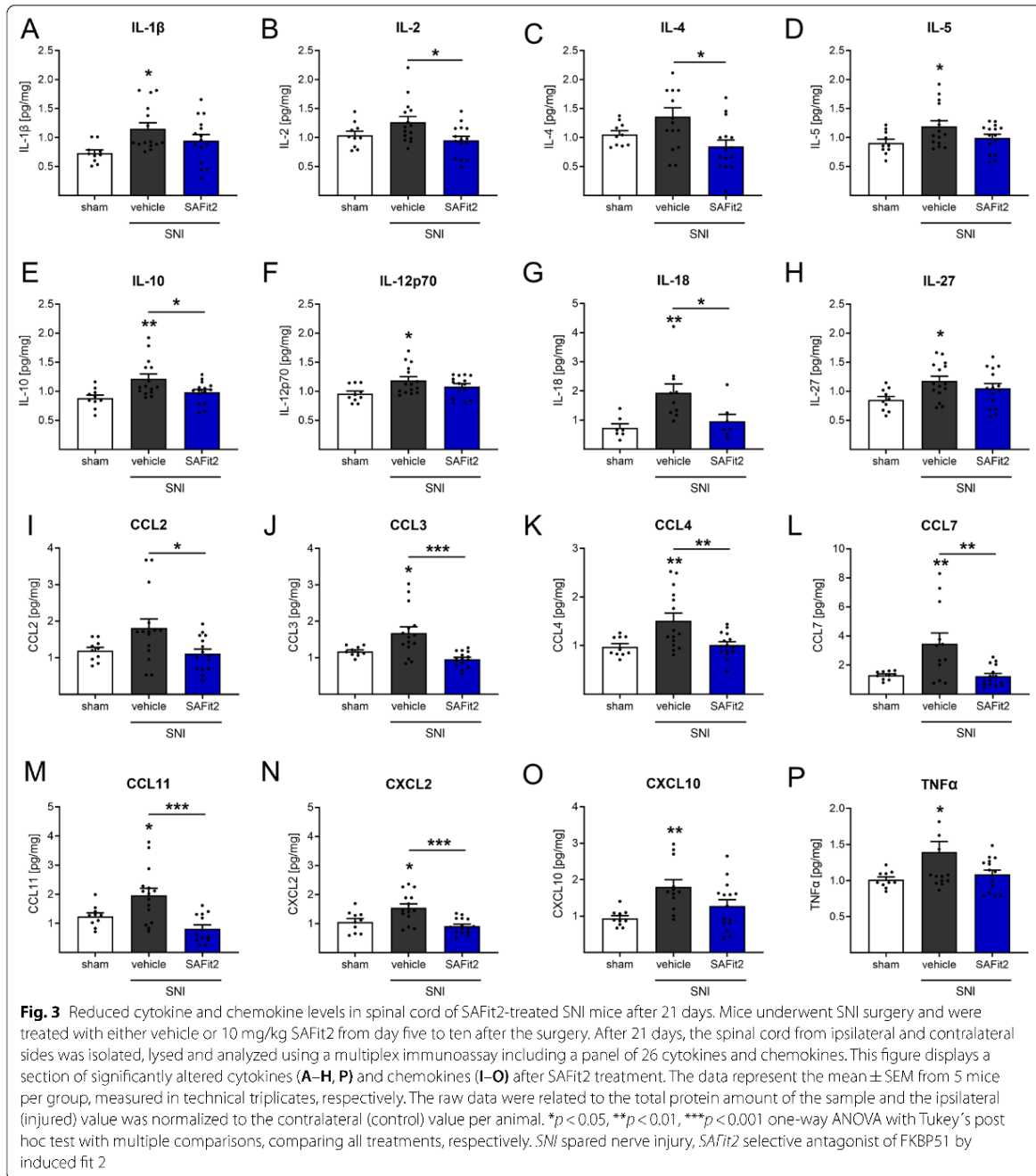
We performed Western blots with respective tissue lysates, in which we analyzed the total and the phosphorylated amount of three crucial protein members of the NF-κB signaling pathway: the inhibitor complex alpha (IκBα), p65 itself and the upstream IκBα kinase beta (IKKβ). These factors were analyzed in both DRGs and spinal cord, respectively (Fig. 4), observing that SAFit2

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**Fig. 2** Reduced cytokine and chemokine levels in lumbar DRGs of SAFit2-treated SNI mice after 21 days. Mice underwent SNI surgery and were then treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, L4–L6 DRGs from ipsilateral and contralateral sides were isolated, lysed and tested in a multiplex immunoassay of 26 cytokines and chemokines. Shown is a section of significantly altered interleukins (**A–G**) and chemokines (**K–P**) after SAFit2 treatment. The data represent the mean ± SEM from 5 mice per group, measured in technical triplicates, respectively. The raw data were related to the total protein amount of the sample and the ipsilateral (injured) value was normalized to the contralateral (control) value per animal. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ one-way ANOVA with Tukey´s post hoc test with multiple comparisons, comparing all treatments, respectively. *SNI* spared nerve injury, *DRGs* dorsal root ganglia, *SAFit2* selective antagonist of FKBP51 by induced fit 2

treatment has no influence on the total protein amount of IκBα (Fig. 4B), p65 (Fig. 4C) and IKKβ (Fig. 4D).

However, SAFit2 significantly affects the phosphorylation state of the respective factors (Fig. 4E–G). We observed that SAFit2 treatment leads to a significant decrease of IκBα phosphorylation (Fig. 4F) and p65 phosphorylation (Fig. 4G) in lumbar DRGs and spinal cord of nerve-injured mice. Based on this, we concluded

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**Fig. 3** Reduced cytokine and chemokine levels in spinal cord of SAFit2-treated SNI mice after 21 days. Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the spinal cord from ipsilateral and contralateral sides was isolated, lysed and analyzed using a multiplex immunoassay including a panel of 26 cytokines and chemokines. This figure displays a section of significantly altered cytokines (**A–H, P**) and chemokines (**I–O**) after SAFit2 treatment. The data represent the mean ± SEM from 5 mice per group, measured in technical triplicates, respectively. The raw data were related to the total protein amount of the sample and the ipsilateral (injured) value was normalized to the contralateral (control) value per animal. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ one-way ANOVA with Tukey´s post hoc test with multiple comparisons, comparing all treatments, respectively. *SNI* spared nerve injury, *SAFit2* selective antagonist of FKBP51 by induced fit 2

that SAFit2 does not change the basal expression of the respective factors, but strongly reduces the NF-κB pathway activation. Besides the NF-κB pathway, the transcription factor NFAT might also have an influence on cytokine and chemokine regulation. However, we detected no alterations in the regulation of NFAT after SAFit2 treatment (Additional file 1: Fig. S1), indicating the NF-κB pathway as primary pathway for cytokine and chemokine production after nerve injury.

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## SAFit2 treatment reduces immune cell infiltration into dorsal root ganglia and spinal cord after nerve injury

Since modulation of the NF-κB signaling pathway activation can influence immune cell migration [19, 28, 39], we next investigated the influence of SAFit2 on the immune cell distribution in neuronal tissue after nerve injury. We hypothesized that a reduced immune cell infiltration would additionally explain reduced cytokine and chemokine levels. Therefore, we performed the same in vivo experiment, an SNI surgery followed by SAFit2 treatment, and collected lumbar DRGs, the respective parts of the spinal cord and both sciatic nerves 14 days after surgery. The tissue was gently homogenized, and the subset of immune cells determined in the respective tissues using a flow cytometry analysis (Fig. 5).

Interestingly, we detected a significant reduction of all immune cells in the DRGs (CD45+, Fig. 5A) which was not the case for the inflamed sciatic nerve which is the core of inflammation (Fig. 5K). Moreover, we observed a significant reduction of dendritic cells (Fig. 5D), regulatory T cells (Fig. 5H), memory T cells (Fig. 5I) and gamma delta T cells (Fig. 5J) in the DRGs after SAFit2 treatment, which we could not observe in the sciatic nerve samples. Instead, a slight upregulation of the respective immune cells was measured in the sciatic nerve samples (Fig. 5N, R, S, T). A significant decrease of monocytes (Fig. 5B) and eosinophils (Fig. 5E) was detected in the spinal cord after SAFit2 treatment. In contrast, these immune cell types were unaltered in the sciatic nerve samples comparing vehicle and SAFit2-treated animals (Fig. 5L and O). Also, the total amount of immune cells was not altered by SAFit2 treatment in the sciatic nerve samples (CD45+, Fig. 5K). In summary, SAFit2 treatment in vivo leads to a reduced infiltration of immune cells into DRGs and spinal cord, but it does not affect the number nor the distribution of immune cells at the sciatic nerve.

## SAFit2 reduces the migration of primary murine and human macrophages

As we have seen significant changes in the number of immune cells in neuronal tissue after SAFit2 treatment and the counteracting effect of the compound on the pro-migratory NF-κB pathway, we next assessed whether SAFit2 has a direct effect on the migratory behavior of antigen presenting cells. In particular, we focused on analyzing the impact of SAFit2 on macrophage migration since macrophages constitute an essential role in neuroinflammation and central sensitization [62]. In addition, the target FKBP51 was previously suggested to be involved in promoting cell migration [32, 54].

For investigating the migration of primary macrophages, we isolated monocytes either from murine bone marrow or from human blood donations and differentiated the monocytes into primary macrophages in cell culture. Afterwards, their migratory behavior was assessed in Transwell assays during the treatment with SAFit2 (Fig. 6). Interestingly, SAFit2 significantly reduced the migration of both murine (Fig. 6B) and human macrophages (Fig. 6C) in a concentration dependent manner. However, ddSAFit2 (Additional file 1: Fig. S3), which corresponds almost to the structure of SAFit2 as chiral analogue and is instead biologically inactive, does not affect the migration of murine and human macrophages (Fig. 6B, C). As a control experiment, we analyzed the impact of SAFit2 on murine macrophage viability and metabolism to exclude any cytotoxic effect which could also reduce the total amount of migrated macrophages (Additional file 1: Fig. S4). However, SAFit2 has neither a detectable influence on cell viability nor on oxidative phosphorylation, adenosine triphosphate (ATP) synthesis and mitochondrial oxygen consumption rate (Additional file 1: Fig. S4).

As we discovered that SAFit2 reduces macrophage migration, we wanted to further investigate whether this effect is on target (i.e., mediate by FKBP51) or a potential off-target effect of the compound (Fig. 6D, E). Therefore, we again performed Transwell assays with primary murine macrophages, however pretreating the cells this time with two FKBP inhibitors from a different chemical series [26, 41]. The concentration and treatment duration for a successful inhibition were chosen based on previous intracellular FKBP51 occupancy studies [16]. These FKBP inhibitors also reduced the murine macrophage migration at low concentrations (Fig. 6D) and in a non-additive manner to SAFit2 (Fig. 6E), strongly suggesting an FKBP as relevant target for the reduction of macrophage migration. Likewise, SAFit2 shows similar effects in low micromolar concentrations after two hours of treatment.

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(See figure on next page.)

**Fig. 4** SAFit2 treatment reduces NF-κB pathway activation in SNI-treated mice after 14 days. Mice underwent SNI surgery and were then treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. Lumbar L4–L6 DRGs and spinal cord were collected from ipsilateral and contralateral sides at day 14. **A** Representative Western blot of the total protein expression of the factors IKKβ, p65 and IκBα after vehicle and SAFit2 treatment. **B–D** Quantification of the total protein expression of IκBα (**B**), p65 (**C**) and IKKβ (**D**). **E** Representative Western blot of phosphorylated IκBα and p65 after vehicle and SAFit2 treatment. **F, G** Quantification of phosphorylated IκBα (**F**) and phosphorylated p65 (**G**) levels in DRGs and spinal cord. The data represent the mean ± SEM of 3–5 Western blots measured with the tissue pooled from 5 mice per group. *$p < 0.05$ Student´s t-test with Welch´s correction. *SNI* spared nerve injury, *DRGs* dorsal root ganglia, *SC* spinal cord, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *IκBα* NF-κB inhibitor alpha, *IKKβ* IκB kinase beta

**Fig. 4** (See legend on previous page.)

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**Fig. 5** Immune cell reduction in DRGs and spinal cord of SAFit2-treated mice 14 days after SNI. Mice underwent SNI surgery and were then treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. L4–L6 DRGs, spinal cord and sciatic nerve were collected from ipsilateral and contralateral sides at day 14 after the surgery. **A** Flow cytometry analysis was performed with tissue lysates to analyze the immune cell distribution after SAFit2 treatment. **A–J** Relative immune cell amount in L4–L6 DRGs and spinal cord. **K–T** Relative immune cell amount in sciatic nerves. The data represent the mean ± SEM from 5 mice per group. *$p < 0.05$, ***$p < 0.001$ two-way ANOVA with Sidak´s post hoc test for DRGs and SC figures, *$p < 0.05$ Student´s *t*-test with Welch´s correction for sciatic nerve figures. *SNI* spared nerve injury, *DRGs* dorsal root ganglia, *SC* spinal cord, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *n.d.* not detected

## SAFit2 causes a calcineurin-dependent desensitization of the TRPV1 channel in sensory neurons which reduces the secretion of CGRP

We observed a beneficial role of SAFit2 in nerve injury-induced neuroinflammation. Neuroinflammation is on the one hand mediated by immune cells, but also on the other by an increased activity of sensory neurons and the subsequent release of pro-inflammatory mediators [6, 7]. We therefore hypothesized that SAFit2 may also have an effect on the activity of sensory neurons and subsequently on the downstream signaling that may involve processes which initiate neuroinflammation. More specifically, sensory neurons express ion channels such as the transient receptor potential cation channel subfamily V member 1 (TRPV1) which plays an important role in the development of neuropathic pain. States of hypersensitivity, as in the SNI experiments, can be associated with TRPV1 modulation. To test whether SAFit2 alters the activity of TRPV1, we dissected DRGs from naïve mice and measured the TRPV1 activity via calcium imaging.

For examining the impact of SAFit2 on TRPV1-mediated calcium flux, we established a protocol in which we stimulated TRPV1 twice: at first with capsaicin alone and second after a preincubation with SAFit2 to determine whether SAFit2 alters the channel-mediated calcium influx. The effect of the compound was verified by comparing the calcium fluxes of both stimuli.

**Fig. 6** SAFit2 reduces murine and human macrophage migration in vitro. Primary monocytes were isolated and differentiated into macrophages in vitro. The effect of SAFit2 on macrophage migration was assessed in Transwell assays in which the cells were treated either with different concentrations of SAFit2 or with ddSAFit2 for two hours. Other structurally different FKBP51 inhibitors (19$^{(S)-Me}$ [26] and cmpd 16 h [41]) were added already 24 h previous the Transwell assays and were kept in medium during the assay. **A** Representative images of stained membranes displaying reduced murine macrophage migration after SAFit2 treatment. **B** Quantification of murine macrophage migration after SAFit2 and ddSAFit2 treatment. **C** Quantification of human macrophage migration after SAFit2 and ddSAFit2 treatment. **D** Quantification of murine macrophage migration comparing SAFit2 treatment with other unspecific FKBP51 inhibitors. **E** Quantification of murine macrophage migration comparing unspecific FKBP51 inhibitor treatment with or without additional SAFit2 treatment. The data represent the mean ± SEM of 45–75 quantified images from 9 to 15 membranes per group, which were measured in three runs from 3 to 6 different biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ one-way ANOVA with Tukey´s post hoc test. *cmpd* compound, *SAFit2* selective antagonist of FKBP51 by induced fit 2

Indeed, SAFit2 treatment concentration dependently reduced the second capsaicin induced calcium influx, at 5 µM to less than half of the vehicle response (Fig. 7A, B). In contrast, the chiral analogue ddSAFit2 did not affect the capsaicin induced calcium influx (Fig. 7C, D).

To further investigate this effect of SAFit2, we performed additional control calcium imaging experiments with FKBP inhibitors from a different chemical series to verify whether this effect is FKBP dependent (Fig. 7E, F). However, none of the structurally unrelated FKBP inhibitors was capable of reducing the TRPV1 channel-mediated calcium fluxes (Fig. 7F). Since the structurally unrelated FKBP inhibitors were shown to potently occupy FKBP51 in human cells [16], we concluded that SAFit2 reduces the activity of TRPV1 in an FKBP51-independent manner.

As the TRPA1 channel is closely related to the TRPV1 channel and co-expressed in a subset of TRPV1 expressing neurons, we also examined the influence of SAFit2 on the TRPA1 channel. However, we did not observe any effect of SAFit2 on the activity of the TRPA1 channel (Additional file 1: Fig. S5).

To examine the impact of SAFit2 on the downstream signaling of TRPV1, we performed a calcitonin gene-related peptide (CGRP) assay. CGRP is a neuropeptide that is released from sensory neurons upon increasing intracellular calcium concentrations and is involved in pain mediation (Fig. 7G). Furthermore, the activation of its receptors can cause vasodilation and neurogenic inflammation [3, 15, 56]. Interestingly, we detected that SAFit2 reduces the CGRP concentrations in the supernatant of capsaicin-treated sensory neurons when they were co-treated with SAFit2 during stimulation (Fig. 7H). Based on these results, we conclude that the influence of SAFit2 on the TRPV1 channel activity also affects the downstream signaling events such as the secretion of CGRP.

Next, we wanted to determine whether SAFit2 antagonizes the TRPV1 directly. This might be problematic since TRPV1 antagonists led to severe side effects when they were tested in human volunteers [59]. Therefore, we assessed the influence of SAFit2 in a heterologous expression system using calcium imaging on TRPV1 transfected HEK-293t cells. There was no indication for TRPV1 inhibition over the large concentration range of 0.15 to 15 µM SAFit2 (Additional file 1: Fig. S6). In addition, we analyzed specifically whether SAFit2 has a direct impact on the channel activity of TRPV1 using whole-cell patch-clamp recordings. In these measurements, we demonstrated that 5 µM SAFit2 alone does not alter the amplitude of capsaicin-activated TRPV1 currents or the kinetics of the channel when added either to the intracellular or extracellular solution (Additional file 1: Fig. S7), ruling out a direct effect of SAFit2 on TRPV1 activity. In summary, SAFit2 does not inhibit the TRPV1 channel but desensitizes TRPV1 by affecting another factor that changes TRPV1 activity in sensory neurons.

As a next step, we tried to examine how the desensitization of TRPV1 is mediated. Since the TRPV1 channel´s open probability is also strongly affected by phosphorylation [34], we suggested that SAFit2 probably has an impact on the most relevant phosphatase in sensory neurons which is the protein phosphatase 3 (PP3/calcineurin). Based on this, we hypothesized that SAFit2 enhances the calcineurin-mediated dephosphorylation of TRPV1 which further leads to a reduced open probability and a reduced calcium influx. To investigate the effect of SAFit2 on calcineurin, we performed calcium imaging with sensory neurons in which we inhibited calcineurin with cyclosporine while treating the cells with SAFit2 (Fig. 8A). Interestingly, we detected that an additional cyclosporine treatment restored calcium flux to vehicle level (Fig. 8B), indicating that SAFit2 may have an effect on the phosphatase activity of calcineurin. Finally, we explored this effect in a calcineurin activation assay in which we measured the phosphate release by calcineurin in the presence of different SAFit2 concentrations. Indeed, we observed an increased phosphate release by calcineurin with increasing SAFit2 concentrations (Fig. 8D). In contrast, the inactive analogue ddSAFit2 as well as structurally unrelated FKBP inhibitor did not enhance the phosphate release by calcineurin (Fig. 8D). Based on these results, we assumed that SAFit2 desensitizes the TRPV1 channel in a calcineurin-dependent manner, which is not mediated by FKBP51, and thereby is probably capable to counteract the increased activity of sensory neurons in neuroinflammation.

## Discussion

SAFit2 has previously been shown to pass the blood–brain barrier [13]. Based on these results, it is quite likely that SAFit2 can also pass the blood spinal cord barrier and may partly mediate its analgesic effect in the central nervous system. As depicted in Fig. 9, we detected that SAFit2 reduces nerve injury-induced mechanical hypersensitivity in vivo by reducing neuroinflammation in neuronal tissue after SNI. On the one hand, SAFit2 has a direct effect on sensory neurons, particularly on the pain-mediating TRPV1 channel, on the other hand it reduces inflammatory processes. Thereby, SAFit2 diminishes enhanced neuronal activity and excessive neuroinflammation that can lead to central sensitization and to enhanced mechanical hypersensitivity. In detail, we showed that SAFit2 enhances the activity of the most relevant phosphatase in sensory neurons which is calcineurin. As a consequence, the phosphorylation state of the pain-mediating TRPV1 channel is decreased, and its open probability reduced. This in turn decreases the

(See figure on next page.)

**Fig. 7** SAFit2 desensitizes the TRPV1 channel and reduces the CGRP secretion in primary sensory neurons. Primary sensory neurons were isolated from mice and the effect of SAFit2 on the TRPV1 channel activity was assessed in calcium imaging experiments. **A, C, E** Representative traces of calcium influxes in sensory neurons, which were pre-incubated with 5 µM SAFit2 (**A**), 1 µM ddSAFit2 (**C**) or 1 µM structurally unrelated FKBP inhibitors (**E**) for 2 min and stimulated with capsaicin (100 nM, 30 s) afterwards and KCl (50 mM) as a positive control for neuronal response at the end of each experiment. **B, D, F** Quantification of the treated calcium response related to the untreated calcium response. **G** Schematic illustration of the release of CGRP from sensory neurons after capsaicin treatment. The scheme was created with images from motifolio. **H** Quantification of the amount of CGRP in the supernatant of treated DRG cultures. **B, D, F** The data represent the mean $\pm$ SEM of 25–49 sensory neurons per group. **H** The data represent the mean $\pm$ SEM of $n = 4$–5 per group $*p < 0.05$, $** p \, 0.01$, one-way ANOVA with Tukey´s post hoc test. *TRPV1* transient receptor potential cation channel subfamily V member 1, *CGRP* calcitonin gene-related peptide, SAFit2: selective antagonist of FKBP51 by induced fit 2, *cap* capsaicin, *KCl* potassium chloride

**Fig. 7** (See legend on previous page.)
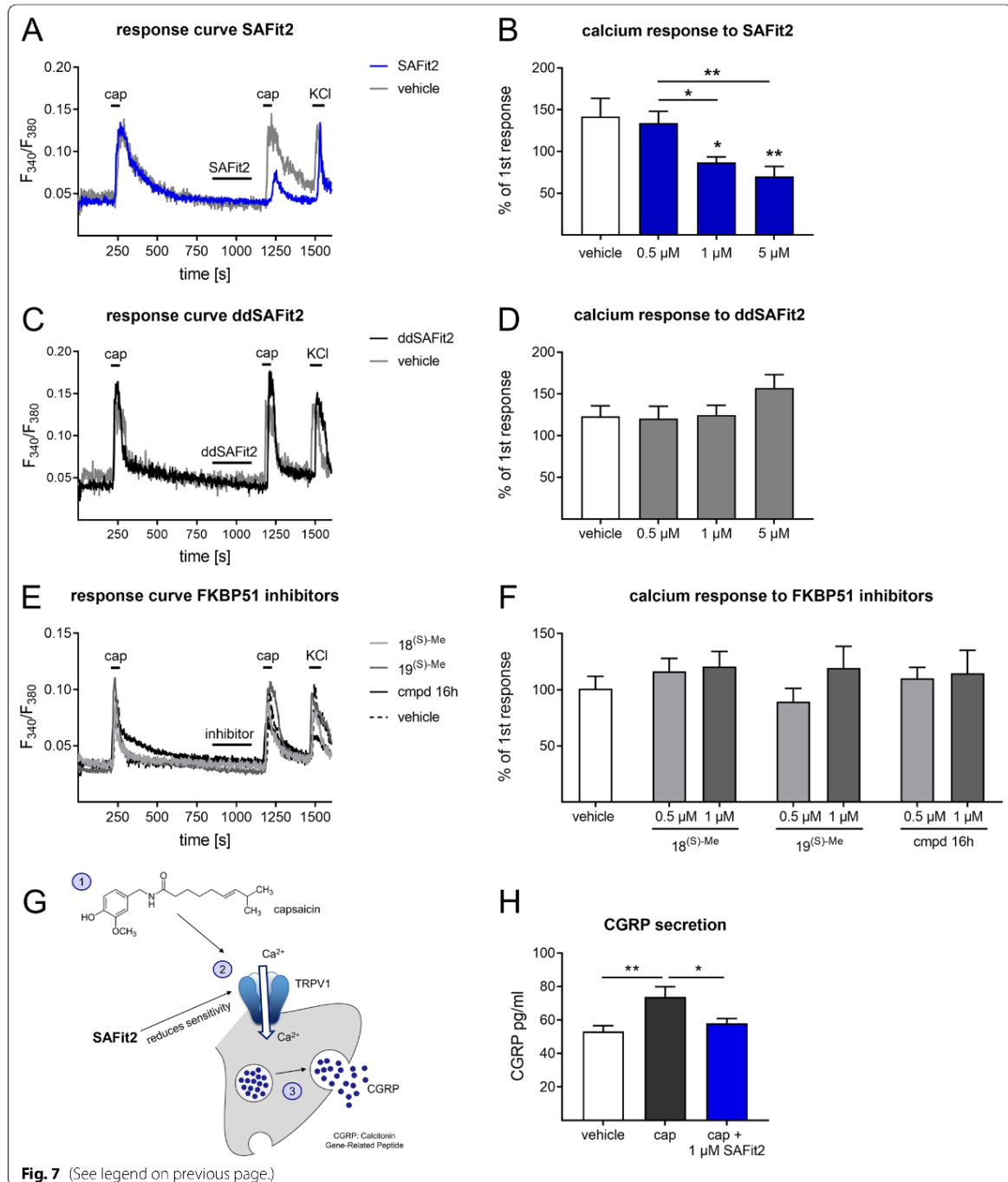
calcium influx and subsequently the transmitter release, including the release of the pro-inflammatory neuropeptide CGRP, leading to a reduction of exacerbated pain transmission (Fig. 9).

Moreover, we observed that SAFit2 ameliorates neuroinflammation in nerve-injured mice by reducing the infiltration of immune cells into neuronal tissue without affecting the inflammation resolution at the site of
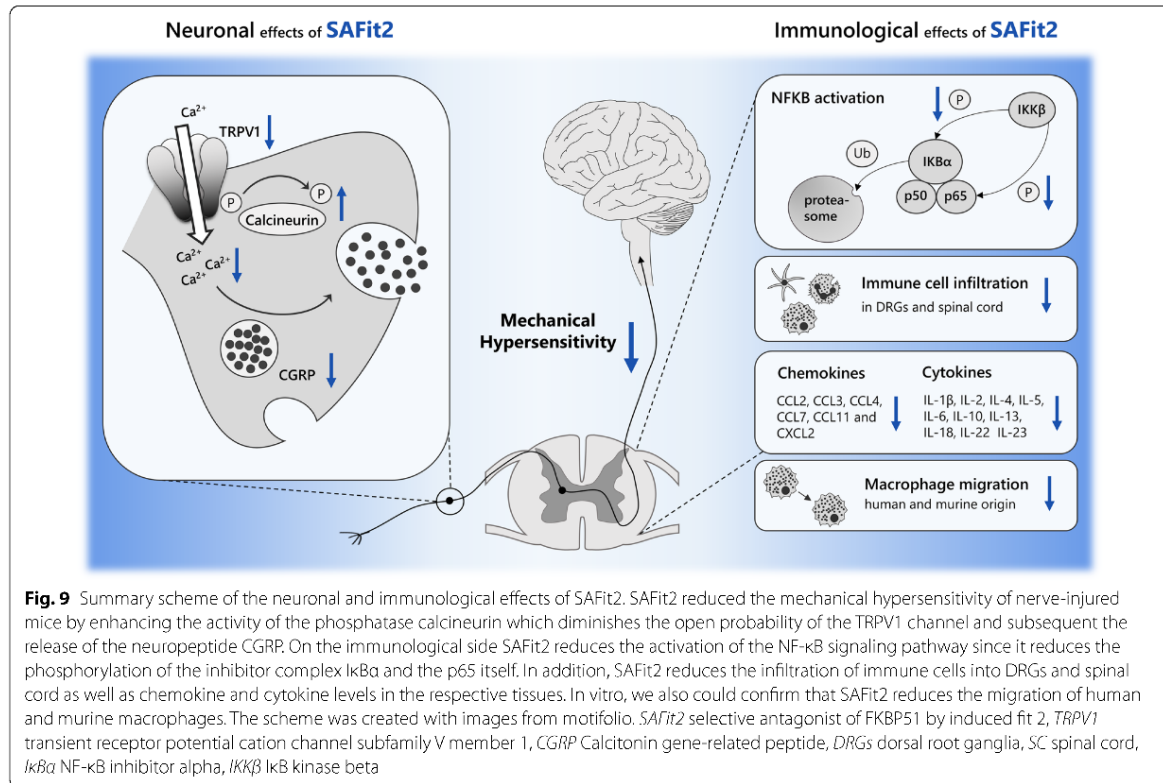
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**Fig. 8** SAFit2 enhances the activity of the phosphatase calcineurin in primary sensory neurons. **A** Representative traces of calcium influxes in sensory neurons, which were pre-incubated with SAFit2 and cyclosporine (200 nM, 2 min) and stimulated with capsaicin (100 nM, 30 s) afterwards and KCl (50 mM) as a positive control for neuronal response at the end of each experiment. **B** Quantification of the calcium response with treatment related to the calcium response without treatment and comparison of SAFit2 treatment alone to SAFit2 and cyclosporine treatment. **C** Schematic illustration of the phosphatase calcineurin dephosphorylating the TRPV1 channel. The scheme was created with images from motifolio. **D** Quantification of the phosphate release after SAFit2 treatment in a calcineurin activation assay. **B** The data represent the mean ± SEM of 25–35 sensory neurons per group. *$p < 0.05$, **$p < 0.01$ one-way ANOVA with Tukey´s post hoc test, $^{a}p < 0.05$, $^{aa}p < 0.01$ Student´s t-test with Welch´s correction. **D** The data represent the mean ± SEM of $n = 3$–11 per group. ***$p < 0.001$ one-way ANOVA with Sidak´s post hoc test. *TRPV1* transient receptor potential cation channel subfamily V member 1, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *cap* capsaicin, *cyclo* cyclosporine, *KCl* potassium chloride

injury. This effect was further confirmed by the reduction of murine and human macrophage migration in vitro, pointing out translational potential to the human disease state. In addition, we detected that SAFit2 reduces the activation of the NF-κB signaling pathway. Both effects of SAFit2 reduced cytokine and chemokine levels in DRGs and spinal cord that contribute to neurogenic inflammation and pain transmission. In summary, SAFit2 reduces neuroinflammation and ameliorates nerve injury-induced mechanical hypersensitivity (Fig. 9).

Interestingly, we observed that SAFit2 showed its anti-hyperalgesic effects after the treatment phase. We therefore assume that SAFit2 interferes rather in the maintenance of neuropathic pain than in its onset, and that it takes a certain time until its cellular and molecular effects are reflected in behavioral changes.

This raises the question whether SAFit2 may serve as preventative or protective therapy for nerve injury-indued neuropathic pain. Mechanistically, we would argue that SAFit2 is a treatment option after injury rather

than a protective/preventative therapeutic, because for the observed effects of SAFIt2 (reduction of nociceptive input and reduction of immune cell migration and mediator secretion) a previous injury is required for their initiation. It seems that SAFIt2 reduces these effects, but does not completely stop them. Therefore, we presume that SAFit2 can reduce these injury-related effects but might not be able to prevent them. However, this is speculation and should be addressed by future studies.

Neuroinflammation comprises the activation of resident immune cells, such as microglia and astrocytes, as well as migration of neutrophils, macrophages and T cells. Moreover, the secretion of pro-inflammatory mediators is a hallmark of neuroinflammation. These mediators can be cytokines, chemokines and growth factors and lead to central sensitization and an increased activity of peripheral sensory neurons, attributing these mechanisms an essential role in the onset and maintenance of neuropathic pain [21]. In this study, we detected that SAFit2 treatment can reduce the number of immune

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**Fig. 9** Summary scheme of the neuronal and immunological effects of SAFit2. SAFit2 reduced the mechanical hypersensitivity of nerve-injured mice by enhancing the activity of the phosphatase calcineurin which diminishes the open probability of the TRPV1 channel and subsequent the release of the neuropeptide CGRP. On the immunological side SAFit2 reduces the activation of the NF-κB signaling pathway since it reduces the phosphorylation of the inhibitor complex IκBα and the p65 itself. In addition, SAFit2 reduces the infiltration of immune cells into DRGs and spinal cord as well as chemokine and cytokine levels in the respective tissues. In vitro, we also could confirm that SAFit2 reduces the migration of human and murine macrophages. The scheme was created with images from motifolio. *SAFit2* selective antagonist of FKBP51 by induced fit 2, *TRPV1* transient receptor potential cation channel subfamily V member 1, *CGRP* Calcitonin gene-related peptide, *DRGs* dorsal root ganglia, *SC* spinal cord, *IκBα* NF-κB inhibitor alpha, *IKKβ* IκB kinase beta

cells in the peripheral nervous system and at the interface between the peripheral and central nervous system. Likewise, the concentrations of pro-inflammatory and proalgesic mediators is reduced in the respective tissues compared to tissues from vehicle-treated mice. SAFit2 treatment also caused a reduction of anti-inflammatory cytokines (IL-4, IL-5 IL-13, IL-22) in DRGs and the spinal cord. These anti-inflammatory cytokines are mainly produced by T helper cells, such as Th2 and Th17 cells [10]. Although we did not directly measure the amount of Th cells by FACS, we see reduced amounts of other T cell species especially in the DRGs following SAFit2 treatment (Treg, Tm, Tγδ). Based on these data, we presume that T-cell migration to the DRGs is reduced after SAFit2 treatment, including Th2 and Th17 cells which leads to the observed decrease of IL-4, IL-5, IL13 and IL-22 concentrations. According to our observations, this is mediated by a reduced activation of the NF-κB signaling pathway in SAFit2-treated mice. The interaction between FKBP51 and the NF-κB pathway has previously been suggested in the context of steroid-refractory inflammation and cancer biology [24, 45] which is in line with our observations. In more detail, we mainly see strong influences of SAFit2 treatment on the phosphorylation state

of p65 and IκBα, which are both central components of the NF-κB pathway.

We started the SAFit2 treatment 5 days after the SNI surgery, a time point when glial cell activation and neuroinflammation have already been initiated [53]. This might be the reason why the mechanical hypersensitivity of the SNI-treated animals is not completely restored to baseline levels and can explain why we still observe immune cells in DRGs and spinal cord, although significantly lower in the DRGs of SAFit2-treated animals. In addition, the difference of immune cell quantity is particularly strong between DRGs from vehicle and SAFit2-treated animals. We also observe a strong reduction of dendritic cells in DRG tissue after SAFit2 treatment. Recently, it was shown that dendritic cells release chemokines which cause an increased activity of peripheral sensory neurons via the activation of the C-C chemokine receptor 4 [48]. This is in line with our observation that SAFit2-treated animals show fewer dendritic cells and reduced levels of proalgesic mediators in DRGs.

In the spinal cord, the total number of immune cells is similar in SAFit2 and vehicle-treated mice, probably because the activated microglia and astrocytes in the spinal cord are resident immune cells, whereas infiltration

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of migrating immune cells occurs much stronger in the DRGs during persistent pain [42, 62]. However, we detected less eosinophils infiltrating into the spinal cord after SAFit2 treatment. Eosinophils are well known to induce several diseases like eosinophilia, eosinophilic vasculitis and many others when they are infiltrating into tissues [5, 35, 55]. Moreover, these cells were also associated with peripheral neuropathy and have been attributed effects on sensory nerve branching [36].

Apart from the immune cell component, neuroinflammation can also be initiated by increased neuronal activity and the subsequent release of neuropeptides, such as calcitonin gene-related peptide (CGRP) which serves as chemoattractant for immune cells [6, 7]. While most anti-inflammatory agents can target the immune cell component of neuroinflammation, they are generally not capable of addressing the neuronal component. However, we observed that SAFit2 causes a desensitization of the neuronal TRPV1 channel that seems to be mediated by a calcineurin-dependent dephosphorylation.

While TRPV1 represents an interesting target in persistent pain, its complex regulatory functions and its requirement for normal maintenance of the body temperature has made it difficult to target and even more than 20 years after its initial discovery, there is still no TRPV1 antagonist available for clinical use [23, 25, 59]. It may thus be more promising to keep TRPV1 activity in the physiological range and reduce its sensitization that occurs in pathophysiological pain states. In this regard, the desensitizing effect of SAFit2 could be an additional beneficial effect of the compound to ameliorate persistent pain. Subsequently, the release of the neuropeptide CGRP triggered by an enhanced TRPV1 channel activation can also be diminished by SAFit2 in cultured sensory neurons, which shows that SAFit2 can reduce neuroinflammation both at the immune cell level, but also at the level of sensory neurons and neuropeptide release.

A limitation of this study is the lack of female mice in the behavioral assays. Previously, it was reported that sex differences occur in development and maintenance of neuropathic pain in vivo [33, 51]. Although the contribution of FKBP51 to neuropathic pain has been found to be sex independent [30], it is conceivable that the effects of SAFit2 during neuropathic pain and in neuroinflammation differ between male and female mice.

Besides the influences of the drug target FKBP51 in the peripheral nervous system, it was previously also revealed as a target for central nervous system (CNS) indications and psychological disorders [46]. In particular, a strong genetic association was validated for FKBP51 in stress-related and endocrinologic mediated diseases as depression, type two diabetes and obesity [46]. Moreover, inhibiting FKBP51 recently revealed an improvement of anxiety related [18] as well as of stress-related disorders [40].

In the context of chronic pain, the target FKBP51 was already shown to be upregulated in spinal cord and DRGs, whereas the knockout of FKBP51 was shown to relieve pain in a chronic ankle joint inflammation model [31]. Moreover, the knockdown of FKBP51 was also shown to ameliorate neuropathic pain after chronic constriction injury [61], revealing FKBP51 as a promising therapeutically target for a broad range of disorders ranging from psychological disorders to chronic pain. However, the development of a possible drug candidate was hampered in the past, due to the lack of a specific and potent FKBP51 inhibitor, highlighting SAFit2 as a novel and promising treatment option for neuropathic pain.

## Abbreviations

BMDM: Bone marrow-derived macrophages; CCL: C-C motif ligand; CGRP: Calcitonin gene-related peptide; CXCL: C-X-C motif ligand; DRGs: Dorsal root ganglia; FKBP51: FK506-binding protein 51; IκBα: NF-κB inhibitor alpha; IKKβ: IKBα kinase beta; IL: Interleukin; SAFit2: Selective antagonist of FKBP51 by induced fit; SC: Spinal cord; SNI: Spared nerve injury; TRP: Transient receptor potential; TRPV1: Transient receptor potential cation channel subfamily V member 1.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02615-7.

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**Additional file 1: Figure S1.** Gene expression of neuronal stress and oxidative stress markers in lumbar DRGs and spinal cord of SAFit2 treated mice 21 days after SNI. **Figure S2.** Cytokine and chemokine levels in lumbar DRGs and spinal cord of SAFit2 treated SNI mice after 21 days. **Figure S3.** Synthesis of ddSAFit2 and competitive fluorescence assay (FPA) of SAFit2 and ddSAFit2. **Figure S4.** Cytotoxic and metabolic influence of SAFit2 on primary bone marrow derived macrophages. **Figure S5.** SAFit2 has no impact on the TRPA1 activity in primary sensory neurons. **Figure S6.** SAFit2 has no direct impact on the human hTRPV1 channel in HEK 293t cells. **Figure S7.** Extracellularly or intracellularly administered SAFit2 has no effect on the amplitude and kinetics of capsaicin activated TRPV1 currents in HEK-293 cells. **Figure S8.** Uncropped Western Blot images for NF-κB signaling pathway.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

# Declarations

## Ethics approval and consent to participate

All experiments involving animals were approved by the local Ethics Committee for Animal Research (Darmstadt) under the permit number F152/1021. Furthermore, all animal experiments were performed according to the recommendations of the Preclinical Pain Research Consortium for Investigating Safety and Efficacy (PPRECISE) Working Group [1], according to the updated ARRIVE guidelines for reporting animal research [38] and guidelines as defined by European Quality In Preclinical Data (EQIPD) [4]. All efforts were made to minimize animal suffering.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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**Publisher's Note**

## 2.3 Supplemental file

# Additional file 1

## SAFit2 reduces neuroinflammation and ameliorates nerve injury-induced neuropathic pain

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Figures: 8
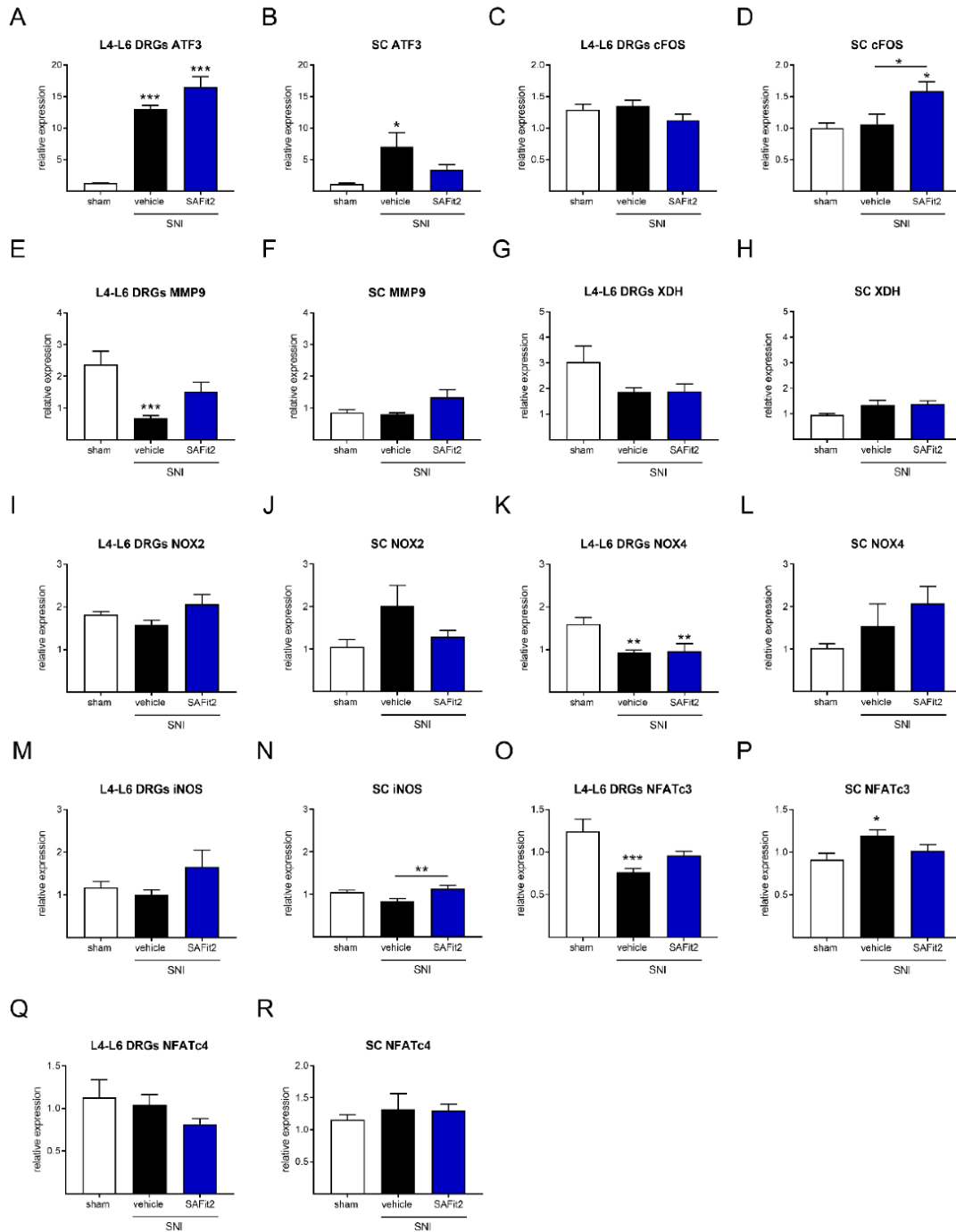
**Figure S1: Gene expression of neuronal stress and oxidative stress markers in lumbar DRGs and spinal cord of SAFit2 treated mice 21 days after SNI.** Mice underwent a SNI surgery and were then treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the expression of neuronal stress

markers: ATF3 **(A,B)**, cFOS **(C,D)**, MMP9 **(E,F)**, oxidative stress markers: XDH **(G,H)**, NOX2 **(I,J)**, NOX4 **(K,L)**, iNOS **(M,N)** and NFAT subunits: NFATc3 and NFATc4 **(O-R)** was measured. The data represents the mean ± SEM of 3-4 mice per group, measured in technical triplicates respectively. $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$ one-way ANOVA with Tukey´s post hoc test. Abbreviations: SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, ATF3: activating transcription factor 3, MMP9: matrix metallopeptidase 9, XDH: xanthine dehydrogenase, NOX2/4: NADPH oxidase 2/4, iNOS: inducible nitric oxide synthase, NFAT: nuclear factor of activated T cells
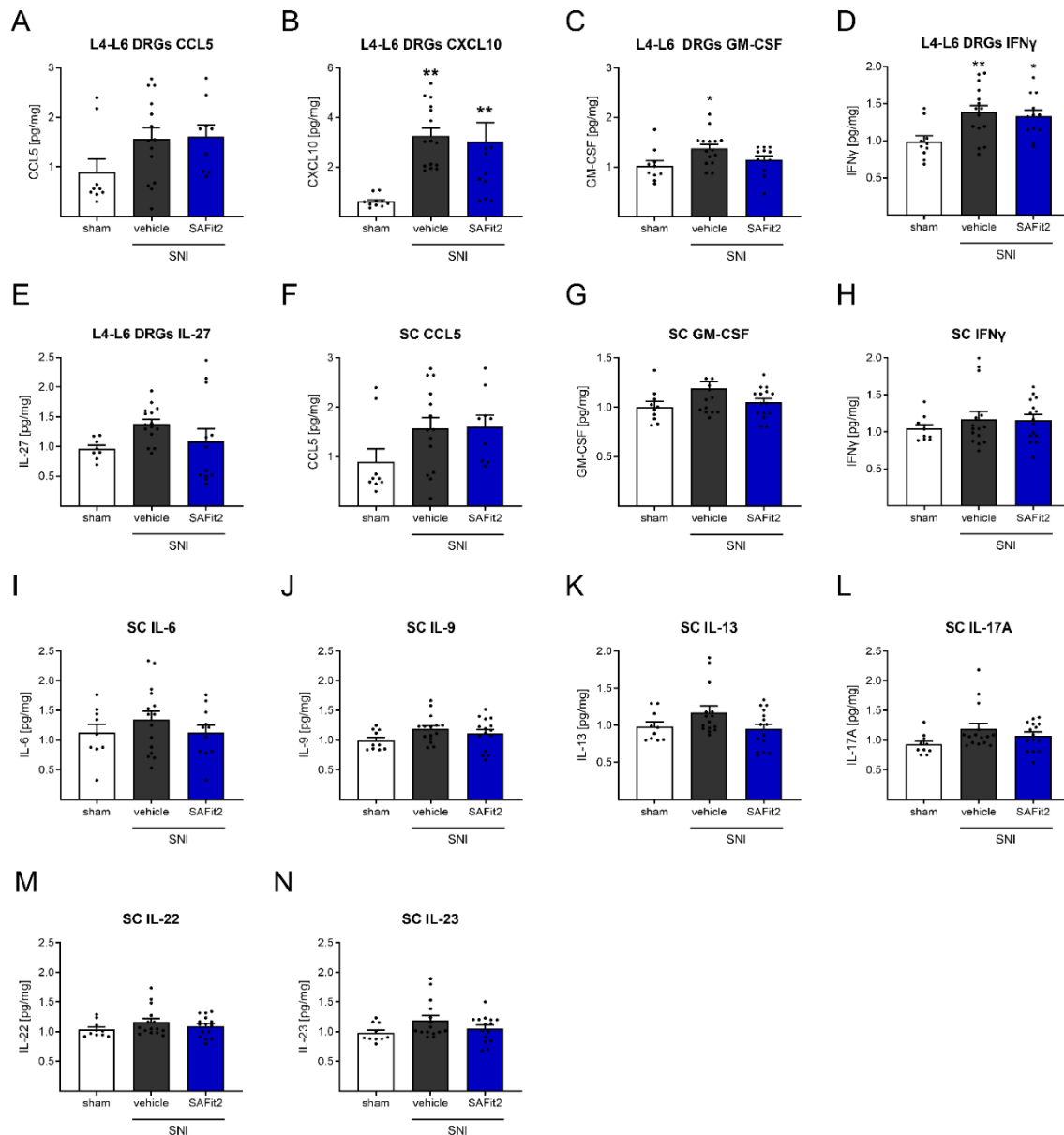
**Figure S2: Cytokine and chemokine levels in lumbar DRGs and spinal cord of SAFit2 treated SNI mice after 21 days.** Mice underwent SNI surgery and were then treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, L4-L6 DRGs and spinal cord were isolated from ipsilateral and contralateral sides with which lysates a multiplex immunoassay was performed including a panel of 26 cytokines and chemokines. Shown is a section of mediators measured in L4-L6 DRGs **(A-E)** and spinal cord **(F-N)** after SAFit2 treatment. The data

represents the mean ± SEM from 5 mice per group, measured in technical triplicates respectively. The raw data was related to the total protein amount of the sample and the ipsilateral (injured) value was normalized to the contralateral (control) value per animal. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with Tukey's post hoc test. Abbreviations: SNI: spared nerve injury, DRGs: dorsal root ganglia, SC: spinal cord, SAFit2: selective antagonist of FKBP51 by induced fit 2

A

B

no protein
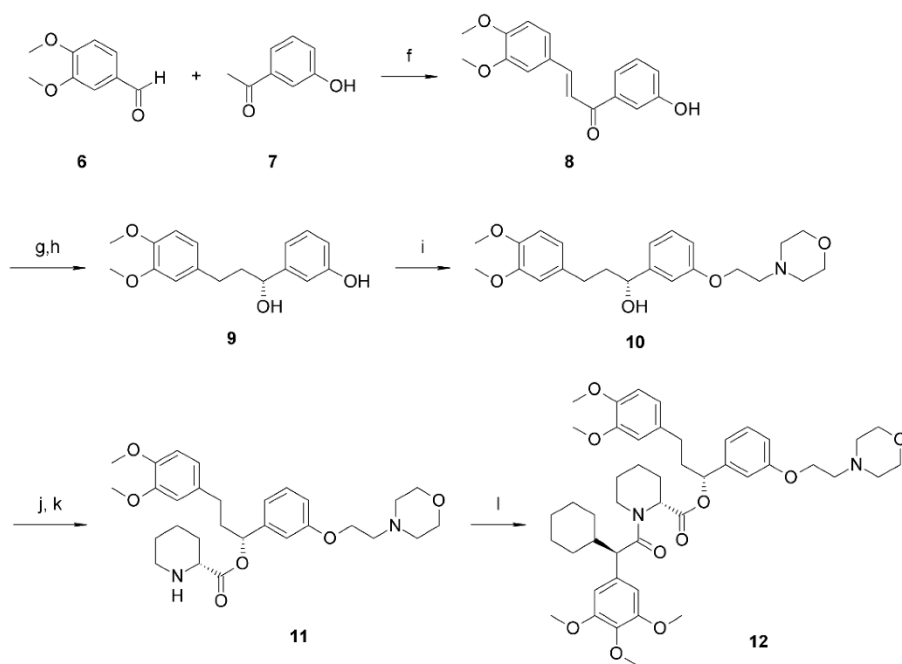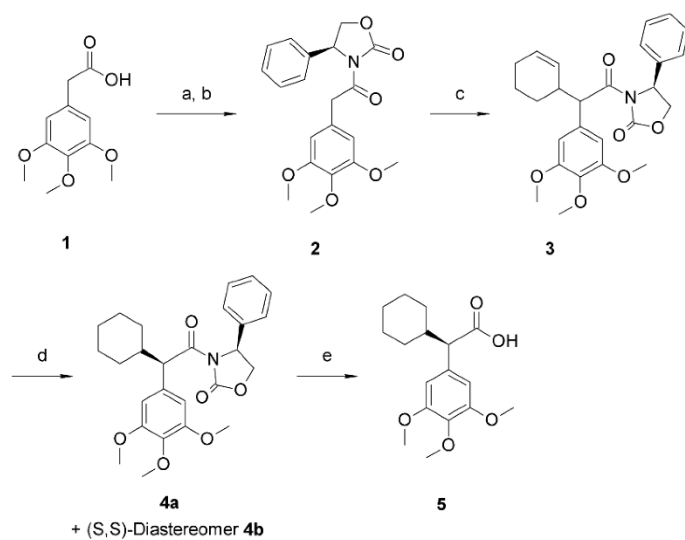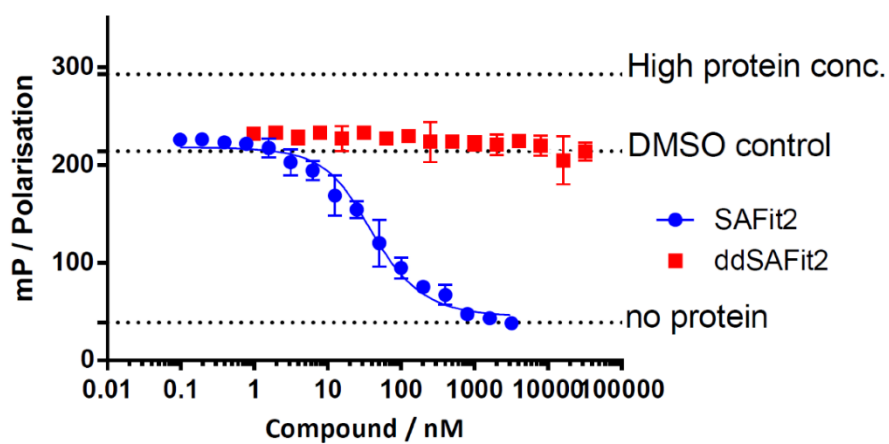
**Figure S3: Synthesis of ddSAFit 12 and competitive fluorescence assay (FPA) of SAFit2 and ddSAFit2. (A)**. **a)** Pentafluorphenol, EDC · HCl, DMAP, DCM 0 °C, 74% yield, **b)** (S)-4-phenyloxazolidin-2-one, n-BuLi, THF,−78 °C, 76% yield, **c)** LiHMDS, 3-bromo cyclohexene, THF -78 °C, 74% yield, **d)** 1 bar $H_2$, Pd/C, MeOH, 19% of 4a and 70% of the diastereomer 4b, **e)** LiOH/$H_2O_2$, THF/$H_2O$ 8:5, 0 °C, 69% yield, **f)** KOH, $H_2O$/EtOH, 0 °C, 99% yield, g) Zn/$NH_4$Ac, MeOH, r.t., 84% yield, **h)** atmospheric $H_2$, KOtBu, Noyori catalyst, IPA, r.t., 95%, **i)** $K_2CO_3$, 2-chloroethylmorpholine hydrochloride, MeCN, reflux, 78% yield, **j)** EDC · HCl, DMAP, R)-1-(tert-butoxycarbonyl)piperidine-2-carboxylic acid, DCM, 0 °C, 78% yield, **k)** DCM/TFA 2:1, r.t., quant. yield, **l)** HATU, 4a, DiPEA, DCM/DMF, r.t., 78% yield. **(B)** Competitive fluorescence polarization assay (FPA) of SAFit2 (blue dots) and ddSAFit2 (red squares), high protein control, DMSO control, and no protein control are indicated by a tick mark and dashed lines. Fitting the curve results in a $K_d$ of 8 nM for SAFit2 and > 80000 nM for ddSAFit2. FPA was performed as described previously (Bauder et al., 2021 (https://doi.org/10.1021/acs.jmedchem.0c02195).
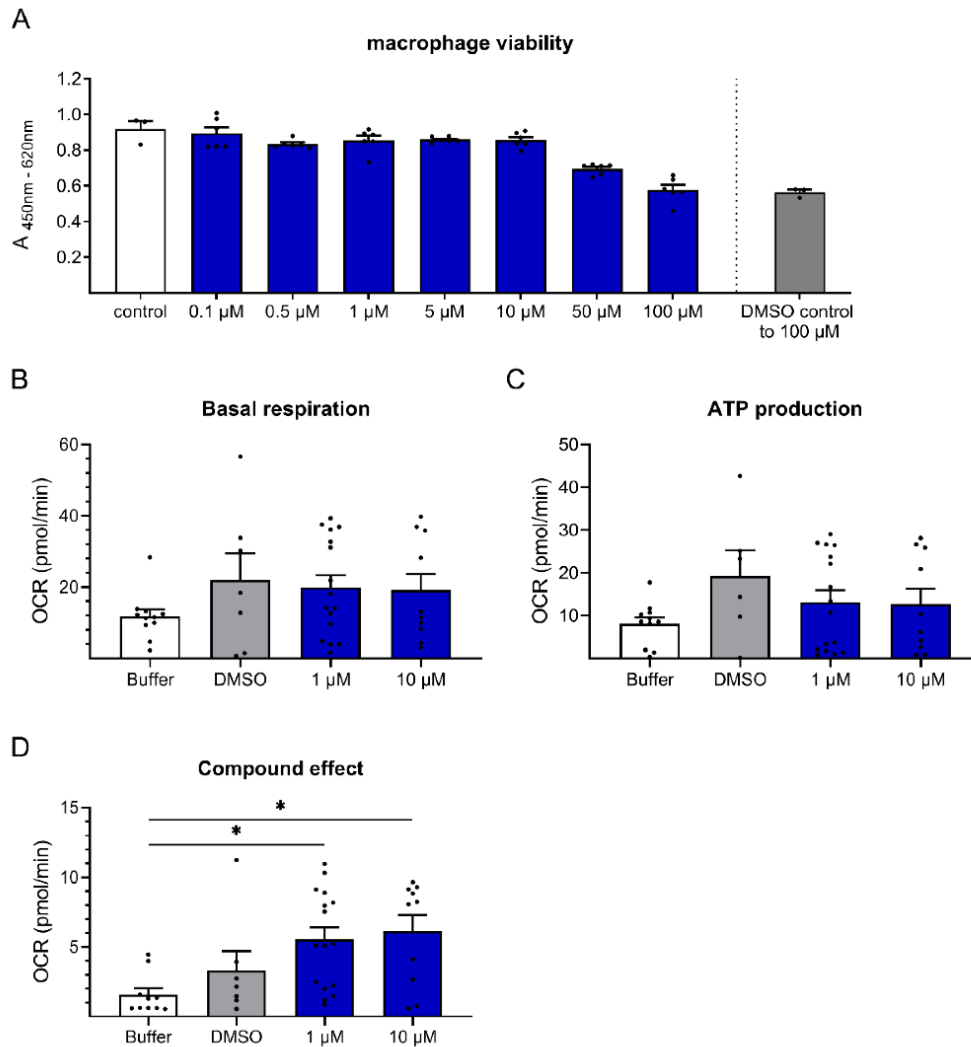
**Figure S4: Cytotoxic and metabolic influence of SAFit2 on primary bone marrow derived macrophages. (A)** The effect of SAFit2 on the macrophage viability was assessed in a WST-1 assay showing that SAFit2 has no influence on the cell viability. The data represents the mean ± SEM of 3-6 per group **(B-D)** The influence of SAFit2 the macrophage metabolism was analyzed with a Seahorse bioanalyzer measuring the oxygen consumption rate (OCR). SAFit2 affects neither the basal respiration **(B)** nor the ATP production **(C)** of macrophages. It slightly increases the total respiration of macrophages compared to the buffer control, which is negligible since the DMSO control is also slightly increased. **(D).** The data represents the mean ± SEM of 1-2 runs

per group, measured in several technical replicates respectively. * p < 0.05 one-way

ANOVA with Tukey´s post hoc test.
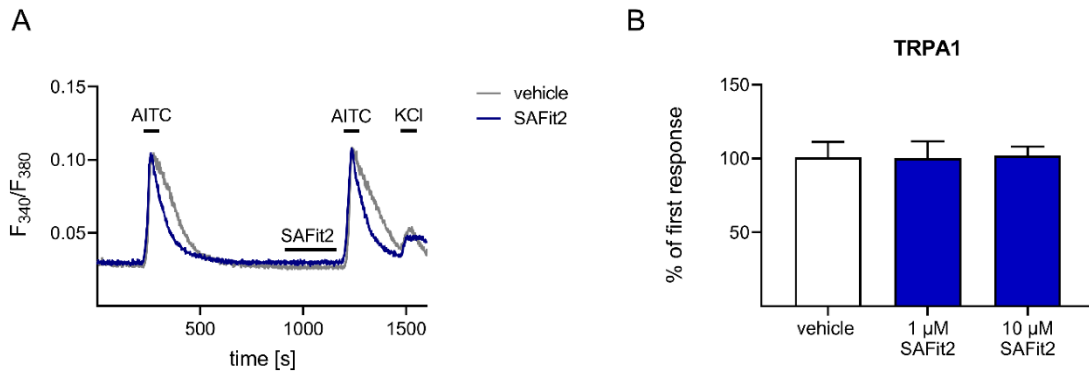
**Figure S5: SAFit2 has no impact on the TRPA1 activity in primary sensory neurons.** Primary sensory neurons were isolated from mice and the effect of SAFit2 on the TRPA1 channel activity was assessed in calcium imaging experiments. **(A)** Representative traces of TRPA1 calcium fluxes from sensory neurons, which were pre-incubated with SAFit2 for 2 minutes and stimulated with the gold standard TRPA1 agonist allyl isothiocyanate (AITC) (100 µM, 45 s) afterwards and KCl (50 mM) as a positive control for neuronal response at the end of each experiment. **(B)** Quantification of the treated calcium response (second stimulus) related to the untreated calcium response (first stimulus). The data represents the mean ± SEM of 37-51 sensory neurons per group.
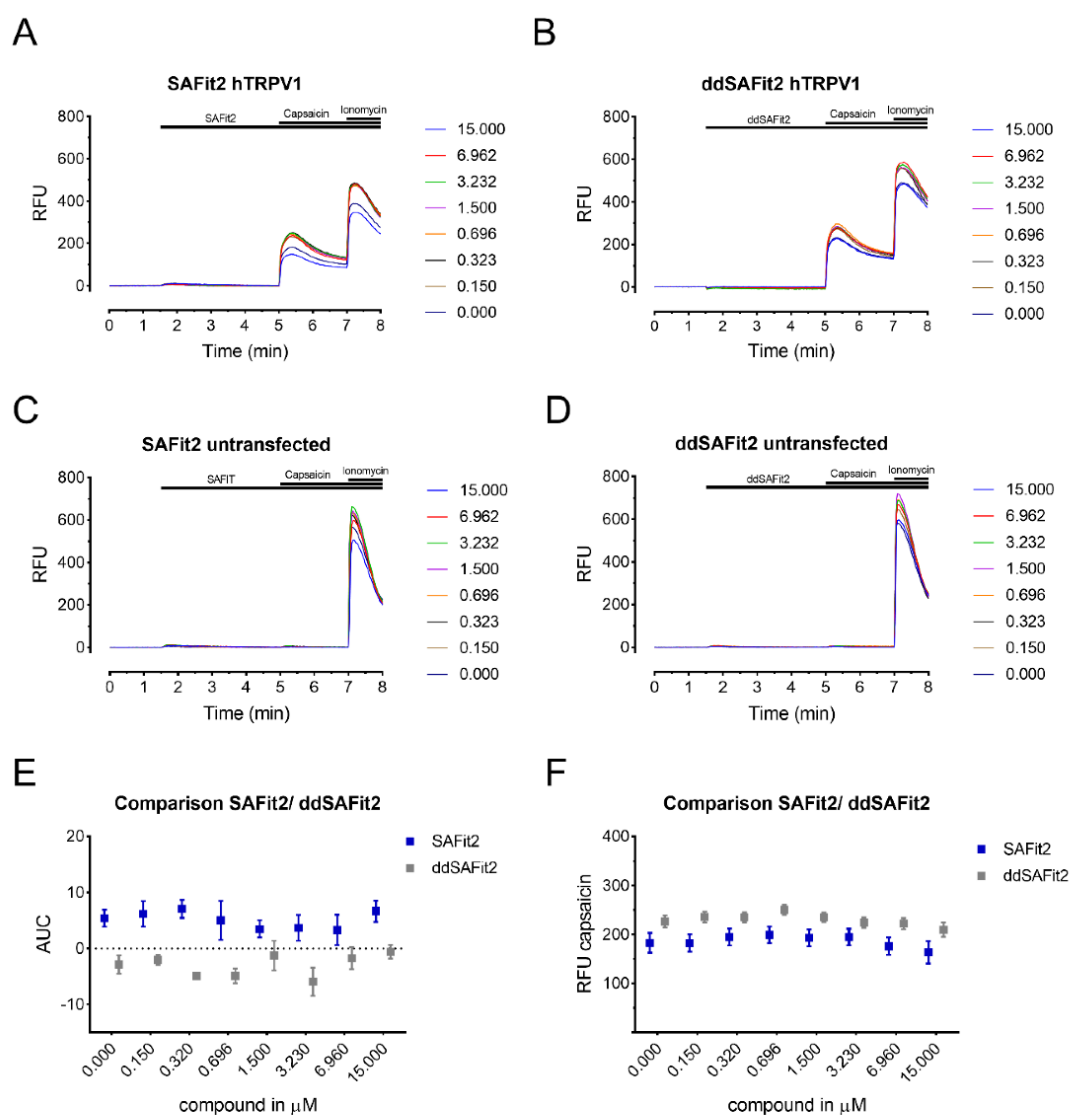
**Figure S6: SAFit2 has no direct impact on the human hTRPV1 channel in HEK-293t cells. (A, B)** Mean relative fluorescence intensity (RFU) traces of hTRPV1 transfected HEK-293t cells after the treatment with up to 15 µM SAFit2 **(A)** and ddSAFit2 **(B)**, followed by a stimulation with 200 nM capsaicin and 2 µM ionomycin as a positive control. **(C, D)** Mean relative fluorescence intensity (RFU) traces of untransfected HEK-293t cells after the treatment with up to 15 µM SAFit2 **(C)** and ddSAFit2 **(D)**, followed by a stimulation with 200 nM capsaicin and a positive control with 2 µM ionomycin. **(E)** Shown is the comparison of the total area under the curve (AUC) values of SAFit2 and ddSAFit2 for respective concentrations. **(F)** Shown is the

calculated RFU with its error for capsaicin stimulations after SAFit2 and ddSAFit2 treatment with respective concentrations. The data represents the mean ± SD of 3 measurements.
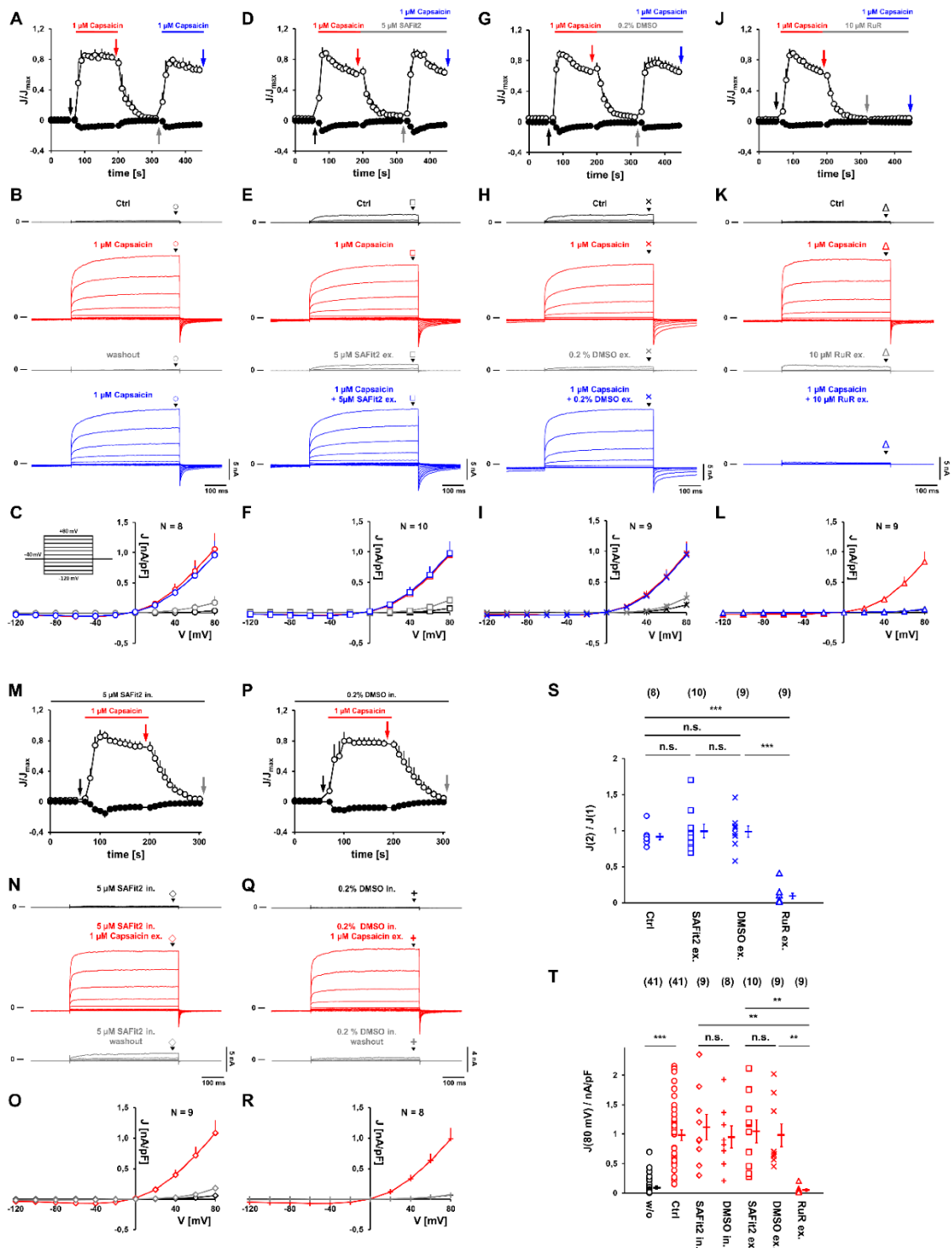
Figure S7: Extracellularly or intracellularly administered SAFit2 has no effect on the amplitude and kinetics of capsaicin-activated TRPV1 currents in HEK-293 cells. Whole-cell patch-clamp experiments were performed on HEK-293 cells transiently expressing TRPV1-GFP. TRPV1 currents were activated by addition of

1 µM capsaicin to the bath solution. **(A-L)** To determine the effect of extracellularly added compounds on TRPV1 activation, capsaicin was applied twice for two minutes each: the first application was used to determine TRPV1 activity in the absence, the second in the presence of the compound. The compound was administered during washout after the first capsaicin application by perfusion with bath solution. The following compounds were tested: no compound **(A-C)**, SAFit2 **(D-F)**, DMSO (vehicle control) **(G-I)**, and Ruthenium Red (RuR, positive control) **(J-K)**. **(A,D,G,J)** Time courses of normalized current densities at -60 mV (closed circles) and +60 mV (open circles) as obtained by repeatedly applying 500 ms voltage ramps from -60 mV to +60 mV. Current densities were normalized for each cell to the maximal current density measured at +60 mV during the first capsaicin application. Data points represent arithmetic mean ± SEM of N = 4-10 cells. **(B,E,H,K)** Representative whole-cell current traces evoked by the voltage-step protocol shown in **(C)**. The time points at which the voltage-step protocol was applied are indicated in the time courses in **A,D,G,J** with arrows of the corresponding color. **(C,F,I,L)** Steady-state current densities obtained from voltage-clamp measurements as shown in **B,E,H,K**. The time points at which the current densities were measured are highlighted in **B,E,H,K** with the corresponding symbols. Data points represent arithmetic mean ± SEM. The number of measured cells N is indicated in the graphs. **(M-R)** To determine whether SAFit2 can directly affect TRPV1 activity from the intracellular side of the plasma membrane, patch-clamp experiments were performed with 5 µM SAFit2 **(M-O)** and with 0.2% DMSO (vehicle) added to the pipette solution **(P-R)**. **(M,P)** Time courses of normalized current densities at -60 mV (closed circles) and +60 mV (open circles) as obtained by repeatedly applying 500 ms voltage ramps from -60 mV to +60 mV. Current densities were normalized for each cell to the maximal current density measured at +60 mV. Data points represent arithmetic mean ± SEM of N = 5-9 cells. **(N,Q)** Representative whole-

cell current traces evoked by the voltage-step protocol shown in **(C)**. The time points at which the voltage-step protocol was applied are indicated in the time courses in **(M,P)** with arrows of the corresponding color. **(O,R)** Steady-state current densities obtained from voltage-clamp measurements as shown in **(N,Q)**. The time points at which the current densities were measured are highlighted in **(N,Q)** with the corresponding symbols. Data points represent arithmetic mean ± SEM. The number of measured cells in **N** is indicated in the graphs. **(S)** Ratios J(2)/J(1) of steady-state current densities measured at +80 mV at the end of the first (J(1)) and second (J(2)) capsaicin application in the voltage-clamp experiments shown in **(A-L)**. **(T)** Steady-state current densities at +80 mV obtained from the voltage-clamp experiments shown in **(A-R)** without (w/o, black circles) and after 2 minutes of capsaicin application (red symbols) to the bath solution in the absence (Ctrl) or presence of 5 µM SAFit2, 0.2% DMSO (vehicle) or 10 µM ruthenium red (RuR) in the pipette (in.) or bath solution (ex.). **(S)** and **(T)** show the values of the individual measurements as well as the arithmetic mean ± SEM. The number of measured cells is given in brackets above each group. Not significant (n.s.) $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ student t-test with Welch's correction.
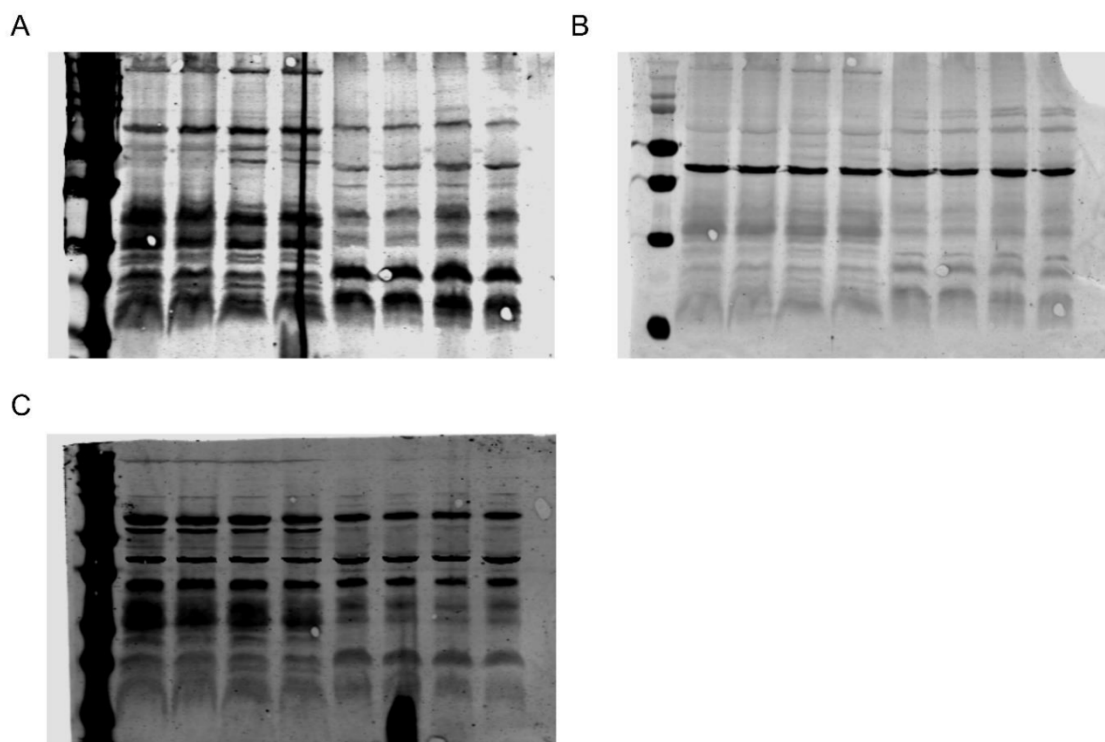
**Figure S8: Uncropped Western Blot images for NF-κB signaling pathway.** Images of **(A)** phosphorylated p65 and IκBα, **(B)** β-Actin loading control for phosphorylated proteins and **(C)** total protein amount of IKKβ, p65, IκBα and the respective loading control β-Actin.

## Methods for the synthesis of ddSAFit2

If not mentioned otherwise, reactions were performed in an oven dried flask under argon atmosphere. $^1$H- and $^{13}$C-NMR spectra were recorded at the NMR department of chemistry of the Technical University of Darmstadt (TUD) on a Bruker DRX500 and at the Thiele lab by Johann Primozic and Matthias Brauser on a Bruker Avance III 600 MHz and Bruker Avance III HD 700 MHz spectrometer. Chemical shifts for $^1$H and $^{13}$C are given in ppm (δ) relative to the tetramethylsilane (TMS) internal standard. Deuterated chloroform (CDCl$_3$ was used as solvent and the spectra calibrated according to their corresponding peak. The multiplicities are abbreviated as follows:

singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), multiplet (m). HRMS spectra were acquired on an LTQ Orbitrap XL that was calibrated using the calmix solution of the manufacturer at least a week before the measurements were conducted. Chiral HPLC was performed using a Beckman System Gold 125S Solvent Module with a Beckman System Gold Diode Array Detector Module 168 recording UV spectra at 220 nm using a Daicel chemical industries Ltd, Chiralcel OD-H, normal phase analytical column, 250 × 4.6 mm, 5 µm with an isocratic gradient given for each compound using n heptane as solvent A and isopropyl alcohol (IPA) as solvent B. UHPLC-MS measurements were performed on an Agilent 1260 infinity II system consisting out of a flexible pump, a Vialsampler, a multicolumn compartment with a column oven, a DAD detector, and a 6125B MSD. Data was acquired with a 50 x 2.1 mm, 1.9 µm EC-C18 Poroshell 120 column using a gradient starting with an isocratic hold of 0.2 min at 5% solvent B up to 100% solvent B over 1.8 min followed by a hold for 1 min with solvent A being water with 0.1% formic acid and solvent B acetonitrile with 0.1% formic acid. Purity is determined by UV detection at 220 nm and given in percent. LC-MS measurements were performed using a Beckman System Gold 125 Solvent Module and Beckman System Gold 199 Detector Module with a YMC Pack Pro C8, 100 × 4.6 mm, 3 µm column using a 0 - 100% B gradient in 19 min with a 1 mL/min flow using solvent A: 95% $H_2O$, 5% MeCN, 0.1% formic acid and solvent B: 95% MeCN, 5% $H_2O$, 0.1% formic acid with a LCQ Deca XP Plus operated in ESI mode. Flash chromatography is performed using an Isolera system from Biotage using the flash columns of the manufacturer with DAD-UV detection. If not mentioned otherwise, separations are performed with a linear 10 column volume gradient. Manual column chromatography was performed using Kieselgel 60 from Roth, 0.04 − 0.063 mm with the solvent mixture mentioned for each compound. Preparative HPLC is performed on an Interchim Puriflash 5.250 system with UV detection.

Purifications are performed using a Luna 250 x 21.2 mm, 5 µm C18 column with a 100 Å pore size using a gradient from 5% B to 100% B in ten column volumes at a flow rate of 30 mL/min unless mentioned otherwise. Solvent A is water with 0.1 TFA and solvent B acetonitrile with 0.1% TFA.

**(E)-3-(3,4-Dimethoxyphenyl)-1-(3-hydroxyphenyl)prop-2-en-1-one** **(8):** 9.0 g (54.2 mmol, 1.0 eq) 3,4-Dimethoxybenzaldehyde and 7.37 g (54.2 mmol, 1.0 eq) 3-Hydroxyacetophenone are dissolved in 90 mL EtOH and the mixture is cooled to 0 °C. 24.5 g (217 mmol, 4.0 eq) potassium hydroxide are dissolved in 60 mL water and to the aforementioned mixture. The mixture is stirred for 2 h and then quenched by adding ice and then 3 M HCl until a pH of 2 is reached. The mixture is crystallized from the quenched reaction mixture to yield 15.3 g (99%) as a yellow solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.77 (dd, J = 15.6, 1.4 Hz, 1H), 7.67 – 7.59 (m, 1H), 7.56 (d, J = 7.7 Hz, 1H), 7.40 – 7.33 (m, 2H), 7.22 (dt, J = 8.4, 1.8 Hz, 1H), 7.15 (t, J = 1.8 Hz, 1H), 7.13 – 7.07 (m, 1H), 6.89 (dd, J = 8.3, 1.5 Hz, 1H), 6.64 (s, 1H), 3.94 (s, 3H), 3.93 (s, 3H). $^{13}$C-NMR (126 MHz, CDCl3): δ 190.9, 156.6, 151.7, 149.4, 145.7, 139.9, 130.0, 127.9, 123.5, 120.9, 120.3, 120.0, 115.3, 111.3, 110.3, 56.2, 56.1. UHPLC-MS: t$_R$ = 1.753 min (99%), m/z: calculated = 285.11 [M+H]$^+$, found = 285.2 [M+H]$^+$.

**3-(3,4-Dimethoxyphenyl)-1-(3-hydroxyphenyl)propan-1-one:** 14.0 g (215 mmol, 10 eq) zinc and 16.5 g (215 mmol, 10 eq) ammonium acetate are suspended in 100 mL MeOH. 6.1 g (21.5 mmol, 1.0 eq) **8** are dissolved in 100 mL MeOH and added dropwise to the aforementioned mixture. The solids are filtered, washed with MeOH and the crude product is precipitated from the filtrate by addition of water. The crude product is purified by recrystallization from water/methanol to yield 5.17 g (84%) as a white solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.54 (dd, J = 2.7, 1.6 Hz, 1H), 7.49 (dq, J = 7.9, 1.2 Hz, 1H), 7.31 (td, J = 8.0, 1.1 Hz, 1H), 7.08 (ddd, J = 8.0, 2.6, 0.9 Hz, 1H), 6.83 – 6.73 (m, 3H), 6.63 (d, J = 42.6 Hz, 1H), 3.84 (s, 3H), 3.84 (s, 3H), 3.26 (dd, J =

8.3, 6.9 Hz, 2H), 3.05 – 2.97 (m, 2H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 200.4, 156.6, 149.0, 147.5, 138.3, 133.9, 130.0, 120.8, 120.6, 120.3, 114.7, 112.1, 111.6, 56.1, 56.0, 40.9, 30.0. UHPLC-MS: t$_R$ = 1.778 min (98 %), m/z: calculated = 287.13 [M+H]$^+$, found = 287.2 [M+H]$^+$.

**(R)-3-(3-(3,4-Dimethoxyphenyl)-1-hydroxypropyl)phenol (9):** 9.70 g (33.9 mmol, 1 eq) **3-(3,4-Dimethoxyphenyl)-1-(3-hydroxyphenyl)propan-1-one** and 50.8 mL (50.8 mmol, 1.5 eq) 1 M potassium butoxide were dissolved in 100 mL IPA. The mixture was degassed with Ar and 270 mg (0.27 mmol, 0.01 eq) RuCl$_2$[(S)-dm-segphos] [(S)-daipen] were added. The mixture was saturated with hydrogen for 15 min before the flask was sealed with a rubber septum and equipped with a hydrogen balloon. The mixture is stirred for 72 h before the reaction is stopped by degassing with Ar and removing volatiles under reduced pressure. The crude product is purified by recrystallization from DCM to yield 9.28 g (95%) as a white solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.14 (t, J = 7.8 Hz, 1H), 6.86 (t, J = 2.0 Hz, 1H), 6.79 (d, J = 7.7 Hz, 1H), 6.76 – 6.70 (m, 2H), 6.69 – 6.61 (m, 2H), 4.58 (dd, J = 7.6, 5.4 Hz, 1H), 3.80 (s, 3H), 3.78 (s, 3H), 2.71 – 2.47 (m, 2H), 2.13 – 1.85 (m, 2H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 156.3, 148.9, 147.2, 146.1, 134.4, 129.8, 120.4, 118.2, 114.9, 112.9, 112.1, 111.5, 74.0, 56.0, 55.9, 40.3, 31.6. LC-MS: t$_R$ = 9.87 min (99%), m/z: calculated = 271.13 [M+H-H$_2$O]$^+$, found = 271.26 [M+H-H$_2$O]$^+$. Chiral HPLC (30% B): t$_R$ = 8.78 min (> 99%)

**(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propan-1-ol (10):** 3.33 g (11.5 mmol, 1 eq) **9**, 2.15 g (11.5 mmol, 1 eq) 2-Chloroethylmorpholine hydrochloride, and 6.38 g (66.1 mmol, 4 eq) potassium carbonate are suspended in 50 mL MeCN. The suspension is refluxed for 24 h before the reaction is stopped by filtering off the solids and removing volatiles under reduced pressure. The crude product is purified by manual silica column (EA/TEA/MeOH 100:3:1) to yield 3.60 g (78%) as a yellow oil. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.28 – 7.21 (m, 1H), 6.92 (dt, J =

6.6, 1.4 Hz, 2H), 6.83 – 6.79 (m, 1H), 6.79 – 6.77 (m, 1H), 6.75 – 6.69 (m, 2H), 4.65 (dd, J = 7.9, 5.2 Hz, 1H), 4.15 – 4.06 (m, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.74 – 3.68 (m, 4H), 2.78 (td, J = 5.7, 1.2 Hz, 2H), 2.75 – 2.56 (m, 2H), 2.58 – 2.53 (m, 4H), 2.14 – 1.93 (m, 2H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 159.1, 149.0, 147.3, 146.6, 134.5, 129.6, 120.3, 118.6, 113.7, 112.3, 111.9, 111.4, 73.8, 67.0, 65.9, 57.8, 56.1, 56.0, 54.2, 40.7, 31.8. UHPLC-MS: $t_R$ = 1.358 min (98%), m/z: calculated = 402.23 [M+H]$^+$, found = 402.2 [M+H]$^+$.

**Perfluorophenyl 2-(3,4,5-trimethoxyphenyl)acetate:** 50 g (221 mmol, 1.0 eq) 3,4,5,-Trimethoxyphenylacetic acid, 40.7 g (221 mmol, 1.0 eq) pentafluorophenol, and 4.96 g (44.2 mmol, 0.2 eq) DMAP are dissolved in 500 mL DCM and cooled to 0 °C, 46.6 g (243 mmol, 1.1 eq) EDC · HCl are added and the mixture is stirred overnight at room temperature. After quenching the reaction by adding brine, the mixture is extracted three times using DCM, the combined organic phases are washed twice with 1 M HCl, dried over MgSO$_4$, and volatiles are removed under reduced pressure. The crude product is recrystallized from MeOH to yield 64.2 g (74%) as a white solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 6.57 (s, 2H), 3.90 (s, 2H), 3.88 (s, 6H), 3.85 (s, 3H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 153.2, 136.4, 127.9, 106.4, 60.8, 56.1. LC-MS: tR = 13.43 min (99%), m/z: calculated = 393.08 [M+H]$^+$, found = 393.07 [M+H]$^+$.

**(S)-4-Phenyl-3-(2-(3,4,5-trimethoxyphenyl)acetyl)oxazolidin-2-one (2):** 28.19 g (173 mmol, 1.1 eq) (S)-4-Phenyloxazolidin-2-one are dissolved in 620 mL THF and cooled to −78 °C. 68.0 mL (173 mmol, 1.1 eq) *n*-butyl lithium are slowly added and the mixture is stirred for 15 min. 61.6 g (157 mmol, 1.0 eq of **Perfluorophenyl 2-(3,4,5-trimethoxyphenyl)acetate** are added and the mixture is stirred for 2 h at −78 °C before the reaction is quenched by addition of isopropyl alcohol (IPA). The mixture was extracted with EA, dried over MgSO$_4$, and volatiles are removed under reduced pressure. The crude product is purified by flash column chromatography (Cy/EA

gradient) to yield 40.0 g (76%) as a white solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.37 – 7.28 (m, 3H), 7.23 – 7.15 (m, 2H), 6.45 (s, 2H), 5.43 (dd, J = 8.8, 4.0 Hz, 1H), 4.69 (t, J = 8.9 Hz, 1H), 4.30 – 4.23 (m, 2H), 4.16 (d, J = 14.8 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 6H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 170.6, 153.7, 153.3, 138.8, 137.3, 134.1, 129.2, 128.9, 126.1, 106.8, 70.0, 61.0, 57.9, 56.2, 41.8. UHPLC-MS: t$_R$ = 1.98 min (96%), m/z: calculated = 372.15 [M+H]$^+$, found = 372.2 [M+H]$^+$.

**(4S)-3-(2-(cyclohex-2-en-1-yl)-2-(3,4,5-trimethoxyphenyl)acetyl)-4-phenyloxazolidin-2-one:** 40.0 g (108 mmol, 1.0 eq) **2** are dissolved in 240 mL THF and cooled to 78 °C. 118 mL (118 mmol, 1.1 eq) of 1 M LiHMDS are added and the mixture is stirred for 2 h at −78 °C. 18.9 mL (162 mmol, 1.5 eq) 3-bromocyclohexene are added and the mixture is stirred for another 2 h before the reaction is quenched by addition of NH$_4$Cl solution. The mixture was extracted with EA, dried over MgSO$_4$, and volatiles are removed under reduced pressure. The crude product is purified by flash column chromatography (Cy/EA gradient) to yield 35.9 g (74%) as a white solid as a mixture of diastereomers. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.43 – 7.37 (m, 2H), 7.36 – 7.30 (m, 3H), 6.67 (d, J = 8.7 Hz, 2H), 5.61 (dqd, J = 9.7, 4.6, 1.8 Hz, 1H), 5.36 (ddd, J = 9.2, 5.6, 3.7 Hz, 1H), 5.04 (dq, J = 10.2, 2.3 Hz, 1H), 4.83 (d, J = 11.3 Hz, 1H), 4.57 (td, J = 8.9, 3.2 Hz, 1H), 4.20 (ddd, J = 8.9, 3.8, 2.1 Hz, 1H), 3.83 (d, J = 2.8 Hz, 6H), 3.81 (d, J = 0.7 Hz, 3H), 2.80 (ddp, J = 16.1, 7.7, 2.6 Hz, 1H), 1.93 (ddq, J = 12.4, 5.9, 3.3 Hz, 2H), 1.70 – 1.58 (m, 1H), 1.49 – 1.28 (m, 2H), 1.11 (tdd, J = 12.7, 6.2, 2.6 Hz, 1H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 173.5, 153.5, 153.4, 153.2, 139.4, 139.4, 137.4, 132.7, 132.6, 129.4, 129.2, 129.1, 128.9, 128.8, 128.5, 128.0, 126.1, 126.0, 106.4, 106.2, 69.5, 69.5, 60.8, 58.2, 58.2, 56.2, 56.2, 53.6, 53.4, 39.7, 39.6, 27.6, 26.1, 25.3, 25.2, 21.1, 20.4. UHPLC-MS: t$_R$ = 1.150 min (99%), m/z: calculated = 452.21 [M+H]$^+$, found = 452.2 [M+H]$^+$.

**(S)-3-((R)-2-cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetyl)-4-phenyloxazolidin-2-one (4a) and (S)-3-((S)-2-Cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetyl)-4-phenyloxazolidin-2-one (4b):** 23.2 g (51.4 mmol, 1.0 eq) **(4S)-3-(2-(cyclohex-2-en-1-yl)-2-(3,4,5-trimethoxyphen-yl)acetyl)-4-phenyloxazolidin-2-one** are dissolved in 220 mL MeOH and the mixture is degassed with Ar for 15 min before 2.73 g (2.57 mmol, 0.05 eq) Pd/C are added. The mixture is then saturated with hydrogen gas for 15 min before the flask is sealed with a rubber septum and equipped with a hydrogen balloon. The reaction is stirred overnight at room temperature and then stopped by degassing the solution for 15 min with Ar before filtering over silica. Volatiles are removed under reduced pressure. The crude product is purified by flash column chromatography (Cy/EA gradient) to yield **11a** 16.38 g (70%) as a white solid and **11b** 4.38 g (19%) as a white solid.

**4a:** $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.22 – 7.12 (m, 3H), 6.85 – 6.79 (m, 2H), 6.27 (s, 2H), 5.44 (dd, J = 9.0, 5.0 Hz, 1H), 4.72 (d, J = 10.7 Hz, 1H), 4.68 – 4.58 (m, 1H), 3.83 (s, 3H), 3.63 (s, 6H), 2.05 – 1.92 (m, 1H), 1.85 (dt, J = 12.4, 3.1 Hz, 1H), 1.70 (dt, J = 14.7, 3.9 Hz, 1H), 1.65 – 1.57 (m, 3H), 1.35 – 1.20 (m, 2H), 1.20 – 1.07 (m, 2H), 1.02 (tdd, J = 12.4, 10.8, 3.6 Hz, 1H), 0.82 (qd, J = 11.8, 3.0 Hz, 1H). $^{13}$C-NMR (126 MHz, CDCl3): δ 172.7, 153.3, 153.0, 138.4, 137.1, 132.1, 128.8, 128.5, 125.9, 106.0, 69.5, 60.9, 57.9, 56.0, 55.9, 39.8, 32.4, 30.3, 26.5, 26.1, 26.1. UHPLC-MS: t$_R$ = 1.186 min (99%), m/z: calculated = 454.23 [M+H]$^+$, found = 454.2 [M+H]$^+$.

**4b:** $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.46 – 7.26 (m, 5H), 5.36 (dd, J = 8.8, 3.6 Hz, 1H), 4.79 (d, J = 10.8 Hz, 1H), 4.58 (t, J = 8.8 Hz, 1H), 4.21 (dd, J = 8.9, 3.6 Hz, 1H), 3.84 (s, 6H), 3.82 (s, 3H), 2.08 – 1.89 (m, 1H), 1.58 (d, J = 16.1 Hz, 4H), 1.36 – 1.20 (m, 3H), 1.09 (d, J = 8.3 Hz, 2H), 1.01 – 0.62 (m, 1H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 174.0, 153.7, 153.1, 139.5, 137.2, 133.2, 129.3, 128.9, 126.0, 106.2, 69.5, 61.0, 58.3, 56.2,

54.7, 42.2, 31.5, 30.3, 26.4, 26.0, 25.9. UHPLC-MS: $t_R$ = 1.238 min (99%), m/z: calculated = 454.23 $[M+H]^+$, found = 454.2 $[M+H]^+$.

**(S)-2-Cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetic acid:** 16.38 g (36.1 mmol, 1.0 eq) **4b** are dissolved in 160 mLTHF and added to a mixture of 2.59 g (108 mmol, 3.0 eq) LiOH and 18.4 mL (181 mmol, 5.0 eq) $H_2O_2$ dissolved in 80 mL water. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of 1 M HCl. The mixture is extracted with ether, dried over $MgSO_4$, and the solvent is removed under reduced pressure. The crude product is purified by flash column chromatography (Cy/EA+1% of formic acid gradient) to yield 9.7 g (87%) as a pale yellow solid. $^1$H-NMR (500 MHz, $CDCl_3$): δ 6.54 (s, 2H), 3.84 (s, 6H), 3.82 (s, 3H), 3.12 (d, J = 10.7 Hz, 1H), 1.95 (tt, J = 11.0, 3.3 Hz, 1H), 1.92 – 1.86 (m, 1H), 1.79 – 1.71 (m, 1H), 1.64 (dq, J = 8.0, 3.9 Hz, 2H), 1.43 – 1.34 (m, 1H), 1.34 – 1.24 (m, 1H), 1.22 – 1.13 (m, 2H), 1.07 (tdd, J = 12.6, 10.8, 3.5 Hz, 1H), 0.81 – 0.69 (m, 1H). $^{13}$C-NMR (126 MHz, $CDCl_3$): δ 180.0, 153.3, 137.5, 133.0, 105.8, 60.9, 59.1, 56.3, 41.0, 32.0, 30.4, 26.4, 26.0. UHPLC-MS: $t_R$ = 1.863 min (99%), m/z: calculated = 309.2 $[M+H]^+$, found = 309.2 $[M+H]^+$.

**(R)-2-Cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetic acid (5):** 4.34 g (9.57 mmol, 1.0 eq) **4a** are dissolved in 80 mL THF. The mixture is cooled to 0 °C and 0.69 g (28.7 mmol, 3.0 eq) LiOH and 4.89 mL (47.9 mmol, 5.0 eq) $H_2O_2$ dissolved in 50 mL are added. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of diluted HCl. The mixture is extracted with EA, dried with $MgSO_4$, and volatiles are removed under reduced pressure. The crude product is purified by flash column chromatography (DCM/MeOH + 1% FA) to yield 2.03 g (69%) as a white solid. $^1$H-NMR (500 MHz, $CDCl_3$): δ 6.54 (s, 2H), 3.84 (s, 6H), 3.82 (s, 3H), 3.12 (d, J = 10.7 Hz, 1H), 2.02 – 1.85 (m, 2H), 1.79 – 1.71 (m, 1H), 1.68 – 1.58 (m, 2H), 1.41 – 1.23 (m, 2H), 1.22 – 1.01 (m, 3H), 0.81 – 0.69 (m, 1H). $^{13}$C-NMR (126 MHz,

CDCl₃): δ 179.9, 153.3, 137.5, 133.0, 129.3, 126.1, 105.8, 61.0, 59.1, 56.3, 41.0, 32.1, 30.4, 26.4, 26.1. UHPLC-MS: $t_R$ = 0.492 min (97%), m/z: calculated = 309.18 [M+H]+, found = 309.2 [M+H]⁺.

**(S)-1-Tert-butyl 2-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)pro-pyl)** **piperidine-1,2-dicarboxylate:** 31.3 g (77.8 mmol, 1.0 eq) **10**, 18.74 g (81.7 mmol, 1.05 eq) (S)-N-Boc-pipecolate, and 1.90 g (15.6 mmol, 0.2 eq) DMAP are dissolved in 350 mL DCM. The mixture is cooled to 0 °C and 15.7 g (81.7 mmol, 1.05 eq) EDC · HCl are added. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of brine. The mixture is extracted with DCM, dried with MgSO₄, and volatiles are removed under reduced pressure. The crude product is purified by flash column chromatography (Cy/EA + 3% TEA) to yield 42.2 g (97%) as a pale yellow oil. ¹H-NMR (500 MHz, CDCl₃): δ 7.24 (t, J = 8.2 Hz, 2H), 6.91 (d, J = 7.8 Hz, 2H), 6.87 (s, 2H), 6.82 (d, J = 8.2 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.67 (q, J = 6.6 Hz, 3H), 5.76 (s, 1H), 5.04 – 4.89 (m, 1H), 4.75 (d, J = 6.0 Hz, 1H), 4.10 (p, J = 6.3 Hz, 5H), 4.06 – 3.90 (m, 1H), 3.84 (d, J = 2.5 Hz, 9H), 3.80 – 3.68 (m, 7H), 2.79 (t, J = 5.7 Hz, 3H), 2.67 – 2.42 (m, 4H), 2.25 (dd, J = 26.2, 13.2 Hz, 4H), 2.04 (d, J = 8.3 Hz, 4H), 1.77 – 1.55 (m, 5H), 1.47 (s, 6H), 1.36 (d, J = 7.2 Hz, 7H), 1.22 – 1.02 (m, 1H). ¹³C-NMR (126 MHz, CDCl₃): δ 171.5, 171.2, 158.9, 155.5, 149.0, 147.5, 141.9, 133.8, 133.6, 129.7, 120.3, 119.2, 114.2, 114.0, 113.1, 111.8, 111.4, 80.0, 76.2, 67.0, 65.9, 60.5, 57.8, 56.0, 55.9, 55.9, 55.1, 54.2, 54.0, 49.2, 42.3, 41.2, 38.4, 38.2, 34.1, 31.5, 31.2, 28.5, 28.4, 26.9, 25.7, 25.1, 25.0, 24.7, 21.1, 21.0, 20.8, 14.3. UHPLC-MS: $t_R$ = 2.072 min (99%), m/z: calculated = 613.35 [M+H]⁺, found = 613.4 [M+H]⁺.

**(S)-(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl** **piperidine-2-carboxylate:** 42.2 g (68.9 mmol, 1.0 eq) **(S)-1-Tert-butyl 2-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)pro-pyl)** **piperidine-1,2-**

**dicarboxylate** are dissolved in 40 mL of DCM and 20 mL TFA. The mixture is stirred for 2 h at room temperature before the reaction is quenched by addition of NaHCO$_3$. The mixture is extracted with DCM, dried with MgSO$_4$, and volatiles are removed under reduced pressure. The crude product (35.3 g quant.) is used without further purification. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.22 − 7.15 (m, 1H), 6.86 (d, J = 7.6 Hz, 1H), 6.82 (d, J = 2.5 Hz, 1H), 6.77 (dd, J = 8.2, 2.5 Hz, 1H), 6.73 (d, J = 8.1 Hz, 1H), 6.65 − 6.56 (m, 2H), 5.70 (dd, J = 8.1, 5.5 Hz, 1H), 4.04 (t, J = 5.6 Hz, 2H), 3.80 (s, 3H), 3.79 (s, 3H), 3.68 (t, J = 4.5 Hz, 4H), 3.32 (dd, J = 10.1, 3.3 Hz, 1H), 3.02 (dt, J = 12.2, 3.8 Hz, 1H), 2.74 (t, J = 5.8 Hz, 2H), 2.65 − 2.54 (m, 1H), 2.54 − 2.44 (m, 6H), 2.18 (ddt, J = 14.7, 9.0, 4.2 Hz, 1H), 2.05 − 1.94 (m, 2H), 1.75 (dt, J = 8.5, 4.8 Hz, 1H), 1.59 − 1.48 (m, 2H), 1.47 − 1.36 (m, 2H), 1.16 − 0.96 (m, 1H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 172.8, 158.8, 148.8, 147.3, 141.8, 133.6, 129.5, 120.1, 120.1, 119.0, 119.0, 113.8, 113.0, 111.7, 111.7, 111.3, 75.5, 67.0, 66.9, 65.7, 58.7, 57.6, 55.9, 55.8, 55.8, 54.1, 52.9, 45.9, 45.6, 38.0, 33.9, 31.3, 31.3, 29.3, 25.8, 25.7, 25.0, 24.1, 9.3, 8.1. UHPLC-MS: t$_R$ = 1.302 min (98%), m/z: calculated = 513.30 [M+H]$^+$, found = 513.2 [M+H]$^+$.

**(S)-(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl 1-((S)-2-cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetyl)piperidine-2-carboxylate (SAFit2):** 13.4 g (43.3 mmol, 1.0 eq) **(S)-2-Cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetic acid**, 18.1 g (47.6 mmol, 1.1 eq) HATU, and 30.2 mL (173 mmol, 4.0 eq) DiPEA are dissolved in 20 mL of DMF. The mixture is stirred for 30 min at room temperature before 33.3 g (64.9 mmol, 1.5 eq) **(S)-(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl piperidine-2-carboxylate** dissolved in 250 mL DCM are added. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of brine. The mixture is extracted with DCM, dried with MgSO$_4$, and volatiles are removed under reduced

pressure. The crude product is purified by preparative HPLC and flash column chromatography (Cy/EA+3% TEA) to yield 19.5 g (56%) as a white solid. ^{1}H-NMR (700 MHz, CDCl$_3$): δ 7.28 (t, J = 7.9 Hz, 0H), 7.09 (t, J = 7.9 Hz, 1H), 6.95 (d, J = 7.6 Hz, 0H), 6.90 (t, J = 2.1 Hz, 0H), 6.88 – 6.84 (m, 0H), 6.78 (d, J = 8.2 Hz, 0H), 6.76 – 6.73 (m, 2H), 6.69 (t, J = 2.0 Hz, 1H), 6.68 (dd, J = 8.2, 2.0 Hz, 0H), 6.65 (d, J = 2.0 Hz, 0H), 6.63 – 6.60 (m, 2H), 6.47 (s, 2H), 6.41 (s, 1H), 6.39 (d, J = 7.6 Hz, 1H), 5.79 (t, J = 7.0 Hz, 0H), 5.55 (dd, J = 8.2, 5.5 Hz, 1H), 5.46 (d, J = 5.6 Hz, 1H), 4.71 (d, J = 5.8 Hz, 0H), 4.55 (d, J = 13.6 Hz, 0H), 4.14 – 4.08 (m, 1H), 4.06 (t, J = 5.7 Hz, 2H), 3.93 (d, J = 13.6 Hz, 1H), 3.85 (s, 1H), 3.84 (s, 1H), 3.84 (s, 3H), 3.83 (s, 5H), 3.82 (s, 1H), 3.76 (s, 3H), 3.71 (t, J = 4.7 Hz, 6H), 3.70 (s, 5H), 3.36 (d, J = 9.8 Hz, 1H), 2.96 (d, J = 9.7 Hz, 0H), 2.81 – 2.78 (m, 1H), 2.77 (t, J = 5.7 Hz, 2H), 2.65 – 2.52 (m, 6H), 2.46 (ddd, J = 14.4, 9.4, 5.3 Hz, 1H), 2.37 (ddd, J = 13.9, 9.1, 7.0 Hz, 1H), 2.28 (d, J = 13.7 Hz, 1H), 2.13 – 2.04 (m, 2H), 2.03 (s, 0H), 1.95 (dtd, J = 14.0, 8.7, 5.3 Hz, 1H), 1.87 (d, J = 11.9 Hz, 1H), 1.85 – 1.75 (m, 1H), 1.72 – 1.64 (m, 2H), 1.64 – 1.61 (m, 2H), 1.61 – 1.52 (m, 2H), 1.45 – 1.37 (m, 1H), 1.36 – 1.27 (m, 2H), 1.27 – 1.21 (m, 1H), 1.19 – 1.07 (m, 3H), 0.99 (t, J = 12.6 Hz, 0H), 0.89 (qd, J = 12.5, 3.5 Hz, 1H), 0.78 – 0.71 (m, 1H), 0.64 – 0.50 (m, 1H). ^{13}C-NMR (176 MHz, CDCl$_3$): δ 172.4, 172.3, 171.2, 170.7, 170.6, 159.1, 158.7, 153.4, 153.1, 149.1, 148.9, 147.6, 147.4, 141.9, 141.6, 137.1, 136.9, 134.4, 133.7, 133.6, 133.4, 129.9, 129.6, 120.3, 120.2, 119.2, 118.5, 114.2, 113.8, 113.4, 113.2, 111.9, 111.8, 111.5, 111.4, 105.9, 105.4, 76.9, 75.7, 67.0, 67.0, 65.9, 65.8, 61.0, 60.9, 60.5, 57.8, 56.4, 56.1, 56.0, 56.0, 56.0, 56.0, 55.9, 55.9, 55.8, 55.1, 54.2, 52.1, 43.7, 41.5, 41.2, 39.5, 38.1, 37.9, 33.1, 32.9, 31.6, 31.1, 30.8, 30.6, 26.9, 26.7, 26.5, 26.3, 26.3, 26.2, 25.6, 24.4, 21.1, 21.1, 20.8, 14.3. UHPLC-MS: t$_R$ = 1.614 min (> 99%), m/z: calculated = 803.45 [M+H]$^+$, found = 803.4 [M+H]$^+$.

**(R)-1-tert-Butyl 2-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)prop-yl) piperidine-1,2-dicarboxylate:** 4.00 g

(10.0 mmol, 1.0 eq) **10**, 2.40 g (10.5 mmol, 1.05 eq) *R*-N-Boc-pipecolate, and 0.24 g (2.0 mmol, 0.2 eq) DMAP are dissolved in 50 mL DCM. The mixture is cooled to 0 °C and 2.01 g (10.5 mmol, 1.05 eq) DCC are added. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of brine. The mixture is extracted with DCM, dried with $MgSO_4$, and volatiles are removed under reduced pressure. The crude product is purified by manual column chromatography (EA/TEA/MeOH 100:3:1) to yield 4.78 g (78%) as a white solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.23 (td, J = 7.9, 5.2 Hz, 1H), 6.90 (q, J = 6.9 Hz, 1H), 6.85 (d, J = 1.8 Hz, 1H), 6.84 – 6.79 (m, 2H), 6.77 (d, J = 8.1 Hz, 1H), 6.68 (dd, J = 8.0, 2.0 Hz, 1H), 6.66 (d, J = 2.0 Hz, 1H), 5.74 (dt, J = 32.3, 6.9 Hz, 1H), 4.98 – 4.88 (m, 1H), 4.77 (s, 0H), 4.09 (t, J = 5.8 Hz, 2H), 3.88 – 3.82 (m, 6H), 3.75 – 3.69 (m, 4H), 2.93 (dt, J = 34.6, 13.1 Hz, 1H), 2.78 (t, J = 5.7 Hz, 2H), 2.66 – 2.47 (m, 6H), 2.22 (qd, J = 15.3, 10.0 Hz, 3H), 2.04 (ddd, J = 16.2, 12.9, 6.4 Hz, 1H), 1.72 – 1.52 (m, 3H), 1.43 (d, J = 32.0 Hz, 11H), 1.22 – 1.02 (m, 1H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 171.5, 171.3, 159.0, 156.1, 155.4, 149.0, 147.5, 142.3, 141.9, 133.8, 133.7, 129.6, 120.3, 118.8, 114.1, 113.9, 113.2, 112.6, 111.9, 111.5, 80.1, 80.0, 76.2, 76.1, 67.0, 65.9, 57.8, 56.0, 56.0, 55.0, 54.2, 53.9, 42.3, 41.2, 38.5, 38.4, 31.6, 31.4, 28.5, 26.9, 26.8, 24.9, 24.7, 20.8, 20.7. UHPLC-MS: $t_R$ = 1.786 min (97%), m/z: calculated = 613.35 [M+H]$^+$, found = 613.2 [M+H]$^+$.

**(R)-(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl-piperidine-2-carboxylate (11):** 4.78 g (7.8 mmol, 1.0 eq) **(R)-1-tert-Butyl 2-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)prop-yl) piperidine-1,2-dicarboxylate** is dissolved in 24 mL DCM and 12 mL TFA. The mixture is stirred for 1.5 h at room temperature before the mixture was concentrated under reduced pressure and the reaction was neutralized with sodium carbonate. The mixture is extracted with DCM, dried with $MgSO_4$, and volatiles are removed under reduced

pressure. The crude product (4.0 g quant.) is used without further purification. $^1$H-NMR (500 MHz, CDCl$_3$): δ 7.22 (td, J = 7.9, 1.4 Hz, 1H), 6.89 (dt, J = 7.7, 1.3 Hz, 1H), 6.85 (dd, J = 2.6, 1.6 Hz, 1H), 6.80 (ddd, J = 8.2, 2.6, 1.0 Hz, 1H), 6.76 (dd, J = 7.9, 1.4 Hz, 1H), 6.69 – 6.62 (m, 2H), 5.74 (dd, J = 7.9, 5.8 Hz, 1H), 4.08 (td, J = 5.8, 2.3 Hz, 2H), 3.84 (d, J = 1.3 Hz, 3H), 3.83 (d, J = 1.3 Hz, 3H), 3.74 – 3.69 (m, 4H), 3.42 – 3.32 (m, 1H), 3.06 (dt, J = 12.2, 3.7 Hz, 1H), 2.77 (td, J = 5.8, 1.4 Hz, 2H), 2.69 – 2.60 (m, 1H), 2.59 – 2.47 (m, 6H), 2.28 – 2.17 (m, 1H), 2.10 – 1.99 (m, 2H), 1.98 – 1.91 (m, 1H), 1.74 (dq, J = 7.7, 3.4 Hz, 1H), 1.60 – 1.53 (m, 1H), 1.51 – 1.33 (m, 3H). $^{13}$C-NMR (126 MHz, CDCl$_3$): δ 172.8, 158.9, 148.9, 147.4, 142.0, 133.7, 129.6, 120.2, 119.0, 113.9, 113.2, 113.1, 111.8, 111.4, 75.7, 67.0, 65.9, 58.7, 57.7, 56.0, 55.9, 54.2, 45.8, 37.9, 31.4, 29.3, 26.0, 24.1. UHPLC-MS: t$_R$ = 1.256 min (97%), m/z: calculated = 513.30 [M+H]$^+$, found = 513.2 [M+H]$^+$.

**(R)-(R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl 1-((R)-2-cyclohexyl-2-(3,4,5-trimethoxyphenyl)acetyl)piperidine-2-carboxylate (ddSAFit, 12):** 2.03 g (6.6 mmol, 1.0 eq) **4a**, and 2.63 g (6.9 mmol, 1.05 eq) HATU, and 4.6 mL (26.3 mmol, 4.0 eq) DiPEA are dissolved in 10 mL of DMF. The mixture is stirred for 30 min at room temperature before 4.0 g (7.8 mmol, 1.2 eq) **11** dissolved in 50 mL DCM are added. The mixture is stirred overnight at room temperature before the reaction is quenched by addition of brine. The mixture is extracted with EA, dried with MgSO$_4$, and volatiles are removed under reduced pressure. The crude product is purified by preparative HPLC and flash column chromatography (Cy/EA+3% TEA) to yield 4.12 g (78%) as a white solid. $^1$H-NMR (700 MHz, CDCl$_3$): δ 7.29 – 7.25 (m, 0H), 7.18 (t, J = 8.0 Hz, 1H), 6.94 (d, J = 7.6 Hz, 0H), 6.93 – 6.83 (m, 1H), 6.80 – 6.73 (m, 3H), 6.71 – 6.64 (m, 1H), 6.62 – 6.56 (m, 2H), 6.49 (s, 1H), 6.41 (s, 1H), 5.78 (dd, J = 7.9, 6.2 Hz, 0H), 5.55 (dd, J = 8.4, 5.2 Hz, 1H), 5.43 – 5.40 (m, 1H), 4.73 (d, J = 5.6 Hz, 0H), 4.66 (d, J = 13.6 Hz, 0H), 4.12 – 4.02 (m, 2H), 3.96 (d, J = 13.8 Hz, 1H), 3.87

– 3.80 (m, 9H), 3.77 (s, 6H), 3.74 – 3.69 (m, 4H), 3.38 (d, J = 9.8 Hz, 1H), 3.10 (d, J = 9.6 Hz, 0H), 2.97 (td, J = 13.4, 2.9 Hz, 1H), 2.78 (dt, J = 17.0, 5.7 Hz, 2H), 2.65 – 2.51 (m, 5H), 2.47 (ddd, J = 14.3, 9.0, 5.5 Hz, 1H), 2.36 (dt, J = 14.6, 7.8 Hz, 1H), 2.26 (dd, J = 12.0, 4.1 Hz, 1H), 2.15 – 2.02 (m, 2H), 1.98 – 1.85 (m, 3H), 1.74 – 1.50 (m, 5H), 1.43 (q, J = 12.9 Hz, 1H), 1.37 – 1.08 (m, 4H), 1.06 – 0.97 (m, 0H), 0.92 (qd, J = 12.4, 3.5 Hz, 1H), 0.82 – 0.55 (m, 1H). $^{13}$C-NMR (176 MHz, CDCl$_3$): δ 172.8, 171.9, 170.6, 170.5, 159.0, 153.4, 153.1, 149.1, 149.0, 147.6, 147.4, 142.3, 141.3, 137.1, 136.9, 134.3, 133.6, 133.6, 133.4, 129.8, 129.7, 120.3, 120.3, 120.2, 119.3, 118.5, 114.1, 113.8, 113.6, 112.7, 111.9, 111.8, 111.5, 111.4, 105.9, 105.4, 77.0, 75.7, 67.0, 67.0, 66.0, 65.9, 61.0, 60.8, 57.8, 57.8, 56.3, 56.2, 56.1, 56.1, 55.9, 55.8, 55.1, 54.2, 54.2, 52.3, 43.7, 41.6, 41.2, 39.7, 38.2, 37.9, 33.2, 32.9, 31.6, 31.3, 30.8, 30.8, 26.9, 26.8, 26.7, 26.7, 26.4, 26.3, 26.3, 26.3, 25.6, 24.6, 21.0, 20.8. UHPLC-MS: $t_R$ = 1.881 min (> 99%), m/z: calculated = 803.45 [M+H]$^+$, found = 803.4 [M+H]$^+$. HRMS: m/z: calculated =803.4477, found = 803.4461 [M+H]$^+$ (1.99 ppm error)

# The FKBP51 Inhibitor SAFit2 Restores the Pain-Relieving C16 Dihydroceramide after Nerve Injury

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## 3.1 Author contributions

Conceptualization: FH, MS and GG conceptualized the project.

Funding acquisition: MS and FH acquired the funding.

Experimental design: **SW**, SO, and MS designed the experiments.

Data acquisition: **SW** performed the qPCRs, calcium imaging experiments and *in vivo* experiments. LH conducted the LC-HRMS measurement and MWA performed the GPCR screen.

Data analysis: **SW** analyzed all the data. LH helped with the LC-HRMS analysis.

Data curation: **SW** performed the data curation.

Original draft preparation: **SW** and MS wrote and revised the manuscript.

All authors approved the final manuscript.

*Article*

# The FKBP51 Inhibitor SAFit2 Restores the Pain-Relieving C16 Dihydroceramide after Nerve Injury

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**Abstract:** Neuropathic pain is a pathological pain state with a broad symptom scope that affects patients after nerve injuries, but it can also arise after infections or exposure to toxic substances. Current treatment possibilities are still limited because of the low efficacy and severe adverse effects of available therapeutics, highlighting an emerging need for novel analgesics and for a detailed understanding of the pathophysiological alterations in the onset and maintenance of neuropathic pain. Here, we show that the novel and highly specific FKBP51 inhibitor SAFit2 restores lipid signaling and metabolism in nervous tissue after nerve injury. More specifically, we identify that SAFit2 restores the levels of the C16 dihydroceramide, which significantly reduces the sensitization of the pain-mediating TRPV1 channel and subsequently the secretion of the pro-inflammatory neuropeptide CGRP in primary sensory neurons. Furthermore, we show that the C16 dihydroceramide is capable of reducing acute thermal hypersensitivity in a capsaicin mouse model. In conclusion, we report for the first time the C16 dihydroceramide as a novel and crucial lipid mediator in the context of neuropathic pain as it has analgesic properties, contributing to the pain-relieving properties of SAFit2.

**Keywords:** neuropathic pain; lipid mediators; ceramides; sensory neurons; nerve injury; FKBP51

## 1. Introduction

Chronic pain and especially neuropathic pain as persistent pain states affect patients worldwide after nerve injuries, chemotherapies, or diseases such as diabetes and multiple sclerosis. However, the available therapeutics for efficient pain relief are currently inadequate, pointing out neuropathic pain as a pathological entity with a lack of treatment possibilities and a broad symptom scope [1,2]. Former studies revealed FK506 binding protein 51 (FKBP51), which is encoded by the FKBP5 gene, as a novel and promising target for various pathologies, since it is involved in many different pathological processes, such as the establishment of chronic pain [3,4], stress endocrinology difficulties [5], and glucocorticoid signaling-related diseases [6]. Moreover, the highly potent FKBP51 inhibitor SAFit2 has been shown to pass the blood–brain barrier [7,8], indicating that SAFit2 is also able to pass the blood spinal cord barrier and can mediate analgesic effects in the central and peripheral nervous system. Likewise, we recently showed that SAFit2 ameliorates neuropathic pain in vivo as it counteracts the enhanced neuronal activity and reduces neuroinflammation after nerve injury [9]. These previous reports highlight the concept of FKBP51 inhibitors as a potential novel treatment approach for neuropathic pain.

Mechanistically, SAFit2 reduces the NF-κB pathway activation after nerve injury, potentially also modulating crucial target genes in the NF-κB downstream signaling [9]. NF-κB itself is a crucial transcription factor in lipid metabolism as it regulates the expression of various enzymes, such as cyclooxygenases, lipoxygenases, and phospholipases [10,11]. These enzymes metabolize and oxidize eicosanoids, linoleic acid metabolites, and sphingolipids, generating lipid mediators that can act as autocrine and paracrine signaling mediators [12]. In addition, previous studies have already demonstrated that lipid mediators can contribute to pain hypersensitivities after chemotherapeutic treatment or nerve injury [13–15]. Importantly, it was shown that lipid mediators can affect the sensitivity or activation threshold of ion channels in sensory neurons, especially of pain-mediating transient receptor potential cation (TRP) channels, which can result in an enhanced neuronal activity, leading to increased pain perception and neuroinflammation [14,16–20]. Moreover, alterations in the subset of lipid mediators were shown to be associated with nerve dysfunctions and chronic pain.

In the development of neuropathic pain, metabolites from the ceramide/sphingolipid pathway were identified as potential targets for treating neuroinflammation, as they were implicated to be crucial regulators at the neuroimmune interface [21]. Particularly, the sphingolipid sphingosine1-phosphate (S1P) is known to sensitize TRP subfamily V member 1 (TRPV1) in inflammatory processes, leading to hypersensitivity and enhanced neuropathic pain [21–23]. However, the influence of other S1P pathway metabolites, such as ceramides and dihydroceramides, on TRP channels has not yet been investigated in the context of neuropathic pain.

In the present study, we focus on the peripheral nervous system and the interface between the peripheral and the central nervous system. We investigated the expression of lipid-generating enzymes and performed lipidomic analysis in sensory neurons of the dorsal root ganglia, as they are the central location of the cell somata of peripheral sensory neurons, and in the dorsal spinal cord. Here, we detected that the neuropathic pain-ameliorating FKBP51 inhibitor SAFit2 also affects the expression of lipid-oxidizing enzymes, such as lipoxygenases, as well as ceramide synthases in the context of nerve injury-induced neuropathic pain in mice. An unbiased LC-HRMS-based lipidomics screening from lumbar dorsal root ganglia (DRGs) and spinal cords revealed that SAFit2 is capable of counteracting the nerve injury-induced alterations in lipid levels after SNI. While the levels of classical eicosanoids were not significantly altered, SAFit2 especially restores the levels of the C16 dihydroceramide (N-palmitoyl-D-erythro-sphinganine) after nerve injury. Neurobiological characterization of the C16 dihydroceramide showed that it significantly reduces the sensitization of TRPV1 in sensory neurons and subsequently the release of the pro-inflammatory neuropeptide CGRP (calcitonin gene-related peptide). Furthermore, the C16 dihydroceramide was shown to significantly decrease the thermal hypersensitivity of mice in an acute pain model, attributing C16 dihydroceramide potential analgesic or antihyperalgesic properties. In summary, we discovered that interfering with the FKBP51 signaling with the inhibitor SAFit2 can influence lipid signaling and metabolism in nervous tissue after nerve injury. In addition, we identified the C16 dihydroceramide as a novel and previously unrelated crucial lipid mediator in the context of neuropathic pain.

## 2. Results

### 2.1. SAFit2 Reduces the Upregulation of Lipid-Oxidizing and -Metabolizing Enzymes after SNI

To investigate the influence of SAFit2 on lipid metabolism after nerve injuries, we first analyzed the expression of lipid-oxidizing enzymes, which play a crucial role in the generation of signaling lipids and lipid metabolites, and which can influence the onset and maintenance of inflammation [12]. Therefore, we performed a spared nerve injury model on mice and treated them intraperitoneally with either 10 mg/kg SAFit2 or vehicle two times daily on six consecutive days (days five to ten) after SNI (Figure 1A). After 21 days, the mice were sacrificed, and lumbar L4-L6 dorsal root ganglia (DRGs) as well as the corresponding section of the spinal cord (SC) were collected to measure the expression

of ceramide synthases, lipoxygenases, cyclooxygenases, phospholipases, and cytochrome-$P_{450}$-oxygenases (Figure 1B–I).
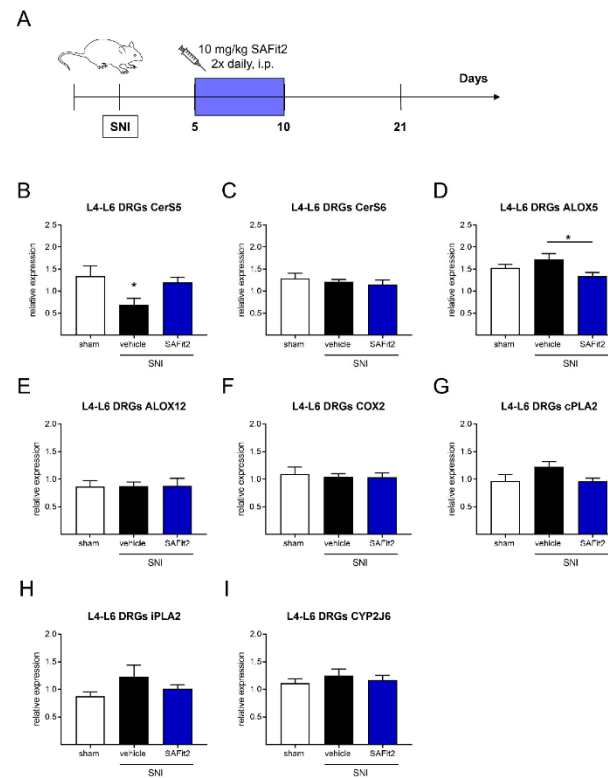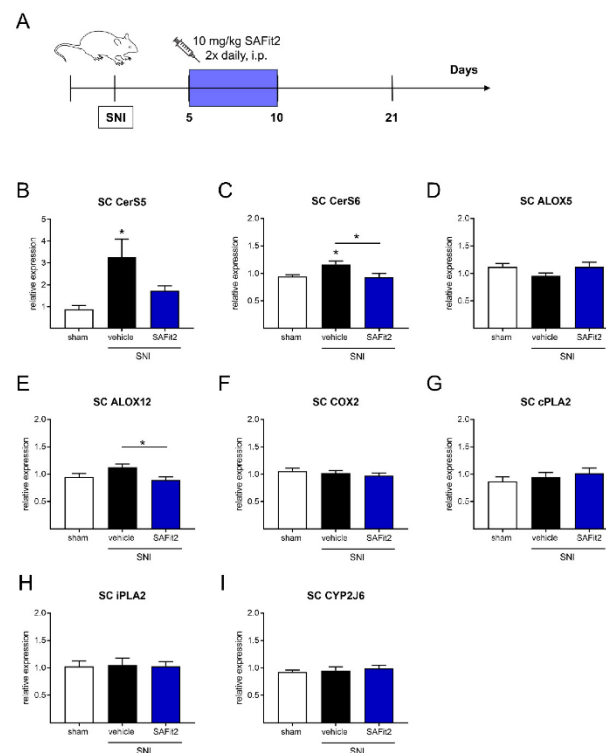


**Figure 1.** Gene expression of ceramide synthases, lipoxygenases, phospholipases, and epoxygenases in lumbar DRGs of nerve-injured mice after additional SAFit2 treatment. (**A**) Schematic illustration of the experimental procedure: SNI surgery was performed followed by a treatment with either 10 mg/kg SAFit2 or vehicle on six consecutive days (days five to ten) after SNI. The gene expression of ceramide synthases (**B,C**), lipoxygenases (**D–F**), phospholipases (**G,H**), and epoxygenase (**I**) was measured 21 days after the surgery. The data are displayed as the mean $\pm$ SEM of technical replicates from three to four mice per treatment. * $p < 0.05$, as per one-way ANOVA with Tukey's post-hoc test. Abbreviations: DRGs: dorsal root ganglia; SAFit2: selective antagonist of FKBP51 by induced fit 2; SNI: spared nerve injury; CerS: ceramide synthase; ALOX: arachidonate lipoxygenase; COX2: cyclooxygenase 2; cPLA2: cytosolic phospholipase A2; iPLA2: calcium-independent phospholipase A2; CYP2J6: cytochrome P450 family 2 subfamily j polypeptide 6.

Interestingly, we detected a significant reduction in ceramide synthase 5 (CerS5), which is required for the synthesis of long-chain ceramides [24], in the lumbar DRGs after SNI (Figure 1B), after conducting a one-way ANOVA with Tukey's post-hoc test. In addition, we observed a significant difference in the expression of the arachidonate lipoxygenase 5 (ALOX5) in DRGs between the vehicle and SAFit2-treated animals (Figure 1D). However, the cyclooxygenase 2 (COX2, Figure 1F), which is typically upregulated in inflammatory processes synthesizing prostaglandins [25], shows an unaltered expression after SNI at our investigated time point. Likewise, the expression of other enzymes, such as ceramide synthase 6 (CerS6, Figure 1C), arachidonate lipoxygenase 12 (ALOX12, Figure 1E), phospholipases cPLA2 and iPLA2 (Figure 1G,H), and cytochrome-$P_{450}$-oxygenase CYP2J6 (Figure 1I), were not significantly altered in the lumbar DRGs after SNI, when comparing treatments using one-way ANOVA with a Tukey's post-hoc test. In addition, the expres-

sion of the arachidonate lipoxygenase 15 (ALOX15) and the cytochrome-$P_{450}$-oxygenase CYP3a11 was below the detection limit in lumbar DRGs.

As we analyzed the expression of the same enzymes in the respective segments of the spinal cord and determined significant alterations with a one-way ANOVA and Tukey's post-hoc test (Figure 2), we again detected an alteration in the expression of ceramide synthases and lipoxygenases. However, this time ceramide synthases 5 and 6 were both significantly upregulated in the vehicle-treated SNI animals (Figure 2B,C). In addition, the expression of the ceramide synthase 6 differs significantly between the vehicle and SAFit2-treated SNI animals, whereas it is quite comparable between SAFit2 and the sham treatment in the spinal cord after SNI (Figure 2C). Furthermore, the expression of arachidonate lipoxygenase 12 (ALOX12) was significantly altered between the vehicle and SAFit2-treated animals in the spinal cord (Figure 2E). The expression of the other lipoxygenases, cyclooxygenases, phospholipases, and cytochrome-$P_{450}$-oxygenases was not changed in the spinal cord after SNI (Figure 2D,F–I). Expression of arachidonate lipoxygenase 15 (ALOX15) and cytochrome-$P_{450}$-oxygenase CYP3a11 was not detectable in the spinal cord.



**Figure 2.** Gene expression of ceramide synthases, lipoxygenases, phospholipases, and epoxygenases in the spinal cord of nerve-injured mice after additional SAFit2 treatment. (**A**) Schematic illustration of the experimental procedure: SNI surgery was performed followed by treatment with either 10 mg/kg SAFit2 or the vehicle on six consecutive days (days five to ten) after SNI, as depicted in Figure 1. The gene expression of ceramide synthases (**B,C**), lipoxygenases (**D–F**), phospholipases (**G,H**), and epoxygenase (**I**) was measured 21 days after the surgery. The data are displayed as the mean ± SEM of technical replicates of three to four mice per treatment. * $p < 0.05$, as per one-way ANOVA with a Tukey's post-hoc test. Abbreviations: SAFit2: selective antagonist of FKBP51 by induced fit 2; SNI: spared nerve injury; CerS: ceramide synthase; ALOX: arachidonate lipoxygenase; COX2: cyclooxygenase 2; cPLA2: cytosolic phospholipase A2; iPLA2: calcium-independent phospholipase A2; CYP2J6: cytochrome P450 family 2 subfamily j polypeptide 6.

### 2.2. The Influence of SAFit2 on Lipid Levels after SNI

Next, we performed an untargeted lipid screening using LC-HRMS to investigate the influence of SAFit2 on lipids after nerve injury. Therefore, we analyzed the lipid distribution in L4-L6 DRGs and the respective parts of the spinal cord 21 days after SNI surgery (Figure 3). In the lipid screening, we detected 249 lipids from 14 classes—fatty acids, ceramides, diglyerides, lysophosphatidylcholines, -ethanolamines, -glycerols, -inositols, phosphatidylcholines, -ethanols, -glycerols, -inositols, -serines, sphingomyelins, and triglycerides—which can be partially further divided into subclasses (Figure 3A). For further analysis, the analytes were sorted into their lipid classes and ipsilateral values and normalized to the contralateral values, performed for every lipid. In addition, the differences between the normalized treatments were compared in heat maps for the spinal cord (Figure 3B) and lumbar DRGs (Figure 3C), separately. For lipids in the spinal cord, only slight changes were observed after nerve injury (Figure 3B). Nevertheless, some lipid classes show minor trends, such as the downregulation of fatty acids or the upregulation of lysophosphatidylethanols and phosphatidylcholines after SNI. However, these effects could be counteracted with a 10 mg/kg SAFit2 treatment after SNI. In contrast, the alterations between the treatments were stronger in the lumbar DRGs comparing the vehicle and SAFit2 treatment after SNI (Figure 3C). The classes of ceramides, diglycerides, fatty acids, lysophosphatidylglycerols, and phosphatidylcholins were clearly downregulated in the vehicle-treated SNI animals compared to the sham-treated animals. Whereas, the SAFit2 treatment counteracted this downregulation after nerve injury, adjusting the concentrations to levels comparable to the sham. In contrast, the lysophosphatidylcholins and lysophosphatidylethanols were upregulated after SNI in the vehicle-treated group while these lipids were less upregulated in the SAFit2-treated group. In summary, SAFit2 seems to compensate the SNI-induced alterations and shifts in lipid synthesis and metabolism in the respective tissues.

For investigating the effect of SAFit2 on individual lipids, we plotted the logarithmic $p$-value, which was calculated with of a two-way ANOVA and Tukey's post-hoc test, of the two treatments against the logarithmic fold change in volcano plots for DRGs, as we have seen major differences in this tissue (Figure 3D). In Figure 3D (top), we compared the vehicle-treated SNI animals with sham animals to elucidate the impact of nerve injury on lipids, discovering four significantly altered lipids. In this comparison, the C16 dihydroceramide (d18:0/16:0), the 36:4 diglyceride, and the polyunsaturated fatty acid docosahexaenoic acid were significantly downregulated, whereas the 16:1 ether-lysophosphatidylcholine was significantly upregulated after nerve injury. Interestingly, the comparison of the vehicle and SAFit2-treated SNI animals revealed only the C16 dihydroceramide (d18:0/16:0) as a significantly altered lipid (Figure 3D (bottom)).

For showing the whole LC-HRMS dataset, the relative amount of each individual lipid is displayed in violin plots in Supplementary Material Figures S1–S3 for DRGs, and for the spinal cord samples, in Supplementary Material Figures S4–S6. The internal standards and the LC-gradient used for the analysis are highlighted in Tables S1 and S2. Summarizing the results from this broad screen, we detected that some lipid mediators, such as the C16 dihydroceramide (d18:0/16:0), 36:4 diglyceride, and docosahexaenoic acid, were significantly downregulated, and the 16:1 ether-lysophosphatidylcholine was significantly upregulated after SNI. However, the C16 dihydroceramide (d18:0/16:0) was the only lipid that was significantly altered comparing the vehicle and SAFit2 treatment after nerve injury. Based on this, we hypothesized that this C16 dihydroceramide might play a crucial role in the analgesic mechanism of SAFit2.
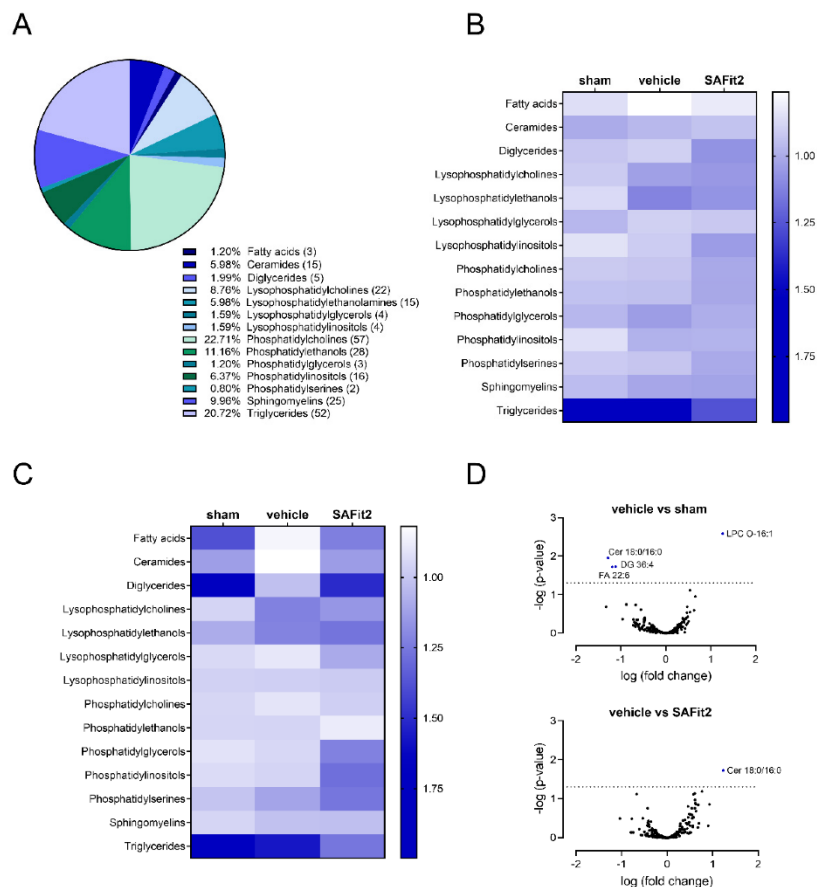
**Figure 3.** LC−HRMS lipid analysis of lumbar DRGs (L4−L6) and the respective segments of spinal cord from nerve-injured mice after additional SAFit2 treatment. (**A**) Distribution of the number of measured analytes per lipid class in LC-HRMS analysis. (**B**) Abundancy of lipid classes in the spinal cord of the vehicle and 10 mg/kg SAFit2 treated SNI animals as well as sham animals. (**C**) Lipid levels of the lipid classes in lumbar DRGs of the vehicle and 10 mg/kg SAFit2-treated animals after SNI, and the sham animals. (**D**) Volcano plots comparing the lipid levels of the sham and vehicle-treated SNI animals (top) and vehicle and SAFit2-treated animals after SNI (bottom). A *p*-value of 0.05 was determined as a threshold for significance, which was calculated with a two-way ANOVA with a Tukey's post-hoc test. All significantly altered lipids were labeled and depicted in blue. Abbreviations: DRGs: dorsal root ganglia; LC−HRMS: liquid chromatography–high-resolution mass spectrometry; SAFit2: selective antagonist of FKBP51 by induced fit 2; SNI: spared nerve injury.

### 2.3. C16 Dihydroceramide Desensitizes the TRPV1 Channel in Sensory Neurons

Based on the previous findings that SAFit2 reduces mechanical hypersensitivity after nerve injury [9], we investigated the influence of the C16 dihydroceramide (d18:0/16:0), which is named as C16 or C16 dihydroceramide in the following, on pain-sensing calcium channels. Firstly, we characterized the impact of C16 on the TRPV1 channel, as it is the most important and most abundantly expressed pain-mediating, ligand-gated calcium channel in sensory neurons, and plays a crucial role in the development of various pain conditions, including neuropathic pain [26].

To investigate the impact of C16 on TRPV1, we isolated sensory neurons from DRGs of naïve mice and analyzed the calcium transients of TRPV1 after C16 treatment in live cell

calcium imaging (Figure 4). For comparing the calcium transients with and without C16 treatment, a protocol was established in which the TRPV1 was stimulated twice: first, only with the agonist capsaicin, and second, with the pre-incubation of C16 (2 min), to verify whether C16 has an influence on the channel-mediated calcium influx (Figure 4A). Interestingly, we detected that C16 concentration-dependently reduced the calcium transients of TRPV1 in sensory neurons (Figure 4B), after comparing the different conditions using a one-way ANOVA with a Tukey's multiple-comparison test.
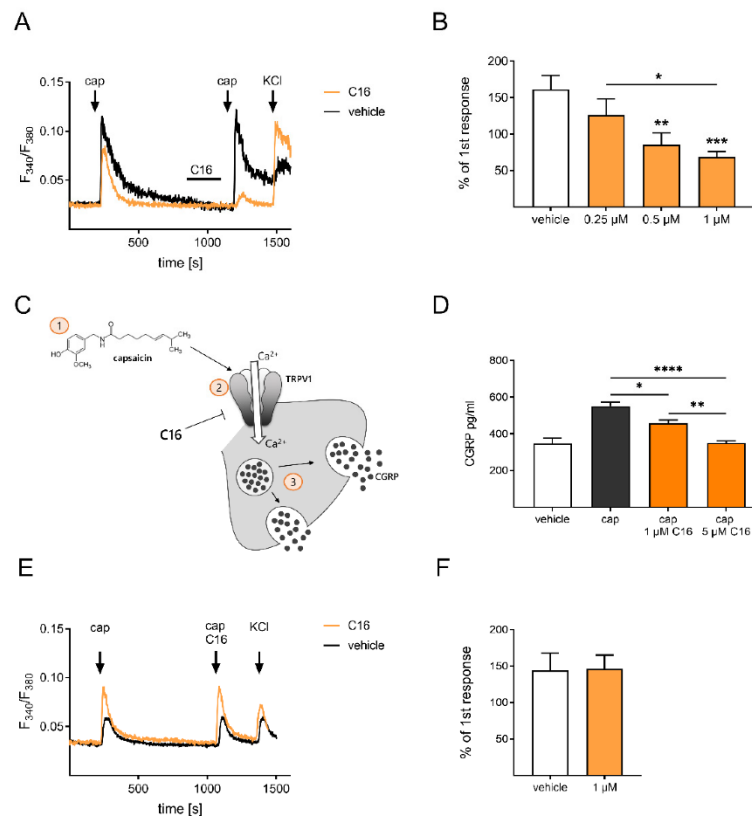


**Figure 4.** C16 dihydroceramide causes a desensitization of the TRPV1 channel and subsequently leads to a reduced CGRP release from primary sensory neurons. (**A**) Representative calcium responses of sensory neurons, which were pre-treated with C16 for 2 min, followed by an activation of the TRPV1 channel with the agonist capsaicin (100 nM, 30 s). Afterwards, the cells were treated with potassium chloride (50 mM, 45s) to discriminate reacting cells as neurons. (**B**) Quantification of the two calcium responses in (**A**) after capsaicin stimulation (untreated and C16 treated). (**C**) The schematic illustration displays the downstream signaling of TRPV1 after capsaicin activation, which results in the release of CGRP from sensory neurons. A scheme depicting the mechanism was designed using images from motifolio. (**D**) CGRP amount measured in the supernatant of C16 and capsaicin treated sensory neurons. (**E**) Representative calcium responses of sensory neurons, which were treated with both capsaicin (100 nM, 45 s) and C16 (1 μM, 45 s) at the same time point. (**F**) Quantification of the double stimulation from (**E**), ruling out antagonist properties of C16. (**B,D**) Each condition displays the mean $\pm$ SEM of 23–38 measured primary sensory neurons. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, and **** $p < 0.0001$, as per one-way ANOVA with a Tukey's post-hoc test. Abbreviations: CGRP: calcitonin gene-related peptide; cap: capsaicin; KCl: potassium chloride; TRPV1: transient receptor potential cation channel subfamily V member 1.

Next, we further evaluated whether this reduced calcium flux has an impact on the TRPV1 downstream signaling pathway. Therefore, the release of the peptide calcitonin gene-related peptide (CGRP) was assessed in primary sensory neuron cultures via ELISA. The neuropeptide CGRP was released from sensory neurons due to increasing intracellular calcium concentrations and functions as a mediator in neuropathic pain (Figure 4C) [27,28]. Interestingly, we observed that the C16 concentration dependently also reduced the CGRP amount in the supernatant of the primary sensory neurons, which were treated with capsaicin beforehand (Figure 4D). Furthermore, the concentration dependency was considered as statistically significant after performing a one-way ANOVA with a Tukey's multiple-comparison test. Based on these results, we concluded that C16 reduces TRPV1-mediated calcium transients and subsequently reduces CGRP-mediated neuroinflammation.

Since many direct TRPV1 antagonists lead to severe side effects and failed in clinical trials [29], we next determined whether C16 inhibits or desensitizes the TRPV1 channel. Therefore, we changed the calcium imaging protocol in which we left out the pre-incubation step with C16, this time applying C16 together with capsaicin at the second stimulation time point (Figure 4E). However, we detected no alterations in the TRPV1-mediated calcium flux between the first and second stimulus (Figure 4F), summarizing that C16 does not directly inhibit the TRPV1 channel.

Next, we tried to elucidate how C16 reduces TRPV1-mediated calcium transients. We hypothesized that the reduced TRPV1 open probability is due to the desensitization of TRPV1, which may be indirectly mediated by a G protein coupled receptor (GPCR). To test this hypothesis, we performed a GPCR screen using three reporter assays to investigate the effect of C16 on 314 different GPCRs (Supplementary Material Figure S7, Table S3). However, we did not detect any C16-induced activation of a GPCR, concluding that C16 probably affects the TRPV1 open probability indirectly, possibly via interacting with the cell membrane.

### 2.4. C16 Dihydroceramide Does Not Affect TRPA1 and P2X3 in Primary Sensory Neurons

For further characterizing C16 in the context of pain, we examined the influence of C16 on other TRP channels. As a first approach, we applied 1 μM and 10 μM C16 to sensory neurons to investigate whether C16 activates any calcium channels in sensory neurons (Supplementary Material Figure S8). However, we did not detect any calcium transients after the stimulation with C16.

Since we detected an influence of C16 on the TRPV1 channel, and since the TRPA1 channel (transient receptor potential ankyrin 1) is both co-expressed in a subgroup of TRPV1-positive sensory neurons as well as closely associated to the TRPV1, we also assessed the impact of C16 on the TRPA1 channel [30]. To examine this, we again performed a protocol involving two stimuli, however this time using allyl isothiocyanate (AITC) as the gold standard agonist of the TRPA1 channel (Figure 5A). We detected a slight increase in calcium transients after a pre-incubation with C16 compared to the vehicle control (Figure 5B). Nevertheless, the calcium transients were also increased after a pre-incubation with cremophor, using cremophor EL as a second, more lipophilic vehicle control. Based on these results, we suggest that C16 and cremophor EL does not sensitize the TRPA1; however, it increases the solubility of the lipophilic agonist AITC in Ringer's solution, which leads to a more efficient stimulation of sensory neurons.

Moreover, we analyzed the effect of C16 on the purinergic ligand-gated calcium channel P2X3, since it also plays a critical role in the development of neuropathic pain [31]. To stimulate the P2X3 channel, we used $\alpha,\beta$-methyleneadenosine 5'triphosphate trisodium salt (meATP) as agonist (Figure 5C). As we compared calcium transients of P2X3 with and without C16 pre-incubation, we detected no differences (Figure 5D). In summary, we observed that C16 does not directly activate any calcium channel, has a desensitizing effect on TRPV1, and no influence on the activity of TRPA1 and P2X3.
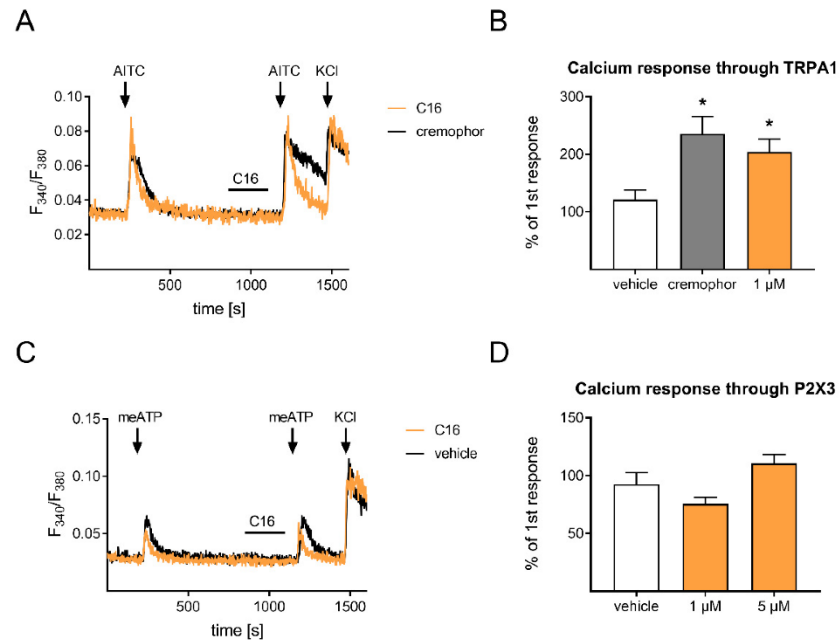
**Figure 5.** C16 dihydroceramide has no influence on the activity of TRPA1 and P2X3 in primary sensory neurons. (**A**) Representative calcium responses of sensory neurons, which were pre-incubated with C16 (2 min) and stimulated with the TRPA1 agonist AITC (100 μM, 45 s) followed by a KCl (50 mM) treatment as a positive control. (**B**) Quantification of the two calcium responses in (**A**) after AITC stimulation (untreated and C16 treated). (**C**) Representative calcium responses of sensory neurons, which were pre-incubated with C16 (2 min) and stimulated with the P2X3 agonist meATP (50 μM, 30 s) followed by a KCl (50 mM) treatment as a positive control. (**D**) Quantification of the two calcium responses in (**C**) after meATP stimulation (untreated and C16 treated). (**B,D**) The data represents the mean ± SEM of 23–30 sensory neurons per group. * $p < 0.05$, as per one-way ANOVA with a Tukey's post-hoc test. Abbreviations: TRPA1: transient receptor potential cation channel subfamily A member 1; AITC: allyl isothiocyanate; meATP: α,β-methyleneadenosine 5′triphosphate trisodium salt; KCl: potassium chloride.

### 2.5. C16 Dihydroceramide Reduces Thermal Hypersensitivity after Capsaicin Treatment

As final step, we wanted to examine whether C16 can influence the thermal hyper-sensitivity of capsaicin-treated mice, as we detected a reduced TRPV1 activity after C16 treatment in vitro. Therefore, the thermal pain threshold of every mouse was assessed with the radiant heat test two times before treatment. Then, the mice were treated intraplanar with either 50 μM C16 or vehicle and 400 μM capsaicin. Afterwards, the thermal sensi-tivity of the mice paws was assessed over 4 h with a radiant heat test. Interestingly, we detected that the C16 treatment prevented the development of thermal hypersensitivity after capsaicin treatment in mice (Figure 6). In addition, the paw withdrawal latencies were significantly different comparing the vehicle and C16-treated mice for the time points 0.5, 1, 2, 3, and 4 h, as per assessment with a two-way ANOVA with Bonferroni's post-hoc test. These results reveal the analgesic properties of C16, pointing out that the desensitization of TRPV1 reduces the thermal hypersensitivity of mice.
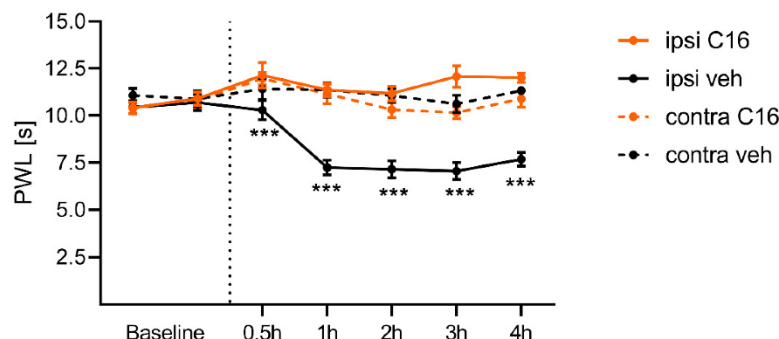
**Figure 6.** C16 dihydroceramide reduces the thermal hypersensitivity after capsaicin treatment in mice. We analyzed the baseline levels the day before and on the day of treatment. The mice were treated intraplantarly with either capsaicin and the C16 dihydroceramide or capsaicin and vehicle to analyze the analgesic properties of C16. The thermal pain threshold was assessed 30 min, 1 h, 2 h, 3 h, and 4 h after treatment. The data represent the mean $\pm$ SEM from seven mice per group., *** $p < 0.001$, as per two-way ANOVA with Bonferroni's post-hoc test.

## 3. Discussion

Neuropathic pain affects around 10 percent of the general population and is induced by lesions and diseases of the somatosensory system, infections, or toxic substances [2]. However, the available therapies are very scarce because of their low efficacy and severe adverse effects [32]. Based on these circumstances, there is a high medical need for novel, safe, effective treatment approaches for neuropathic pain [32]. Nevertheless, the development of many therapy strategies failed, having faced the challenge that neuropathic pain is a multifunctional event mediated by various mediators with pleiotropic effects. In addition, the underlying mechanisms of this complex pain state are barely understood yet. In line with this, investigation of the endogenous mechanisms and alterations, for example, after nerve injury, is becoming increasingly important. Therefore, we tried to discover the alterations in lipids after SNI and assessed the influence of the potential therapeutic approach SAFit2 on these alterations.

The distribution and abundance of lipids affects various mechanisms and conditions, such as the structure of the myelin sheath, the fluidity of membranes, energy storage, and signaling pathways. In the last decades, a growing number of endogenous lipid mediators has been described to be involved in several signaling pathways and to modulate the activity of the TRP channels in sensory neurons [33]. Likewise, these endogenous lipid mediators play a crucial role in the context of persistent pain states, such as neuropathic pain and chronic pain [12,19,33,34]. Especially alterations and dysregulations in specific lipids were associated with nerve dysfunctions and neuropathic pain. A prominent example for lipid metabolism dysregulation, which results in neuropathic pain, is treatment with the cytostatic bortezomib, which significantly alters the sphingosine/ceramide pathway [20]. In contrast, the FDA-approved, S1P receptor-inhibiting substance fingolimod was shown to reduce neuropathic pain after nerve injury and in multiples sclerosis as it targets the sphingosine/ceramide pathway [35,36]. Nevertheless, alterations in the anandamide metabolism were previously associated with the development of chronic pain [37]. Furthermore, several studies have pointed out the importance of anti-inflammatory and analgesic lipid mediators, such as resolvins, in the resolution of inflammation and pain [38–41].

Here, we reveal that the selective FKBP51 inhibitor SAFit2 is capable to counteract pathophysiological alterations in lipid metabolism after SNI. More specifically, we observed that SAFit2 counteracts alterations in the expression of lipid-oxidizing enzymes as well as dysregulations in lipid classes. Likewise, the lipid classes of free fatty acids and ceramides were markedly downregulated in lumbar dorsal root ganglia and spinal cord after SNI, which were not downregulated in SAFit2-treated SNI animals. Interestingly, we could not

observe any difference in arachidonic acid levels after SAFit2 treatment, indicating that the synthesis of eicosanoids, such as prostanoids, EETs (epoxyeicosatrienoic acids), and HETEs (hydroxyl-eicosatetraenoic acids), is unaffected.

Nevertheless, we detected a significant reduction in the free fatty acid (and precursor of several specialized pro-resolving mediators (SPMs)) docosahexaenoic acid (FA 22:6, DHA) in the vehicle-treated SNI mice. However, a SAFit2 treatment was capable to restore the physiological level of DHA after SNI to a level comparable to sham mice. Since docosahexaenoic acid metabolites were identified as endogenous TRP inhibitors, which can contribute to the relief of inflammation and pain [33], these results indicate that SAFit2 can restore omega-3 polyunsaturated fatty acids as precursors for potentially antihyperalgesic lipids, thereby resolving mechanical hypersensitivity after SNI [9]. Nonetheless, synthesis, signaling, and occurrence of SPM in tissues have been the subject of controversies, because of the inability and inconsistency of their detection with analytical methods in biological samples [42].

Interestingly, we detected one lipid in the untargeted screening that shows both a significant downregulation after SNI and a significantly increased level after SAFit2 treatment. These alterations reveal the C16 dihydroceramide as a novel and crucial lipid mediator in the context of neuropathic pain. However, the knowledge about ceramides is very limited in the research field of pain, although the group of sphingosines from the sphingosine/ceramide pathway are well characterized in the context of neuropathic pain [21–23,43–48]. Based on that, we decided to assess the impact of the C16 dihydroceramide on pain mediating TRP channels and its function in acute pain. We showed that the C16 dihydroceramide reduces the sensitivity of the mechanical and thermal pain-mediating TRPV1 channel. However, it does not inhibit the calcium channel directly, which is quite a crucial property, since many TRPV1 antagonists lead to hyperthermia as they disturbed the complex regulatory role of TRPV1 for normal body temperature maintenance [13,29,49]. Based on that, it might be beneficial to maintain the TRPV1 activity in a physiological state and to reduce its sensitization, which mainly occurs in inflammation-related pain states. In conclusion, the desensitizing effects of the C16 dihydroceramide further contribute to the analgesic effects of SAFit2, which are capable of mediating pain relief after neve injury [9].

It is still unclear how SAFit2 alters the levels of the C16 dihydroceramide. Possibly, SAFit2 is involved in regulating the expression of ceramide synthase 5 (CerS5) in sensory neurons. This may be caused by the interference of SAFit2 in NF-κB signaling [9]. In addition, it has previously been shown that NF-κB signaling can be influenced by the ceramide synthase 4 [50]. SAFit2 may also enhance or restore activity or expression of phospholipases, leading to enhanced release and availability of free fatty acids after treatment.

Since we detected no direct effect of the C16 dihydroceramide on TRPV1, we analyzed whether the lipid mediator might desensitize the TRPV1 indirectly via a G protein coupled receptor. However, we also did not detect any activation of a G protein coupled receptor, when screening a library consisting of 314 GPCRs. Based on these results, we suggest that the C16 dihydroceramide modulates the TRPV1 indirectly, as it alters the biophysical plasma membrane properties or may act as an allosteric modulator of TRPV1, as was previously described for cholesterol and other lysophospholipids [51–53]. In addition, we observed an influence of the C16 dihydroceramide on the downstream signaling of TRPV1, as it reduces the release of the pro-inflammatory neuropeptide CGRP in sensory neurons. CGRP is secreted upon enhanced neuronal activity and can cause neuroinflammation via GPCRs, leading to the development of persistent pain states [27,28,54]. We further confirmed these analgesic properties in an acute mouse model in which we could show that the C16 dihydroceramide significantly reduced capsaicin-induced thermal hypersensitivity to a physiological normal level. Here, we identify the C16 dihydroceramide as a novel antihyperalgesic lipid mediator and observe the effects of SAFit2 on the expression of ceramide synthase 5 and on the free fatty acid levels in nervous tissue. These findings help explain the mechanism of SAFit2-meditated analgesic effects and they provide additional

evidence for the involvement of lipids as a regulatory interface for antihyperalgesia in nervous tissue in the context of neuropathic pain.

A limitation of the study is the lack of female mice in our in vivo experiments. Previous studies revealed evidence for sex differences in inflammatory pain models and the development of neuropathic pain [55]. However, the SAFit2-inhibiting target FKBP51 was previously shown to be sex-independently involved in the development of neuropathic pain [4]. In addition, the sex dimorphism in inflammatory and neuropathic pain models was attributed to the involvement of different immune cells in the development of pain [56]. However, our in vivo study focused on acute TRPV1-mediated nociception, which is sex independent and does not involve the influence of any immune cells.

In summary, we report that the highly selective FKBP51 inhibitor SAFit2 is capable of counteracting the alterations in lipid metabolism and signaling after SNI. More specifically, SAFit2 restored the levels of the C16 dihydroceramide, which reduced the TRPV1 activity, as well as the subsequent secretion of CGRP in primary sensory neurons. Both effects indicate the analgesic properties of the C16 dihydroceramide, revealing this lipid as a novel and crucial lipid mediator in the context of neuropathic pain.

Collectively, these findings can mechanistically explain the analgesic effects of SAFit2 in vivo. They also raise the question whether C16 dihydroceramide itself can serve as a treatment for neuropathic pain. However, due to the short half-life and limited distribution and bioavailability of this lipid [57,58], we do not consider it as a direct therapeutic option for the treatment of neuropathic pain.

## 4. Materials and Methods

### 4.1. Spared Nerve Injury Surgery and SAFit2 Treatment

For all animal experiments, we obtained wild type mice with a C57BL/NRj background from commercial breeding companies, such as Janvier and Charles River. Mice were matched regarding age (8–10 weeks) for the in vivo treatments. For analyzing the effect of SAFit2 after nerve injury, we conducted the well-characterized and robust spared nerve injury (SNI) model. Therefore, surgery was performed under anesthesia, in which we firstly ligated the common peroneal and tibial branches of the sciatic nerve and then dissected the sciatic nerve distally from the ligature on the level of the knee joint. The third nerve branch was left intact [59]. After surgery, we treated the mice intraperitoneally with either 10 mg/kg SAFit2 or vehicle (PBS supplemented with 5% Tween, 5% PEG400, and 0.7% ethanol) twice daily from Day 5 to 10.

### 4.2. Tissue Isolation

On Day 21 after surgery, the mice were sacrificed, and lumbar dorsal root ganglia (DRGs) (L4–L6) and the respective segments of the spinal cord (SC) were dissected. The respective tissue was isolated from injured (ipsilateral) and unimpaired (contralateral) sites and frozen in liquid nitrogen for either RNA isolation or LC-HRMS analysis.

### 4.3. Quantitative Real-Time PCR

The total RNA was isolated from lumbar DRGs (L4-L6) and the respective segments of spinal cord using the mirVana miRNA Isolation Kit (Applied Biosystems, Waltham, MA, USA). The isolation was performed according to the manufacturer's instructions. As a next step, we measured the RNA concentrations using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The reverse transcription was conducted with 200 ng RNA for DRG samples and 400 ng RNA for spinal cord samples using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), compliant with the manufacturer's instructions. Quantification of gene expression was assessed with a quantitative real-time PCR, which was performed using the TaqMan® Gene Expression Assay System with respective assay primers (Thermo Fisher Scientific) and the QuantStudio™ Design & Analysis Software v 1.4.3 (Thermo Fisher Scientific). The experimental settings were adjusted compliant to the manufacturer's instructions and using the assay primers

listed in Table 1. The raw data were evaluated with the ΔΔC(T) method to calculate the relative expression of the target genes, as described previously [60,61].

**Table 1.** List of used TaqMan® Gene Expression Assays.

| Target | Gene | Article Number | Company |
|---|---|---|---|
| ALOX12 | Arachidonate 12-lipoxgenase | Mm00545833_m1 | Thermo Fisher |
| ALOX15 | Arachidonate 15-lipoxgenase | Mm00507789_m1 | Thermo Fisher |
| ALOX5 | Arachidonate 5-lipoxgenase | Mm01182747_m1 | Thermo Fisher |
| CerS5 | Ceramide synthase 5 | Mm00556165_m1 | Thermo Fisher |
| CerS6 | Ceramide synthase 6 | Mm00510998_m1 | Thermo Fisher |
| COX2 | Cytochrome c oxidase subunit II | Mm03294838_g1 | Thermo Fisher |
| CYP2J6 | Cytochrome P450, family 2, subfamily j, polypeptide 6 | Mm01268197_m1 | Thermo Fisher |
| CYP3a11 | Cytochrome P450, family 3, subfamily a, polypeptide 11 | Mm00731567_m1 | Thermo Fisher |
| GAPDH | Glceraldehyde-3-phosphate dehydrogenase | Mm99999915_g1 | Thermo Fisher |
| PLA2g4a | Phospholipase A2, group 4a | Mm00447040_m1 | Thermo Fisher |
| PLA2g4c | Phospholipase A2, group 4c | Mm01195718_m1 | Thermo Fisher |

### 4.4. Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS)

For LC-HRMS purposes, DRGs and spinal cord (SC) were homogenized using a pellet pestle mixer (Thermo Fisher scientific). A volume of 79 or 237 μL of ethanol:water (1:9, *v/v*) was added to approximately 1 mg (DRGs) or 3 mg (SC) of tissue and grinded for 30 to 60 s, including short breaks to avoid overheating.

For lipid extraction, 75 μL of internal standards, solved in methanol (see Supplementary Material), were added to 20 μL of tissue homogenate. Next, 250 μL of methyl-tert-butyl-ether (MTBE, HPLC-grade) and 50 μL of 50 mM ammonium formate (for mass spectrometry, ≥99.0 was purchased from Sigma-Aldrich, Munich, Germany) were added and the samples mixed for 1 min. After 5 min of centrifugation at ambient temperature and $20,000 \times g$, the upper phase was transferred and the lower phase reextracted using 100 μL of MTBE:methanol:water (10:3:2.5, *v/v/v*, upper phase), again followed by centrifugation. The combined upper phases were dried under a nitrogen stream at 45 °C, stored at −80 °C and redissolved in 100 μL of methanol prior to analysis. For analysis, an Exploris 480 Orbitrap mass spectrometer coupled with a Vanquish horizon LC-system (both Thermo Fisher Scientific) was used.

Chromatographic separation was performed using a Zorbax RRHD Eclipse Plus C8 column (1.8 μm 50 × 2.1 mm ID, Agilent) with a precolumn of the same type and a 14-min binary gradient. The samples were analyzed in positive and negative ionization mode with a scan range from 180 to 1500 m/z and 120,000 mass resolution. Additionally, data-dependent spectra were acquired at a 15,000 resolution and a total cycle time of 600 ms. Acquisition was performed using XCalibur software v4.4, and for evaluation, TraceFinder software v5.1 was applied (both Thermo Fisher Scientific). Lipids were identified as previously described, with a mass error of 5 ppm [62]. The first ten spinal cord samples were pooled, and multiple replicates were analyzed over the run-time to verify system stability. The results were normalized to the protein concentration, which was determined after tissue homogenization with a Bradford assay.

### 4.5. Isolation of Dorsal Root Ganglia (DRGs)

For performing assays with primary sensory neurons, naïve mice were sacrificed and DRGs isolated, which were stored in precooled HBSS, supplemented with $CaCl_2$ and $MgCl_2$ (Gibco), on ice during dissection until further purification. For purification purposes, we substituted HBSS with a collagenase/dispase solution containing 500 U/mL collagenase and 2.5 U/mL dispase diluted in neurobasal medium (Gibco). The isolated DRGs were incubated with the collagenase/dispase solution for 75 min at 37 °C. Next, we removed the

enzyme solution by centrifuging and discarding the supernatant, followed by two washing steps with neurobasal medium, supplemented with 10% FCS ($v/v$). Afterwards, the DRGs were further enzymatically dissociated via an incubation step with 0.05% trypsin ($v/v$) (Gibco) for 10 min at 37 °C. The DRGs were again washed twice with neurobasal medium containing 10% FCS. After the enzymatic dissociation, we dissociated the DRGs mechanically in neurobasal medium (Gibco) supplemented with L-glutamine (2 mM; Gibco), penicillin (100 U/mL; Gibco), streptomycin (100 µg/mL; Gibco), B-27 supplement (Gibco), and gentamicin (50 µg/mL; Gibco). Finally, 30 µL of the primary sensory neuron suspension were plated on poly-L-lysine-coated cover slips or 48-well plates and incubated at 37 °C for two hours to allow adhesion of the sensory neurons. After the attachment phase, we added another 2 mL of neurobasal medium to each dish.

### 4.6. Calcium Imaging

To analyze the calcium levels in primary sensory neurons, we stained sensory neuron cultures with Fura-2-AM for at least 45 min prior the measurement. Afterwards, the cells were washed twice with freshly prepared Ringer's solution. We also used Ringer's solution during live cell calcium imaging as we measured the baseline levels. In addition, we applied Ringer's solution after and in between stimulations for wash-out purposes. The Ringer's solution consisted of 145 mM NaCl, 1.25 mM $CaCl_2 \times 2\,H_2O$, 1 mM $MgCl_2 \times 6\,H_2O$, 5 mM KCl, 10 mM D-glucose, and 10 mM HEPES, and its pH was adjusted to a physiological level of 7.3 using NaOH. To analyze the influence of the C16 dihydroceramide on different calcium channels, we pre-incubated primary sensory neurons with the C16 dihydroceramide for two minutes (0.25–5 µM) and stimulated the cells with the following agonists, respectively: 100 nM capsaicin for 30 s (TRPV1 agonist), 100 µM allyl isothiocyanate for 45 s (AITC, TRPA1 agonist), and 50 µM α,β-methyleneadenosine 5′triphosphate trisodium salt (meATP, P2X3 agonist). Control experiments were performed with the respective volume of the corresponding vehicle, which was either DMSO or cremophor EL. The agonists as well as the C16 dihydroceramide were diluted from stock solutions in Ringer's solution to their final concentrations. The calcium live cell imaging measurements were conducted using an automated perfusion system (ValveBank II, AutoMate Scientific, San Diego, CA, USA) and recorded with a DMI4000 B Microscope, with a compact light source CTR550 HS (Leica Microsystems, Wetzlar, Germany).

### 4.7. Calcitonin Gene-Related Peptide Assay

To investigate the influence of the C16 dihydroceramide on calcitonin gene-related peptide (CGRP) secretion, the amount of CGRP was measured in the supernatant of sensory neurons after TRPV1 stimulation. Therefore, sensory neurons were isolated from DRGs as previously described and stimulated with a mixture of capsaicin and C16 dihydroceramide the next day. To prepare the primary sensory neurons for the secretion assay, we washed them with HBSS previously. Afterwards, we treated the cells with either vehicle as negative control, capsaicin as positive control, or a mixture of capsaicin and C16 dihydroceramide for 15 min. All compounds were diluted in HBSS. The amount of CGRP in the supernatant was detected with an ELISA kit from Bertin Bioreagent, in accordance with the manufacturer's instructions, measuring the absorption at 405 nm. The quantification was performed with linear regression, as described in the manufacturer's manual.

### 4.8. Screen for C16 Dihydroceramide Activated GPCRs
#### 4.8.1. β-Arrestin Assay

In total, 5000 HTLA cells were seeded into a white, transparent, and ploy-L-Lysin-coated 384-well plate from PerkinElmer. The cells were co-transfected after 6 h with a plasmid from the PRESTO-Tango GPCR library (Addgene, Watertown, MA, USA, [63]). We used a mixture of 10 ng plasmid and 0.04 µL Lipofectamine 2000 per well when performing the transfection, as described by [63]. We used GFP as a transfection control and 100 µM carbachol and the muscarinic M5 receptor as a positive control. After 24 h, the medium

was replaced by 45 µL of serum-free medium. The ligand (5 µL) was then added at a final concentration of 30 µM for approximately 24 h. Afterwards, the medium was aspirated, and the cells lysed using 50 µL of bright-Glo reagent (Promega, Madison, WI, USA) diluted 10 times with PBS. After 15 min of incubation with lysis buffer, the luminescence (endpoint, 1500 ms integration time) was measured using a flexstation 3 plate reader.

### 4.8.2. cAMP Reporter Assay

In total, 5000 HEK293 cells were seeded in a white, transparent and ploy-L-Lysin-coated 384-well plate from PerkinElmer. The cells were co-transfected after 6 h with the cAMP reporter pGL4.29 plasmid (Promega) and a plasmid from the PRESTO-Tango GPCR library (10 ng from each plasmid and 0.04 µL Lipofectamine 2000 per well). As a transfection control, we used GFP and as a positive control we used 10 nM GLP-1 acting on the GLP-1 receptor. After 48 h, the medium was replaced by 45 µL of serum-free medium for one hour. The ligand (5 µL) was then added at a final concentration of 15 µM for 5 h. Afterwards, the medium was aspirated, and the cells were lysed using 50 µL of a mixture of bright-Glo reagent (Promega) diluted 10 times with PBS. After 15 min of incubation with lysis buffer, the luminescence (endpoint, 1500 ms integration time) was determined using a flexstation 3 plate reader.

### 4.8.3. RhoA Reporter Assay

In total, 5000 HEK293 cells were seeded in a white, transparent and ploy-L-Lysin coated 384-well plate from PerkinElmer. The cells were co-transfected after 6 h with the pGL4.34 plasmid (Promega) and a plasmid from the PRESTO-Tango GPCR library (10 ng from each plasmid and 0.04 µL Lipofectamine 2000 per well). As a transfection control, we used GFP and as a positive control we used FBS on cells transfected only with the reporter plasmid pGL4.34. The medium was aspirated after 24 h, and serum-free medium was added instead. After 48 h, the medium was replaced by 45 µL of serum-free medium, and cells were incubated for one more hour. The ligand (5 µL) was then added at a final concentration of 15 µM for 5 h. Afterwards, the medium was aspirated, and the cells were lysed using 50 µL of a mixture of bright-Glo reagent (Promega) diluted 10 times with PBS. After 15 min of incubation with lysis buffer, the luminescence (endpoint, 1500 ms integration time) was measured using a flexstation 3 plate reader.

### *4.9. Radiant Heat Assay*

During the behavioral experiment, the experimenter was blinded. All animals were transferred into respective test cages for at least one hour before the measurement to allow habituation, as described previously [64]. For assessing the thermal withdrawal latency, a radiant heat test was performed using a Plantar Analgesia Meter with a high-intensity projector lamp (IITC Life Science, Woodland Hill, CA, USA). The cut-off time for this experiment was set to 20 s.

### *4.10. Data Analysis and Statistics*

All animal numbers and in vitro replicates are depicted in the figure legends. In addition, the data are displayed as the mean ± SEM. The statistical analysis was performed with GraphPad Prism software version 9. The normal distribution of the raw data was verified and confirmed with the Shapiro–Wilk test. We performed an unpaired and heteroskedastic Student's *t*-test with Welch's correction for comparing two groups for the in vitro experiments. To determine the significance between more than two groups, we conducted a one-way analysis of variance (ANOVA). In line with this, we used a two-way ANOVA when comparing more than three groups. To evaluate the significance levels for behavioral assays, we performed a two-way ANOVA with Bonferroni's multiple-comparisons post-hoc test. We considered a *p*-value lower than 0.05 as statistically significant.

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## 3.3 Supplemental file

# Supplemental Information

## The FKBP51 inhibitor SAFit2 restores the pain relieving
## C16 dihydroceramide after nerve injury

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Supplementary Figures: 8

Supplementary Tables: 3
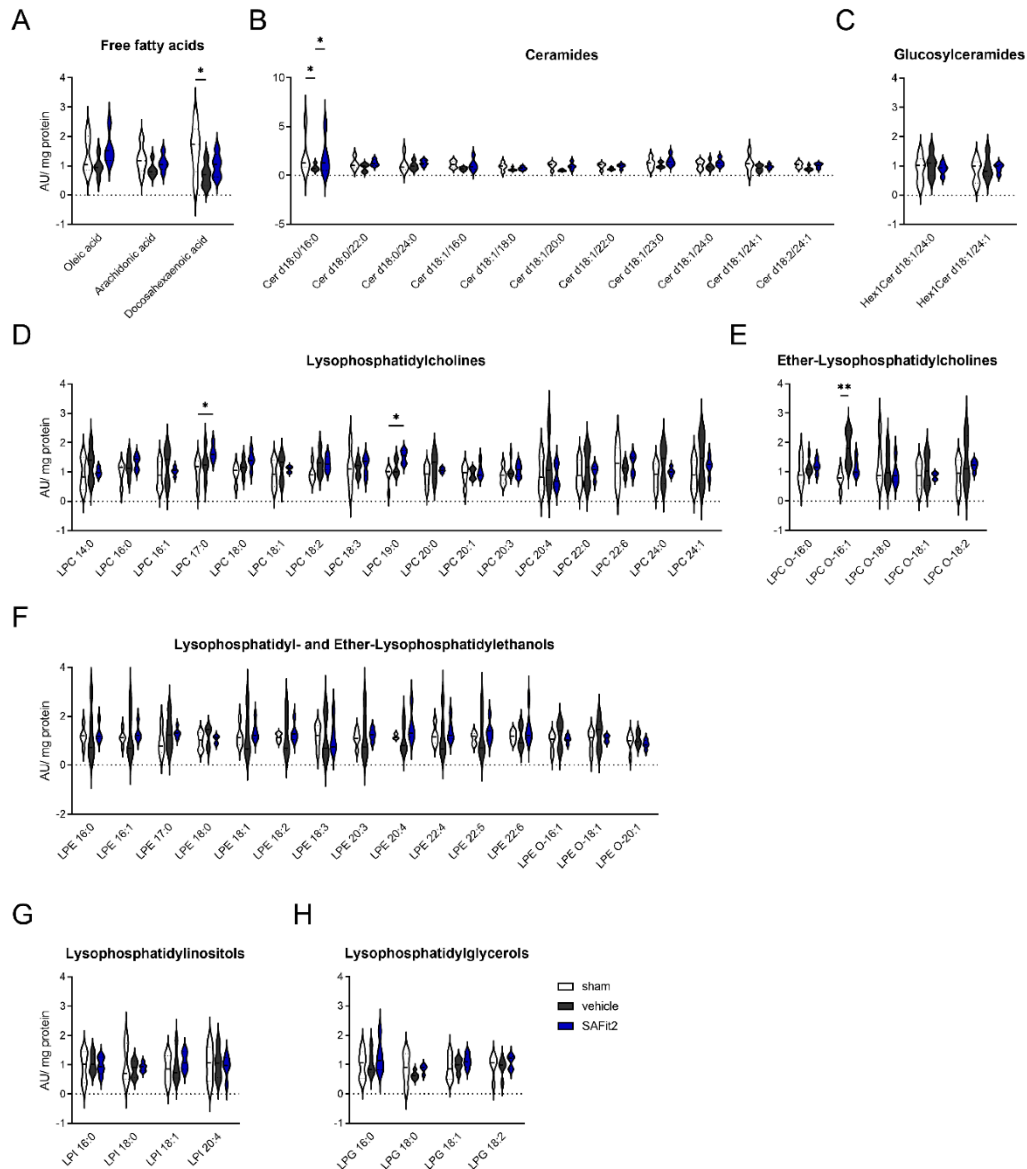
**Figure S1: Normalized lipid concentrations of free fatty acids, ceramides and lysophospholipids in lumbar DRGs 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the lumbar DRGs were isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screening. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are further grouped in classes: **(A)** fatty acids **(B)** ceramides, **(C)** glucosylceramides, **(D)** lysophohsphatidylcholines, **(E)** ether-

lysophosphatidylcholines, **(F)** lysophosphatidyl- and ether-lysophosphatidylethanols, **(G)** lysophosphatidylinositols, **(H)** lysophosphatidylglycerols. * p < 0.05 two-way ANOVA with Tukey´s post hoc test. Abbreviations: DRGs: dorsal root ganglia, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry

A

Phosphatidylcholines

B

Phosphatidylcholines

C

Ether-Phosphatidylcholines

D

Phosphatidylglycerol

E

Phosphatidylserine

F

Phosphatidyl- and Ether-Phosphatidylethanols

G

Phosphatidylinositol

sham
vehicle
SAFit2
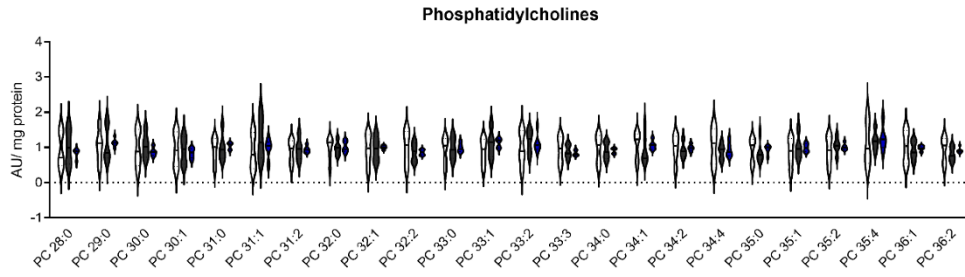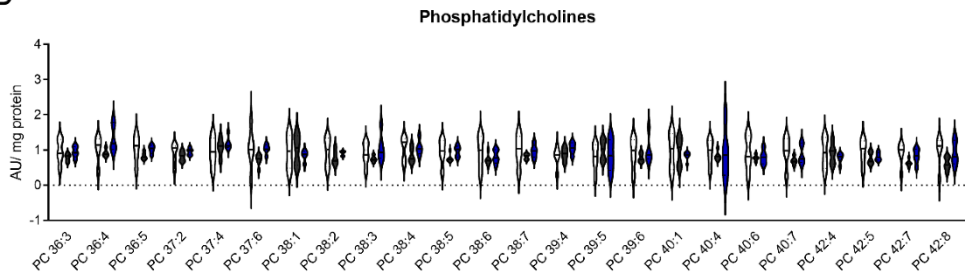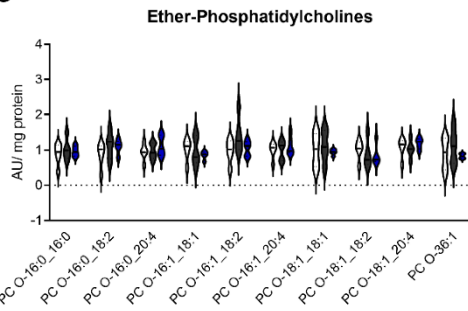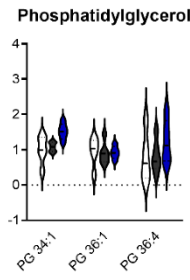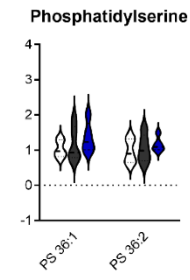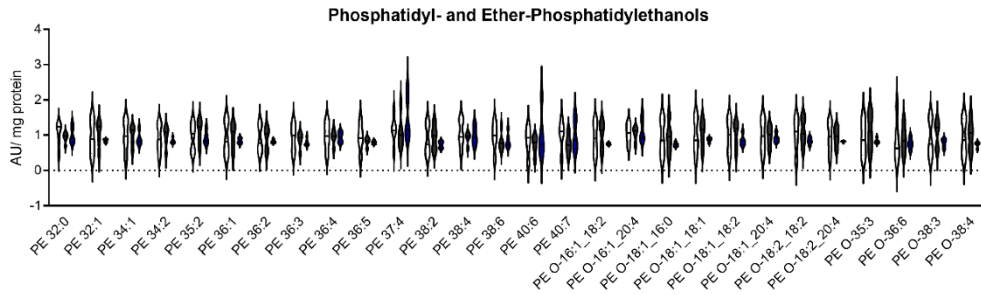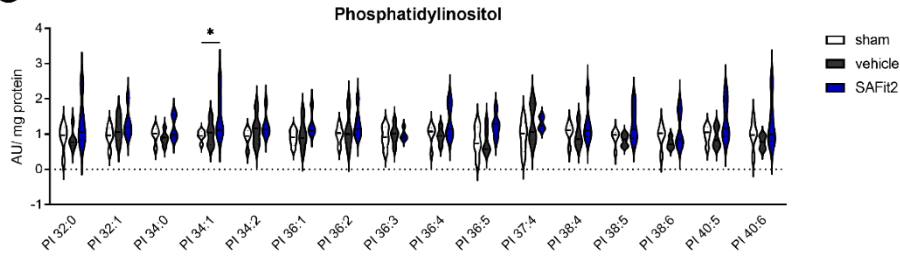
**Figure S2: Normalized lipid concentrations of phosphatidylcholines, -glycerols, -serines, -ethanols and -inositols in lumbar DRGs 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the lumbar DRGs were isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screen. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are further grouped in classes: **(A, B)** phosphatidylcolines, **(C)** ether-phosphatidylcholines, **(D)** phosphatidylglycerols, **(E)** phosphatidylserines, **(F)** phosphatidyl- and ether-phosphatidylethanols, **(G)** phosphatidylinositols. * $p < 0.05$ two-way ANOVA with Tukey´s post hoc test. Abbreviations: DRGs: dorsal root ganglia, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry

**A**
Sphingomyelins

**B**
Triglycerides

**C**
Triglycerides

**D**
Triglycerides

**E**
Diglycerides

**F**
Acyl carnitines

sham
vehicle
SAFit2

**Figure S3: Normalized lipid concentrations of sphingomyelins, triglycerides, diglycerides and acyl carnitines in lumbar DRGs 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the lumbar DRGs were isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screen. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are further grouped in classes: **(A)** sphingomyelins, **(B-D)** triglycerides, **(E)** diglycerides, **(F)** acyl carnitines. * $p < 0.05$ two-way ANOVA with Tukey´s post hoc test. Abbreviations: DRGs: dorsal root ganglia, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry
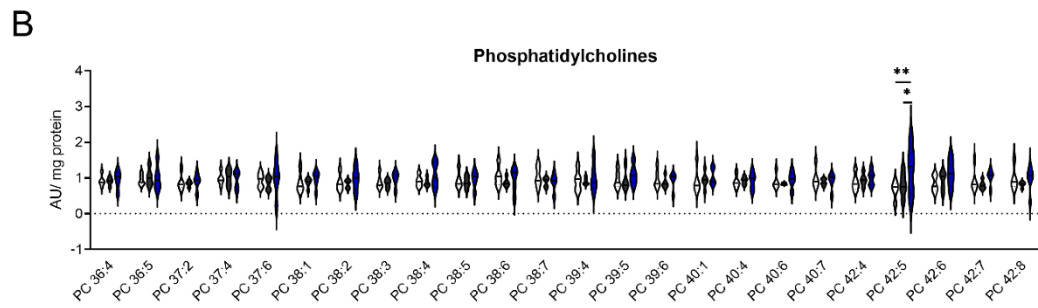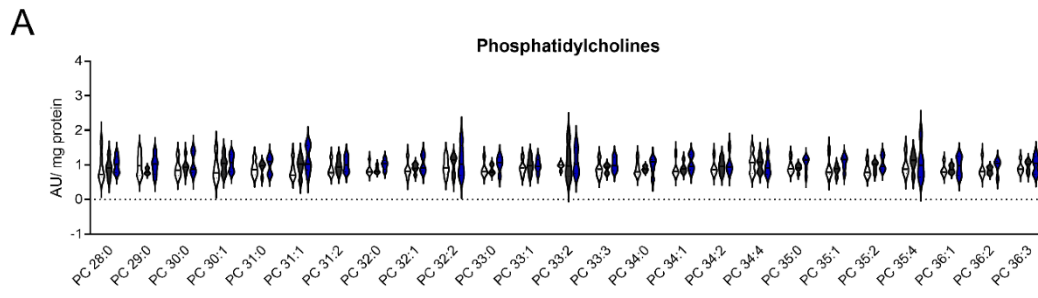
**Figure S4: Normalized lipid concentrations of free fatty acids, ceramides and lysophospholipids in spinal cord 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the spinal cord was isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screen. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are grouped in classes: **(A)** fatty acids **(B)** ceramides, **(C)** glycosylceramides, **(D)** lysophohsphatidylcholines, **(E)** ether-lysophosphatidylcholines, **(F)** lysophosphatidyl-
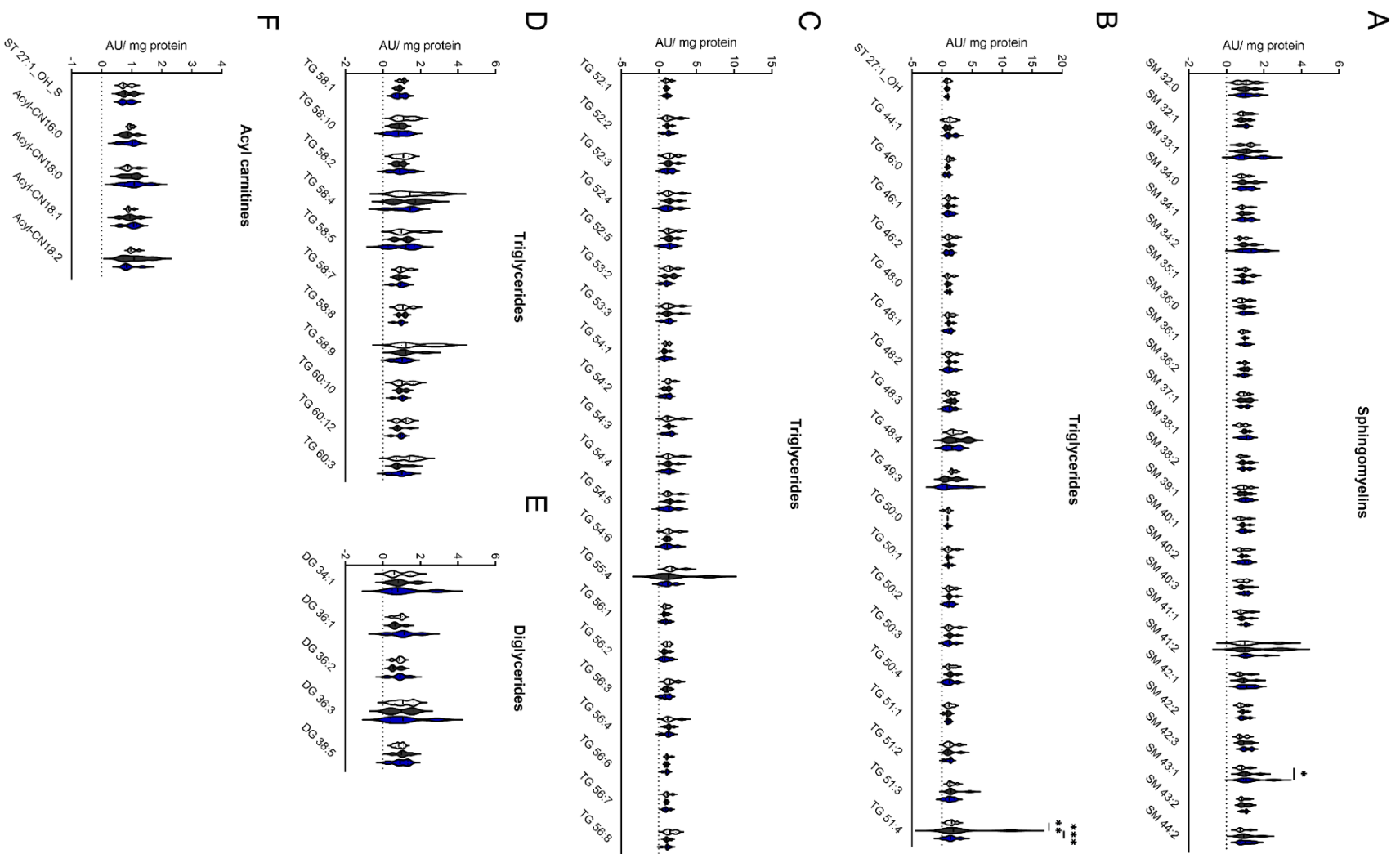
and ether-lysophosphatidylethanols, **(G)** lysophosphatidylinositols, **(H)** lysophosphatidylglycerols. * p < 0.05 two-way ANOVA with Tukey´s post hoc test. Abbreviations: SC: spinal cord, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry
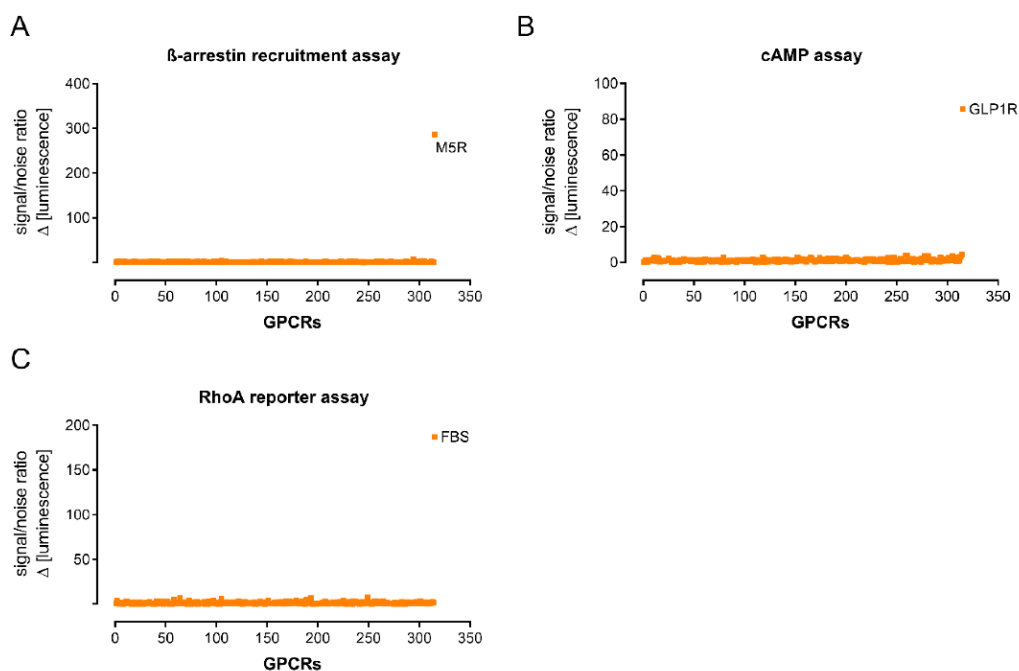
**A** Phosphatidylcholines

**B** Phosphatidylcholines

**C** Ether-Phosphatidylcholines

**D** Phosphatidylglycerols

**E** Phosphatidylserines

**F** Phosphatidyl- and Ether-Phosphatidylethanols

**G** Phosphatidylinositols
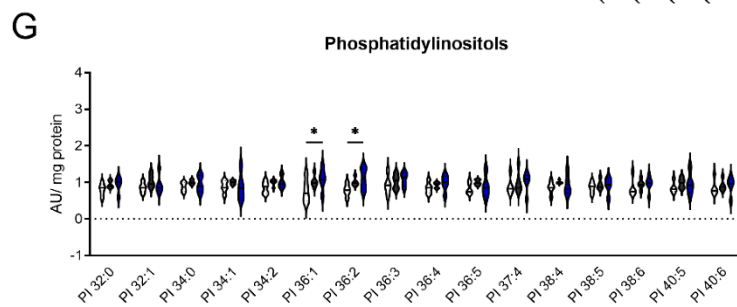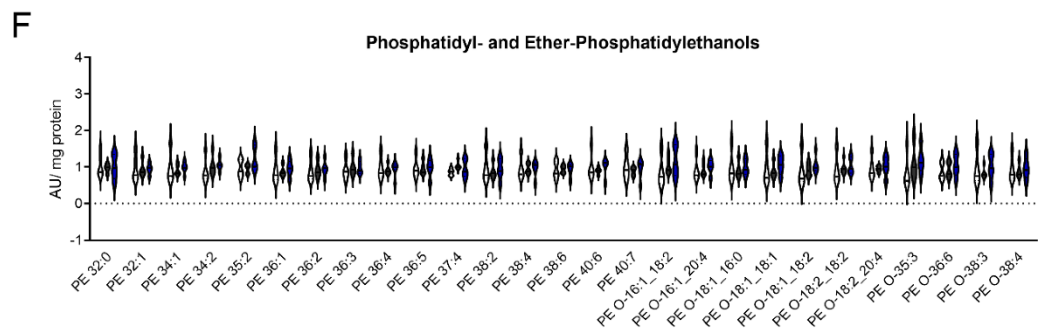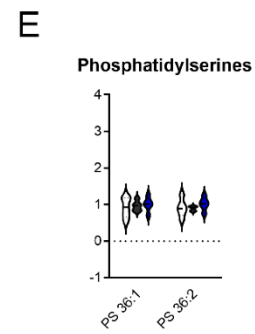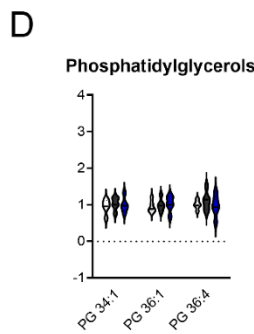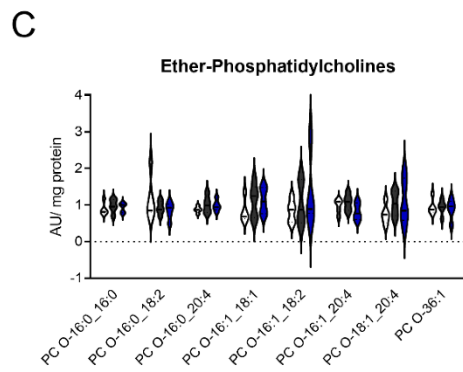
**Figure S5: Normalized lipid concentrations of phosphatidylcholines, -glycerols, -serines, -ethanols and -inositols in spinal cord 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the spinal cord was isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screen. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are grouped in classes: **(A, B)** phosphatidylcholines, **(C)** ether-phosphatidylcholines, **(D)** phosphatidylglycerols, **(E)** phosphatidylserines, **(F)** phosphatidyl- and ether-phosphatidylethanols, **(G)** phosphatidylinositols. * $p < 0.05$ two-way ANOVA with Tukey´s post hoc test. Abbreviations: SC: spinal cord, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry

**Figure S6: Normalized lipid concentrations of sphingomyelins, triglycerides, diglycerides and acyl cyanides in spinal cord after 21 days after SNI.** Mice underwent SNI surgery and were treated with either vehicle or 10 mg/kg SAFit2 from day five to ten after the surgery. After 21 days, the spinal cord was isolated from ipsilateral and contralateral sides, lysed and lipids were measured in an untargeted LC-HRMS screen. The distribution of normalized lipids is displayed treatment-wise for each lipid, which are grouped in classes: **(A)** sphingomyelins, **(B-D)** triglycerides, **(E)** diglycerides, **(F)** acyl carnitines. * $p < 0.05$ two-way ANOVA with Tukey´s post hoc test. Abbreviations: SC: spinal cord, SAFit2: selective antagonist of FKBP51 by induced fit 2, SNI: spared nerve injury, LC-HRMS: liquid chromatography-high-resolution mass spectrometry

**Figure S7: Results of a GPCR screen using C16 dihydroceramide as a potential interaction partner.** To screen a library of 314 GPCRs in search for a potential receptor activated by the C16 dihydroceramide, a β-arrestin recruitment assay **(A)**, a cAMP assay **(B)**, and a RhoA reporter assay **(C)** were performed. The C16 was added at a final concentration of 30 µM **(A)** or 15 µM **(B,C)**. As positive controls 100 µM carbachol and the muscarinic M5 receptor **(A)**, 10 nM GLP-1 and the GLP-1 receptor **(B)**, and FBS **(C)** were used. For details see method section. The GPCRs analyzed are listed in table S1.
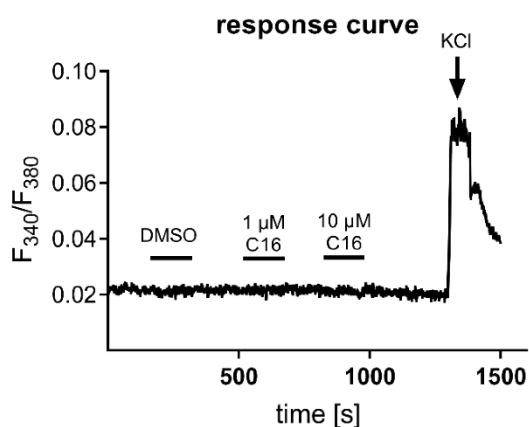
**Figure S8: Representative response curve of calcium fluxes in C16 dihydroceramide treated sensory neurons.** The primary cells were treated with 1 and 10 µM C16 dihydroceramide and the corresponding solvent control (DMSO) followed by a KCl (50 mM) treatment as a positive control.

**Table S1: Internal standards and their concentrations in the working solutions for the lipid screening** (all from Avanti Polar Lipids, Alabaster, AL, USA)

| Analyte | Concentration (µg/mL) |
|---|---|
| Arachidonic acid-d8 | 0.1 |
| CE 18:1-d7 | 5 |
| Cer d18:1/16:0-d7 | 0.02 |
| Cholesterol-d7 | 7.5 |
| DG 15:0/18:1-d7 | 0.3 |
| LacCer d18:1/17:0 | 0.06 |
| LPC 18:1-d7 | 0.3 |
| LPC O-16:0-d4 | 0.02 |
| LPE 18:1-d7 | 0.02 |
| LPG 17:1 | 0.02 |
| LPI 17:1 | 0.02 |
| PC 15:0/18:1-d7 | 2 |
| PC O-18:0/18:1-d9 | 0.2 |
| PE 15:0/18:1-d7 | 0.1 |
| PG 15:0/18:1-d7 | 0.1 |
| PI 15:0/18:1-d7 | 0.1 |
| PS 15:0/18:1-d7 | 0.025 |
| SM d18:1/18:1-d9 | 0.4 |
| TG 14:0/16:1/14:0-d5 | 0.6 |
| TG 15:0/18:1-d7/15:0 | 0.6 |
| TG 20:0/20:1/20:0-d5 | 0.6 |

**Table S2: LC-gradient**

| Time (min) | Sol. A (%) | Sol. B (%) |
|---|---|---|
| 0.00 | 75.0 | 25.0 |
| 0.30 | 75.0 | 25.0 |
| 1.50 | 20.0 | 80.0 |
| 11.00 | 0.0 | 100.0 |
| 12.00 | 0.0 | 100.0 |
| 12.50 | 75.0 | 25.0 |
| 14.00 | 75.0 | 25.0 |

**Instrumentation**

| | |
|---|---|
| Mass spectrometer | Orbitrap Exploris 480 (Thermo Fisher Scientific, Dreiech, Germany) |
| | HESI-source |
| HPLC | Vanquish Horizon (Thermo Fisher Scientific, Dreiech, Germany) |
| | Vanquish Compartment H |
| | Vanquish Split Sampler HT |
| | Vanquish Pump H |
| LC-column | Zorbax RRHD Eclipse Plus C8 1.8 µm 50 x 2.1 mm ID (Agilent, Waldbronn, Germany), heated at 40 °C, flow rate 300 µL/min |
| | Precolumn: ZORBAX Eclipse Plus C8, 2.1 mm, 1.8 µm (Agilent, Waldbronn, Germany) |
| Solvent A | Water + 0.1% formic acid + 10 mM ammonium formate |
| Solvent B | Acetonitrile:isopropanole 2:3 (v/v) + 0.1% formic acid |
| Injection volume | 2 µL (positive ion mode), 5 µL (negative ion mode) |

## Table S3: List of GPCRs which were screened for potential activation by the C16 dihydroceramide

| n | description | n | description | n | description | n | description | n | description | n | description |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ADCYAP1R1 | 51 | CX3C1 | 101 | GPR119 | 151 | GPR4 | 201 | HTR1F | 301 | TA1 |
| 2 | ADORA1 | 52 | CXCR1 | 102 | GPR12 | 152 | GPR44 | 202 | HTR2A | 302 | TAAR2 |
| 3 | ADORA2A | 53 | CXCR2 | 103 | GPR120 | 153 | GPR45 | 203 | HTR2B | 303 | TAAR5 |
| 4 | ADORA2B | 54 | CXCR3 | 104 | GPR123 | 154 | GPR50 | 204 | HTR2C | 304 | TAAR6 |
| 5 | ADORA3 | 55 | CXCR4 | 105 | GPR124 | 155 | GPR52 | 205 | HTR4 | 305 | TAAR8 |
| 6 | ADRA1A | 56 | CXCR5 | 106 | GPR125 | 156 | GPR55 | 206 | HTR5 | 306 | TAAR9 |
| 7 | ADRA1B | 57 | CXCR6 | 107 | GPR126 | 157 | GPR56 | 207 | HTR6 | 307 | TACR1 |
| 8 | ADRA1D | 58 | CXCR7 | 108 | GPR132 | 158 | GPR6 | 208 | HTR7 | 308 | TACR2 |
| 9 | ADRA2A | 59 | CYSLTR1 | 109 | GPR133 | 159 | GPR61 | 209 | KISSPEPTIN | 309 | TACR3 |
| 10 | ADRA2B | 60 | CYSLTR2 | 110 | GPR135 | 160 | GPR62 | 210 | LHCGR | 310 | TBXA2R |
| 11 | ADRA2C | 61 | DRD1 | 111 | GPR141 | 161 | GPR63 | 211 | LPAR1 | 311 | TSHR |
| 12 | ADRB1 | 62 | DRD2 | 112 | GPR142 | 162 | GPR64 | 212 | LPAR2 | 312 | UTS2R |
| 13 | ADRB2 | 63 | DRD3 | 113 | GPR143 | 163 | GPR65 | 213 | LPAR4 | 313 | VIPR1 |
| 14 | ADRB3 | 64 | DRD4 | 114 | GPR144 | 164 | GPR68 | 214 | LPAR5 | 314 | VIPR2 |
| 15 | AGTR1 | 65 | DRD5 | 115 | GPR146 | 165 | GPR75 | 215 | LPAR6 | | |
| 16 | AGTR2 | 66 | EDNRA | 116 | GPR148 | 166 | GPR77 | 216 | LTB4R | | |
| 17 | APJ | 67 | EDNRB | 117 | GPR149 | 167 | GPR78 | 217 | LTB4R2B | | |
| 18 | AVPR1A | 68 | ELTD1 | 118 | GPR15 | 168 | GPR82 | 218 | MAS1 | | |
| 19 | AVPR1B | 69 | F2R | 119 | GPR150 | 169 | GPR83 | 219 | MAS1L | | |
| 20 | AVPR2 | 70 | F2RL1 | 120 | GPR151 | 170 | GPR84 | 220 | MC1R | | |
| 21 | BB3 | 71 | F2RL2 | 121 | GPR152 | 171 | GPR85 | 221 | MC2R | | |
| 22 | BDKRB1 | 72 | F2RL3 | 122 | GPR153 | 172 | GPR87 | 222 | MC3R | | |
| 23 | BDKRB2 | 73 | FFA1 (GPR40) | 123 | GPR156 | 173 | GPR88 | 223 | MC4R | | |
| 24 | C3AR1 | 74 | FFA2 (GPR43) | 124 | GPR157 | 174 | GPR97 | 224 | MC5R | | |
| 25 | C5A | 75 | FFA3 (GPR41) | 125 | GPR158 | 175 | GPRC5A | 225 | MCHR1 | | |
| 26 | CALCRb | 76 | FPR1 | 126 | GPR160 | 176 | GPRC5B | 226 | MCHR2 | | |
| 27 | CALCRL | 77 | FPR2 | 127 | GPR161 | 177 | GPRC5C | 227 | MLNR | | |
| 28 | CASR | 78 | FPR3 | 128 | GPR162 | 178 | GPRC5D | 228 | MRGPRD | | |
| 29 | CCKAR | 79 | FSHR | 129 | GPR17 | 179 | GPRC6A | 229 | MRGPRE | | |
| 30 | CCKBR | 80 | GABBR1 | 130 | GPR171 | 180 | GRM1 | 230 | MRGPRF | | |
| 31 | CCR10 | 81 | GAL1 | 131 | GPR173 | 181 | GRM2 | 231 | MRGPRG | | |
| 32 | CCR2 | 82 | GAL2 | 132 | GPR174 | 182 | GRM4 | 232 | MRGPRX1 | | |
| 33 | CCR3 | 83 | GAL3 | 133 | GPR18 | 183 | GRM5 | 233 | MRGPRX2 | | |
| 34 | CCR4 | 84 | GCGR | 134 | GPR182 | 184 | GRM6 | 234 | MRGPRX3 | | |
| 35 | CCR5 | 85 | GHRHR | 135 | GPR183 | 185 | GRM7 | 235 | MRGPRX4 | | |
| 36 | CCR6 | 86 | GHSR | 136 | GPR19 | 186 | GRM8 | 236 | MTNR1A | | |
| 37 | CCR7 | 87 | GIPR | 137 | GPR20 | 187 | GRPR | 237 | MTNR1B | | |
| 38 | CCR8 | 88 | GLP1R | 138 | GPR21 | 188 | HCA1 | 238 | NMBR | | |
| 39 | CCRL2 | 89 | GLP2R | 139 | GPR22 | 189 | HCA2 | 239 | NMUR1 | | |
| 40 | CD97 | 90 | GNRHR | 140 | GPR25 | 190 | HCA3 | 240 | NMUR2 | | |
| 41 | CHRM1 | 91 | GPBA | 141 | GPR26 | 191 | HCTR1 | 241 | NPBW1 | | |
| 42 | CHRM2 | 92 | GPER | 142 | GPR27 | 192 | HCTR2 | 242 | NPBW2 | | |
| 43 | CHRM3 | 93 | GPR1 | 143 | GPR3 | 193 | HRH1 | 243 | NPFF1 | | |
| 44 | CHRM4 | 94 | GPR101 | 144 | GPR31 | 194 | HRH2 | 244 | NPFF2 | | |
| 45 | CHRM5 | 95 | GPR110 | 145 | GPR32 | 195 | HRH3 | 245 | NPS | | |
| 46 | CMKLR1 | 96 | GPR111 | 146 | GPR34 | 196 | HRH4 | 246 | NPY1R | | |
| 47 | CNR1 | 97 | GPR113 | 147 | GPR35 | 197 | HTR1A | 247 | NPY2R | | |
| 48 | CNR2 | 98 | GPR114 | 148 | GPR37 | 198 | HTR1B | 248 | NPY4R | | |
| 49 | CRHR1 | 99 | GPR115 | 149 | GPR37L1 | 199 | HTR1D | 249 | NPY5R | | |
| 50 | CRHR2 | 100 | GPR116 | 150 | GPR39 | 200 | HTR1E | 250 | NTSR1 | | |

## 4 Publication 3

# SAFit2 ameliorates paclitaxel-induced neuropathic pain by reducing spinal gliosis and elevating pro-resolving lipid mediators

Saskia Wedel[1], Lisa Hahnefeld[1,2], Yannick Schreiber[2], Christian Namendorf[3], Tim Heymann[4], Manfred Uhr[3], Mathias V. Schmidt[3], Natasja de Bruin[2], Felix Hausch[4], Dominique Thomas[1,2], Gerd Geisslinger[1,2] and Marco Sisignano[1,2*]

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## 4.1 Author contributions

Conceptualization: FH, MS and GG conceptualized the project.

Funding acquisition: MS, FH, and NdB acquired the funding.

Experimental design: **SW,** NdB, MU and MS designed the experiments.

Data acquisition: **SW** performed the behavioral experiments and isolated the tissue. **SW** further performed multiplex assays, qPCRs, immunohistochemistry stainings, PLA2 assays and prepared tissue samples for measurements. NdB supervised the behavioral study and the pharmacokinetic study together with MVS. CN performed LC-MS/MS measurements for the pharmacokinetic study and LH conducted the LC-HRMS as well as LC-MS/MS for lipid measurements. DT established the LC-MS/MS oxylipin method. TH synthesized the batch of SAFit2.

Data analysis: **SW** analyzed all the data. LH helped with the LC-HRMS as well as CN and LH helped with the LC-MS/MS analysis. NdB helped with the pharmacokinetic study analysis.

Data curation: **SW** performed the data curation.

Original draft preparation: **SW** and MS wrote and revised the manuscript.

All authors approved the final manuscript

**RESEARCH**                                                                     **Open Access**

# SAFit2 ameliorates paclitaxel-induced neuropathic pain by reducing spinal gliosis and elevating pro-resolving lipid mediators

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## Abstract

**Background** Chemotherapy-induced neuropathic pain (CIPN) describes a pathological pain state that occurs dose-dependently as a side effect and can limit or even impede an effective cancer therapy. Unfortunately, current treatment possibilities for CIPN are remarkably confined and mostly inadequate as CIPN therapeutics themselves consist of low effectiveness and may induce severe side effects, pointing out CIPN as pathological entity with an emerging need for novel treatment targets. Here, we investigated whether the novel and highly specific FKBP51 inhibitor SAFit2 reduces paclitaxel-induced neuropathic pain.

**Methods** In this study, we used a well-established multiple low-dose paclitaxel model to investigate analgesic and anti-inflammatory properties of SAFit2. For this purpose, the behavior of the mice was recorded over 14 days and the mouse tissue was then analyzed using biochemical methods.

**Results** Here, we show that SAFit2 is capable to reduce paclitaxel-induced mechanical hypersensitivity in mice. In addition, we detected that SAFit2 shifts lipid levels in nervous tissue toward an anti-inflammatory and pro-resolving lipid profile that counteracts peripheral sensitization after paclitaxel treatment. Furthermore, SAFit2 reduced the activation of astrocytes and microglia in the spinal cord as well as the levels of pain-mediating chemokines. Its treatment also increased anti-inflammatory cytokines levels in neuronal tissues, ultimately leading to a resolution of neuroinflammation.

**Conclusions** In summary, SAFit2 shows antihyperalgesic properties as it ameliorates paclitaxel-induced neuropathic pain by reducing peripheral sensitization and resolving neuroinflammation. Therefore, we consider SAFit2 as a potential novel drug candidate for the treatment of paclitaxel-induced neuropathic pain.

**Keywords** SAFit2, FKBP51, Neuropathic pain, Chemotherapy, Oxylipins

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## Background

Paclitaxel is a chemotherapeutic agent, which is used for the treatment of malignancies as it stabilizes microtubule formation and thereby disrupts cell proliferation and cytoskeleton related cellular functions [1, 2]. Based on this mechanism of action, paclitaxel became a very important cytostatic, which enables the treatment of many different tumor types. However, the microtubule-stabilizing property of paclitaxel also affects the axonal transport of sensory neurons in the peripheral nervous system, which leads to axonal degeneration, neuropathy and neuropathic pain [3].

Neuropathic pain is a pathophysiological pain state, which is generally induced by nerve lesions or damages in the somatosensory system and can become chronic [4, 5]. A specific type of neuropathic pain is the chemotherapy-induced peripheral neuropathy (CIPN), which arises during chemotherapy as a severe side effect [6]. CIPN is a dose-dependent side effect that can even limit or impede an effective cancer therapy as chemotherapy often has to be reduced in dose or frequency or interrupted after the occurrence of CIPN [7, 8]. Unfortunately, CIPN affects up to 80 percent of all cancer patients and the majority of paclitaxel-treated patients [3]. Nonetheless, the available treatment possibilities for CIPN are extremely confined and mostly inadequate as the therapeutics of CIPN itself consist of a low effectiveness and may induce severe side effects, pointing out CIPN as pathological entity with an emerging need for novel treatment targets [9]. In addition, CIPN comprises a large symptom scope, which needs to be further investigated and underlying mechanisms to be revealed in order to develop appropriate therapeutic drugs.

Interestingly, previous studies revealed the FK506 binding protein 51 (FKBP51, encoded by the FKBP5 gene) as a novel and potential target for several pathological disorders such as chronic pain [10, 11], stress endocrinology [12] and glucocorticoid signaling related diseases [13]. In the context of chronic pain, FKBP51 was shown to be significantly upregulated in sensory neurons of the dorsal horn in an ankle joint knee inflammation model, indicating its involvement in the development of persistent pain states [10]. Based on these previous findings, we synthesized a highly potent and FKBP51 specific inhibitor named SAFit2 to address the scarcity of therapeutic approaches for chronic and especially paclitaxel-mediated neuropathic pain [14, 15].

To investigate whether or not the novel and specific FKBP51 inhibitor SAFit2 may reduce paclitaxel-induced neuropathic pain and improve paclitaxel-induced dysregulations of endogenous mechanisms, we performed a multiple low-dose paclitaxel mouse model. Previous findings already discovered that paclitaxel mediates axonal degeneration and its cumulative toxicity induces neuroinflammation and spinal cord gliosis [3]. Here, we confirm that a paclitaxel treatment induces a release of proalgesic mediators in nervous tissue and mediates spinal cord gliosis. In addition, we show that SAFit2 counteracts spinal cord gliosis after paclitaxel treatment and ameliorates paclitaxel-induced mechanical hypersensitivity in vivo. Previous studies have already shown alterations in the distribution of lipids and the levels of lipid mediators in nerve disorders or chronic pain states [4, 16, 17]. However, the role and precise mechanisms of endogenous lipid mediators in the context of paclitaxel-mediated neuropathic pain are still unclear. Based on this lack of understanding, we performed an unbiased lipidomics screen (LC–HRMS) and a targeted LC–MS/MS oxylipin screen from dorsal root ganglia (DRGs) and spinal cord (SC) samples to elucidate changes in the distribution of lipids after paclitaxel treatment. Furthermore, we assessed the influence of SAFit2 on paclitaxel-mediated alterations in lipid levels. Investigating these lipid levels in paclitaxel-treated mice, we observed that a SAFit2 treatment leads to an increase of anti-inflammatory and pro-resolving lipid mediators, which play a crucial role in the resolution of pain states.

## Materials and methods

### Animals

For all experimental approaches, wild-type animals with a C57BL/NRj background were purchased from the breeding companies Janvier and Charles River. All animal experiments, which included treated animals, were started with nine weeks old mice and naïve mice were matched in age and sex, respectively.

### Paclitaxel and SAFit2 treatment

To induce paclitaxel-mediated neuropathic pain in mice, a multiple low-dose model was performed including four injections of 2 mg/kg paclitaxel, which was administered every second day, adding up to a cumulative dose of 8 mg/kg. [18]. The cytostatic paclitaxel was dissolved in a 50:50 (v/v) ethanol–cremophor EL (238470, Millipore) solution and stored at $-20$ °C until further usage. Before the intraperitoneal injection, the paclitaxel stock-solution was thawed and freshly diluted 1:2 (v/v) with saline solution (NaCl 0.9% (w/v)) to a final concentration of 0.4 mg/ml. For assessing the influence of SAFit2 on paclitaxel-induced neuropathic pain and endogenous mechanisms, the mice were treated intraperitoneally with either 10 mg/kg SAFit2 or vehicle (PBS supplemented with 5% PEG400, 5% Tween and 0.7% ethanol) two times daily on six consecutive days, starting on day five after the first paclitaxel injection. The experimental treatment schedule is displayed in Fig. 1A.

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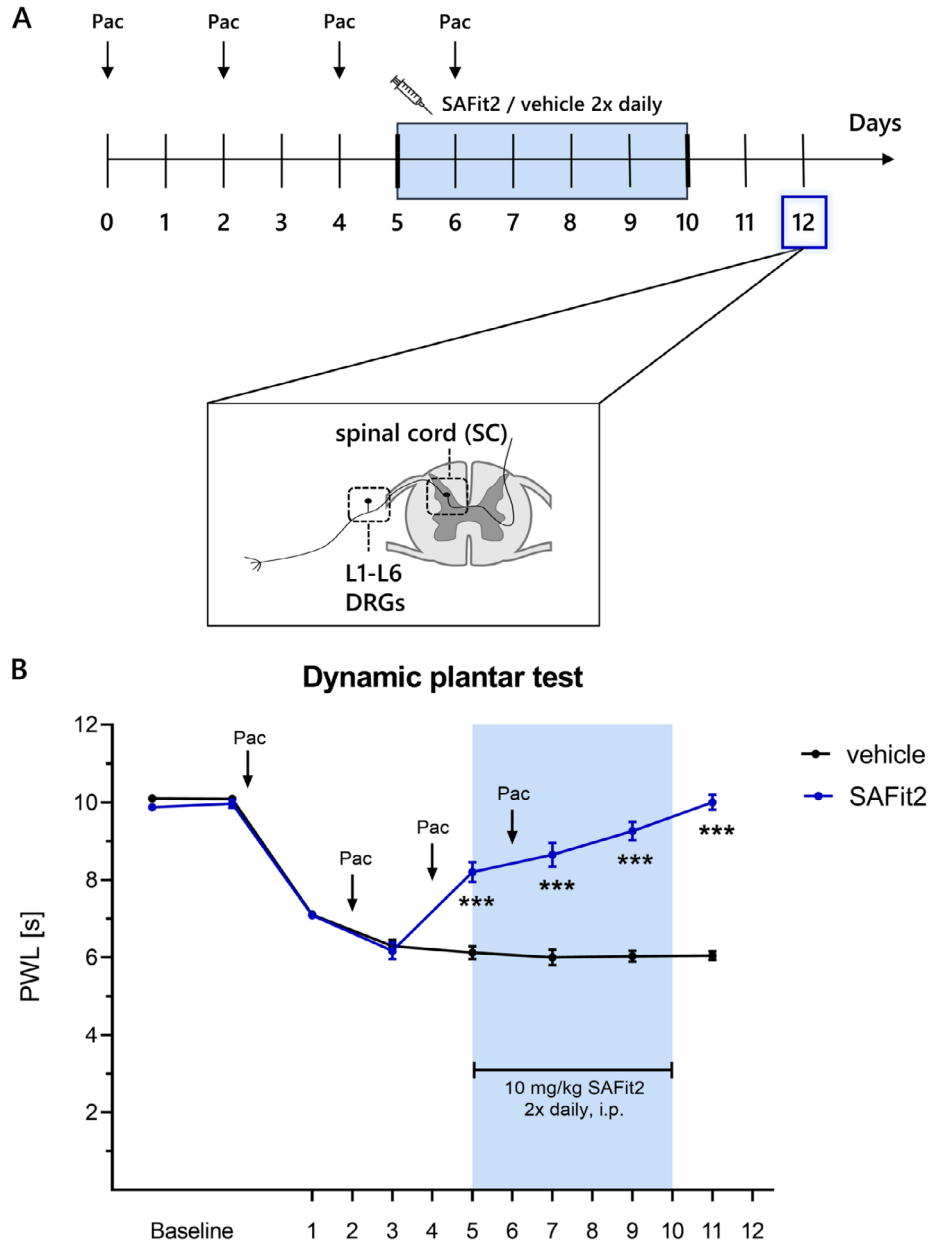
**Fig. 1** SAFit2 reduces paclitaxel-induced mechanical hypersensitivity. **A** Schematic illustration of the animal treatment: neuropathic pain was induced with a multiple low-dose paclitaxel model, including four intraperitoneal injections with 2 mg/kg paclitaxel on day zero, two, four and six. Afterwards, the animals were treated with 10 mg/kg SAFit2 intraperitoneally from day five to ten two times daily. At day twelve, the mice were killed and DRGs and spinal cord samples collected. **B** Baseline measurements were performed on two different days before paclitaxel treatment. The mechanical pain threshold was assessed over 12 days every second day and the behavioral readout within the SAFit2 treatment was recorded between the SAFit2 treatments, approximately three hours after the first dose of SAFi2. The data represent the mean ± SEM from 13–14 mice per group. ***$p < 0.001$ two-way ANOVA with Bonferroni´s post hoc test. *Pac* paclitaxel, *DRGs* dorsal root ganglia, *PWL* paw withdrawal latency, *SAFit2* selective antagonist of FKBP51 by induced fit 2

### Behavioral experiments

Before starting the behavioral experiments and treatment phases, the locomotor function of all animals was verified using a rotarod assay [19]. During all behavioral experiments, the experimenter was blinded. For assessing the mechanical pain threshold, the animals were placed at least one hour before the measurement in the respective test cages to allow habituation. After this habituation phase, the mechanical paw withdrawal threshold was assessed using a Dynamic Plantar Aesthesiometer (Ugo Basile) as described previously [20]. The baseline measurements were performed on two consecutive days before the treatment phase and the mechanical pain thresholds were assessed every second day.

### Tissue isolation

For tissue isolation, mice were euthanized by isoflurane anesthesia (2–2.5% isoflurane in carbogen), cardio puncture, and cervical dislocation 12 days after the first paclitaxel injection. The lumbar (L1–L6) dorsal root ganglia (DRGs) and the respective innervated segments of the spinal cord were dissected followed by freezing tissue samples in liquid nitrogen for either RNA isolation, a multiplex assay or LC–HRMS and LC–MS/MS analysis. For immunohistochemistry staining, the spinal cord was dissected and fixed with 2% paraformaldehyde (PFA) solution until further usage.

### Pharmacokinetic study

In the pharmacokinetic study, a single dose of 40 mg/kg SAFit2, formulated as described above, was applied intraperitoneally. Likewise, a single dose of 100 mg/kg SAFit2, formulated as a slow-release formulation with vesicular phospholipid gel (VPG) [21], was applied subcutaneously. For the first formulation, plasma and brain samples were collected after 15 min, 30 min, 1 h, 3 h, 6 h and 24 h from three animals. For the second formulation, plasma and brain samples were collected after 2 h, 8 h, 24 h, 48 h, 72 h and 96 h from three animals. Mice were euthanized by isoflurane anesthesia (2–2.5% isoflurane in carbogen), cardiac puncture, and cervical dislocation. Brains were weighed and then homogenized in tenfold PBS volume containing Complete Protease Inhibitor Cocktail Tablets (Roche), using a Dispomix Drive (Medic Tools AG). Plasma samples were analyzed using the combined high-performance liquid chromatography/mass spectrometry (HPLC/MS–MS) technique. Analysis was performed using a Shimadzu Nexera X2 (Shimadzu) liquid chromatograph, which was interfaced to the ESI source of a Sciex QTrap 5500 (Sciex) triple quadrupole mass spectrometer. All samples were prepared using Ostro protein precipitation and phospholipid removal plates (Waters).

Chromatography was accomplished using a gradient elution in an Accucore RP-MS column ($100 \times 2.1$ mm, 2.6 μm Thermo Scientific) at a flow rate of 0.5 ml/min and 30 °C. The composition of eluent B was methanol with 10 mM ammonium formate with 0,1% formic acid and water with 10 mM ammonium formate with 0,1% formic acid was eluent A. The gradient was 0–3 min 60% B, 3–4.5 min 60–90% B, 0.5 min held at 90% B, 5–5.2 min 90–60% B and 5.2–6 min 60% B. The total run time was 6 min and the injection volume was 2 μl. The ion source was operated in the positive mode at 500 °C, and multiple reaction monitoring (MRM) collision-induced dissociation (CID) were performed using nitrogen gas as the collision gas. Deuterated SAFit2 (SAFit2-D3) was used as internal standard.

### Quantitative real-time PCR

To isolate the RNA from lumbar DRGs and the respective segments of the spinal cord, the mirVana miRNA Isolation Kit (Applied Biosystems) was used according to the manufacturer´s instructions. Afterwards, RNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and a cDNA synthesis was conducted with 200 ng RNA from DRGs and 400 ng RNA from spinal cord. The reverse transcription was performed with the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. The quantitative real-time PCR was performed with the QuantStudio™ Design & Analysis Software v 1.4.3 (Thermo Fisher Scientific) in a TaqMan® Gene Expression Assay System (Table 1, Thermo Fisher Scientific) according to the manufacturer´s instructions. The raw data were evaluated using the $\Delta\Delta C(T)$ method, as described previously [22, 23].

### Multiplex assay

For performing the ProcartaPlex multiplex immunoassay (Thermo Fisher), proteins were isolated with a manufacturer recommended cell lysis buffer, which was further supplemented with a phosphatase inhibitor cocktail (PhosSTOP, Roche) and a protease inhibitor cocktail (cOmplete, Roche). Each lumbar DRG (L1–L6 per animal) sample was suspended in 100 μl and each spinal cord sample, which comprises the respective innervated segments of the spinal cord, in 200 μl cell lysis buffer. The spinal cord samples were further processed by a cell grinder. Afterwards, the tissue was homogenized two times per sample using a Sonopuls Sonicator (Bandelin) with the setting $6 \times 10\%$. During sonication, the samples were cooled in an ice bath, preventing proteins from degradation. Finally, the samples were centrifuged with $16,000 \times g$ at 4 °C for 10 min, followed by collecting the

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**Table 1** List of used TaqMan® gene expression assays

| Target | Gene | Article number | Company |
|--------|------|----------------|---------|
| ALOX12 | Arachidonate 12-lipoxygenase | Mm00545833_m1 | Thermo Fisher |
| ALOX15 | Arachidonate 15-lipoxygenase | Mm00507789_m1 | Thermo Fisher |
| ALOX5 | Arachidonate 5-lipoxygenase | Mm01182747_m1 | Thermo Fisher |
| ATF3 | Activating transcription factor 3 | Mm00476033_m1 | Thermo Fisher |
| CerS5 | Ceramide synthase 5 | Mm00556165_m1 | Thermo Fisher |
| CerS6 | Ceramide synthase 6 | Mm00510998_m1 | Thermo Fisher |
| cFOS | FBJ osteosarcoma oncogene | Mm00487425_m1 | Thermo Fisher |
| COX2 | Cytochrome c oxidase subunit II | Mm03294838_g1 | Thermo Fisher |
| CYP2J6 | Cytochrome P450, family 2, subfamily j, polypeptide 6 | Mm01268197_m1 | Thermo Fisher |
| CYP3a11 | Cytochrome P450, family 3, subfamily a, polypeptide 11 | Mm00731567_m1 | Thermo Fisher |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Mm99999915_g1 | Thermo Fisher |
| iNOS | Inducible nitric oxide synthase 2 | Mm00440502_m1 | Thermo Fisher |
| MMP9 | Matrix metallopeptidase 9 | Mm00442991_m1 | Thermo Fisher |
| NOX2 | NADPH oxidase 2 | Mm01287743_m1 | Thermo Fisher |
| NOX4 | NADPH oxidase 4 | Mm00479246_m1 | Thermo Fisher |
| PLA2g4a | Phospholipase A2, group 4a | Mm00447040_m1 | Thermo Fisher |
| PLA2g4c | Phospholipase A2, group 4c | Mm01195718_m1 | Thermo Fisher |
| XDH | Xanthine dehydrogenase | Mm00442110_m1 | Thermo Fisher |

supernatant for a protein concentration determination via Bradford.

The ProcartaPlex multiplex immunoassay was performed according to the manufacturer's recommendations. Briefly, a dark wall 96-well plate was prepared by several washing steps and coating steps with respective magnetic beads. Afterwards, standards were prepared in a serial dilution (1:4, (v/v)) and added to the plate, followed by further washing steps. Lastly, the samples were diluted (1:2, (v/v)) and added to the plate, which was sealed and incubated for 40 min with 500 rpm on an orbital shaker at room temperature, overnight at 4 °C and further 50 min with 500 rpm at room temperature. On the next day, the detection antibody mixture was added after a washing step and the plate was further incubated on an orbital shaker for 30 min at room temperature. Again, the plate was washed and streptavidin phycoerythrin (SAPE) was added to the plate and incubated for 30 min as described above. After the last washing step, the plate was prepared with reading buffer, incubated for 5 min with 500 rpm on an orbital shaker, and measured with the Luminex 200 system (Bio-Rad).

**Immunohistochemistry**

Before the immunohistochemical staining, the spinal cord samples were fixed with 2% PFA for 5 h at room temperature, as previously described in the tissue isolation section. As a next step, the tissue was dehydrated with a concentration series of 20% (w/v) and 30% (w/v) sucrose solutions. Therefore, spinal cord samples were firstly incubated in a 20% sucrose solution at 4 °C overnight and then further dehydrated in a 30% sucrose solution for five hours at 4 °C. Next, the spinal cord samples were embedded in Tissue-Tek and frozen at − 80 °C. The frozen tissue samples were serially sliced into 14-µm tissue slices with a cryostat (Leica Biosystems).

For staining the slices, the samples were fixed with 2% PFA for ten minutes. Afterwards, the fixative was removed and the slices rinsed with PBS. Next, slices were permeabilized with PBS (pH 7.4) containing 0.1% Triton X (v/v) for 15 min. After removing the PBST, the slices were blocked with a 3% BSA (w/v) solution for two hours at room temperature. The primary antibodies anti-GFAP (ab7260, Abcam), anti-IBA1 (019-19741, FUJIFILM Wako Pure Chemical Corporation), anti-ATF3 (sc-188, Santa Cruz) and anti-cFOS (9F3, Cell Signaling) were diluted 1:1000, 1:500, 1:100 and 1:200, respectively, in 1% BSA solution and applied for an overnight incubation at 4 °C. The primary antibody anti-FKBP51 (sc-271547, Santa Cruz) was diluted 1:50 as recommended in 3% BSA solution to minimize cross-reactivity as the host species is mouse.

On the next day, the excess of non-specific binding primary antibodies was removed via three washing steps with PBS, this time incubating the slices with PBS for 5 min. Afterwards, the secondary antibodies, goat

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anti-rabbit Alexa Fluor 488 (ab150077, Abcam) and donkey anti-rabbit Alexa Fluor 647 (A-31573, Thermo Fisher) were diluted 1:1000 in 1% BSA solution and applied for one hour at room temperature. The secondary antibodies goat anti-mouse Alexa Fluor 488 (CF488A, Biotium) and sheep anti-mouse Cy3 (C2181, Sigma Aldrich) were diluted in 3% BSA solution. Next, the excess of non-specific binding secondary antibodies was removed, the slices rinsed with PBS and further incubated with DAPI 1:1000 (6335.1, Carl Roth) for ten minutes at room temperature. The slices were mounted with Fluoromount-G mounting medium from Southern Biotech. Images were taken with the fluorescence microscope Observer. Z1 (Carl Zeiss) and processed as well as quantified with ImageJ software. Three to five representative images were taken per stained spinal cord slice, resulting in a total number of 10–20 images per animal. In total, we quantified the number of cells for the markers GFAP and IBA1 by counting cells in 70–80 pictures per condition. For the marker cFOS, we counted the cells in 40 images per condition and for FKBP51, we quantified the mean intensity per image for 40 images per condition.

### Liquid chromatography–high-resolution mass spectrometry (LC–HRMS)

For LC–HRMS purposes, an approximately 3 mg lumbar DRG sample and 30 mg of the respective parts of the spinal cord (SC) were homogenized using a pellet pestle mixer (Thermo Fisher scientific). The tissue samples were grinded for 30 to 60 s, with short breaks included to avoid overheating, in an ethanol:water (1:9, v/v) solution, using 119 µl ethanol for DRG samples and 1190 µl ethanol for spinal cord samples. Afterwards, lipid extraction and chromatographic separation was performed as well as the measurement settings and internal standards chosen as previously described [24]. Instrumental setup consisted of a Vanquish Horizon coupled to an Exploris 480 (both Thermo Fisher Scientific) with Zorbax RRHD Eclipse Plus C8 column (2.1×50 mm, 1.8 µm, Agilent) and a pre-column of the same type. The measured lipid levels were normalized to the respective protein amount of the sample. For all lipidomic measurements, including extraction and analysis, the experimenter was blinded.

### PLA₂ activity assay

To measure $PLA_2$ activity in primary sensory neuron lysates, DRGs were collected from two naïve mice and washed with ice-cold PBS. Next, the cells were lysed in 1 ml of ice-cold PBS and homogenized using a Sonopuls Sonicator (Bandelin) three times with the setting 6×10%. Afterwards, the homogenate was centrifuged with 10,000 rpm for 15 min at 4 °C and the supernatant collect for the $PLA_2$ activity assay (Abcam).

The assay was performed according the manufacturer's recommendations.

### Oxylipin screen

For oxylipin analysis, dorsal root ganglia (DRG) were homogenized in 10% ethanol with addition of 10 M indomethacin (0.04 mg tissue/µl) with a pellet pestle mixer (fisher scientific). Similarly, spinal cord samples were homogenized in 10% ethanol with addition of 10 M indomethacin (0.075 mg tissue/µl) and 7 zirconium oxide beads using a Precellys 24-Dual tissue homogenizer coupled with a Cryolys cooling module (both Bertin Technologies) that was kept at < 10 °C. The Precellys program encompassed three rounds at $6500 \times g$ for 20 s with 60 s pauses in between to avoid overheating. Tissue homogenates were stored on ice during extraction. For both tissue types, to 200 µl of tissue homogenate, 10 µl of 4.4% formic acid, 20 µl internal standard solution and 20 µl of 0.1% BHT in methanol were added and vortexed for 30 s. Proteins were precipitated by addition of 200 µl cold acetonitrile:methanol (8:2, v/v). After vortexing for 120 s and 600 s of centrifugation at approximately $15,000 \times g$, the supernatant was used for solid phase extraction using EVOLUTE EXPRESS ABN 30 mg/1 ml cartridges (Biotage) as previously described [25]. Calibration standards and quality control samples were prepared accordingly with 20 µl of standard solution in methanol with 0.1% BHT and 200 µL of PBS. Additionally, DRG homogenates were diluted by adding 180 µl of 10% ethanol with 10 M indomethacin to 20 µl of homogenate and processed as described above. LC–MS/MS analysis was then conducted on a QTrap 6500 + (Sciex) coupled to a 1290 Infinity II LC-System (Agilent) using an Acquity UPLC BEH C18 column (1.7 µm 100×2.1 mm ID) with a pre-column of the same type. For all measurements, including extraction and analysis, the experimenter was blinded. Further details including method parameters can be found in a previous publication [25].

### Data analysis and statistics

All data are presented as mean ± SEM. Normal distribution was confirmed using the Shapiro–Wilk test. The statistical analysis for behavioral experiments was conducted using a two-way analysis of variance (ANOVA) with Bonferroni's post hoc correction. For all ex vivo experiments, an unpaired and heteroskedastic Student's $t$ test with Welch correction was performed, when comparing only two groups with each other. For the comparison of more than two groups, a one-way ANOVA was used and for examining the effect of two independent variables on a continuous dependent variable a two-way ANOVA was conducted. For all statistical analysis

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the software GraphPad Prism 9.5 was used. A $p$ value of $< 0.05$ was considered as statistically significant.

## Results

Previous studies already suggested a crucial role of FKBP51 in the context of chronic pain states [10, 11]. However, its upregulation in chemotherapy-induced neuropathic pain is not confirmed yet. Therefore, we performed an immunohistochemistry staining to investigate its expression after paclitaxel treatment in L4–L5 dorsal root ganglia as well as in spinal cord slices (Additional file 1: Fig. S1). For both neuronal tissues, we observed a significant increase of FKBP51 after paclitaxel, when comparing the expression to naïve animals. These data confirm the relevance of FKBP51 in the context of chronic pain states and especially chemotherapy-induced neuropathic pain.

### SAFit2 ameliorates paclitaxel-induced mechanical hypersensitivity

To investigate the influence of SAFit2 on paclitaxel-induced neuropathic pain and underlying mechanisms, we chose a well-established multiple low-dose paclitaxel model as a multiple low-dose model corresponds more to a human chemotherapy, than a single high-dose model. The respective model consists of four intraperitoneal injections of paclitaxel (2 mg/kg) every other day, resulting in a cumulative dose of 8 mg/kg to induce paclitaxel-mediated neuropathic pain (Fig. 1A) [26].

As a first approach, the bioavailability and brain permeability of SAFit2 was monitored in a pharmacokinetic study. Here, we tested two SAFit2 doses and two formulations, measuring SAFit2 concentrations in plasma and brain after treatment (Additional file 1: Fig. S2). SAFit2 was either formulated in PBS supplemented with 5% PEG400, 5% Tween and 0.7% ethanol or as slow-release formulation in vesicular phospholipid gel (VPG, Additional file 1: Fig. S2). We observed that the slow-release VPG formulation led to a strong but transient brain permeability of SAFit2 that did not differ between the two doses or vehicles at later time points.

To analyze the effect of SAFit2 on paclitaxel-induced peripheral neuropathy, we aimed to achieve moderate to high SAFit2 plasma concentrations. However, a single injection of the slow-release formulation was not sufficient to achieve sustained moderate to high plasma concentrations and a single dose of 40 mg/kg SAFit2 led to exceedingly high plasma concentrations of SAFit2 at early time points (Additional file 1: Fig. S2). Based on these results and previous studies in which SAFit2 had been administered in vivo, we decided to treat the animals intraperitoneally with either 10 mg/kg SAFit2 or vehicle two times daily from day five to ten to investigate the therapeutic effect of SAFit2 on paclitaxel-treated mice [14, 24, 27]. After 12 days, the animals were killed and the lumbar DRGs (L1–L6) as well as the respective innervated segments of the spinal cord were isolated and collected separately from each individual animal for further biochemical analysis (Fig. 1A).

To investigate whether SAFit2 has an effect on paclitaxel-mediated neuropathic pain, the pain behavior of mice was assessed before the initiation of the model and during the treatment phase until day twelve by recording the mechanical paw withdrawal threshold (Fig. 1B). Both groups (vehicle and SAFit2) developed a mechanical hypersensitivity over time due to the paclitaxel treatment. However, the SAFit2-treated animals recovered from the mechanical hypersensitivity as their paw withdrawal thresholds significantly increased after the first dose of SAFit2 (Fig. 1B). In addition, the amelioration of pain sensation was sustained after the last treatment of SAFit2 until day twelve (Fig. 1B). In summary, SAFit2 significantly reduced the paclitaxel-induced mechanical hypersensitivity in vivo.

### SAFit2 reduces neuronal stress marker expression

As we detected a significant and sustained pain relief through the treatment with SAFit2, we analyzed the expression of neuronal stress markers (Fig. 2A–C) and reactive oxygen species (ROS) (Fig. 2D–G) markers in DRG and spinal cord samples in order to understand how SAFit2 affected underlying mechanisms. In addition, we compared the gene expression of both paclitaxel-treated groups with the basal expression of naïve mice, termed control group in the following.

For the neuronal stress marker activating transcription factor 3 (ATF3), we detected a significantly reduced expression in the DRGs of SAFit2-treated animals compared to vehicle-treated animals (Fig. 2A). In addition, the downregulation of ATF3 was confirmed via an immunohistochemistry staining in L4 and L5 DRGs of SAFit2-treated animals (Additional file 1: Fig. S3). Furthermore, we detected no significant changes in the expression of matrix metallopeptidase 9 (MMP9) (Fig. 2B). In contrast, we detected significantly diverging changes in the expression of the neuronal activity marker cFOS for both tissues (Fig. 2C). However, we observed on a protein level a significant downregulation of cFOS for both tissues after SAFit2 treatment, which we suggest to occur due to posttranscriptional modifications (Additional file 1: Fig. S4). In general, cFOS is known to be biphasically expressed and to contribute to the resolution of neuronal damage as its expression is enhanced in the formation of neuronal and synaptic plasticity [28], however its early upregulation was associated with nociceptive activity. In summary, we detected an enhanced expression of cFOS

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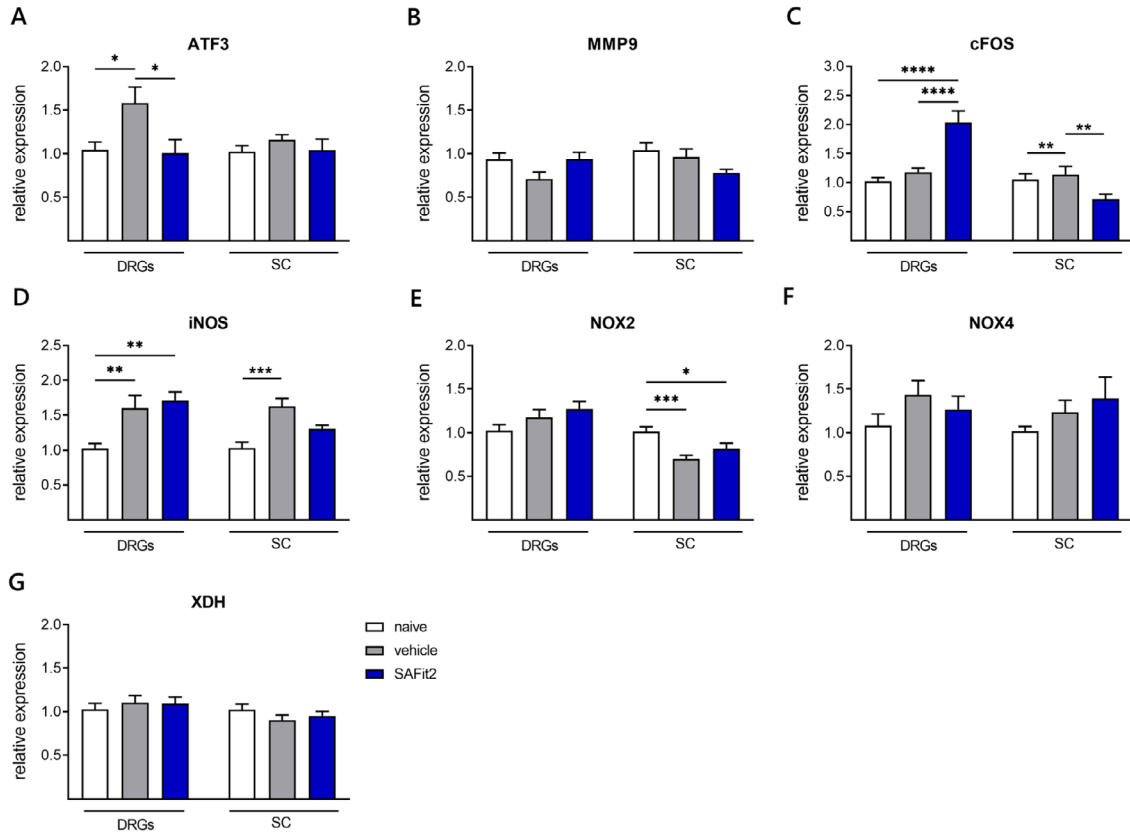


**Fig. 2** Neuronal stress and ROS marker expression in DRGs and spinal cord. After 12 days, the expression of neuronal stress markers (**A–C**) and ROS markers (**D–G**) was assessed via qPCR. The expression of vehicle and SAFit2-treated animals were normalized to the expression of naïve animals. The data represent the mean ± SEM from 3–4 mice per group, measured in technical triplicates, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with Tukey's multiple comparison test per tissue. *DRGs* dorsal root ganglia, *SC* spinal cord, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *ROS* reactive oxygen species, *ATF3* activating transcription factor 3, *MMP9* matrix metallopeptidase 9, *XDH* xanthine dehydrogenase, *NOX2/4* NADPH oxidase 2/4, *iNOS* inducible nitric oxide synthase

on RNA level in the DRGs and a reduced expression of cFOS on protein level in both tissues after SAFit2 treatment. Furthermore, we observed a reduced expression of ATF3 as neuronal stress marker on RNA and protein level in the DRGs after SAFit2 treatment, which indicates a reduction of neuronal stress after paclitaxel treatment.

Furthermore, we analyzed the expression of ROS markers in lumbar DRGs and spinal cord as the cytostatic paclitaxel is known to induce oxidative stress in sensory neurons [29]. However, we only detected a significant increase in the inducible nitric oxide synthase (Fig. 2D). In line with that, we observed no significant differences in the expression of NADPH oxidase two in the DRGs (Fig. 2E) as well as NADPH oxidase four (Fig. 2F) and the xanthine dehydrogenase (Fig. 2G) in both tissues. From this data, we suggested that the time point of investigation, which was day 12 and already 6 days after the last

paclitaxel treatment, may be too late to measure alterations in ROS responding genes and that their expression changes may be more transient after paclitaxel treatment.

### SAFit2 reduces pain-mediating chemokine levels and increases anti-inflammatory cytokine levels in DRGs and spinal cord after paclitaxel treatment

Since we did not observe strong differences in the gene expression of neuronal stress and ROS markers except for ATF3 and cFOS after SAFit2 treatment, we next measured the concentrations of inflammatory and proalgesic mediators in the DRGs and the respective segments of the spinal cord. Therefore, we performed a multiplex immunoassay assay, comprising a panel of 26 mediators, to determine differences in the neuroinflammation between vehicle and SAFit2-treated animals in a multiple

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low-dose paclitaxel model (Fig. 3, Additional file 1: Figs. S5, S6, S7).

Analyzing these mediators, we observed a significant upregulation of the anti-inflammatory interleukin 22 in the DRGs (Fig. 3A) as well as a significant downregulation of several pain-mediating chemokines such as CCL 2, CCL3, CCL4, CCL5, CCL7 and CXCL10 after SAFit2 treatment (Fig. 3B–G). Moreover, we detected the same tendencies in the spinal cord, since we observed a significant upregulation of the anti-inflammatory cytokine

interleukin 10 (Fig. 3H) and the significant down-regulation of CCL7 (Fig. 3I). Overall, SAFit2 seems to decrease the levels of pain-mediating chemokines and to increase anti-inflammatory mediators in both neural tissues after paclitaxel treatment.

### SAFit2 treatment decreases activation of astrocytes and microglia in the spinal cord of paclitaxel-treated mice

Based on the strong effect of SAFit2 on pain-mediating chemokines in the lumbar DRGs and spinal cord, we
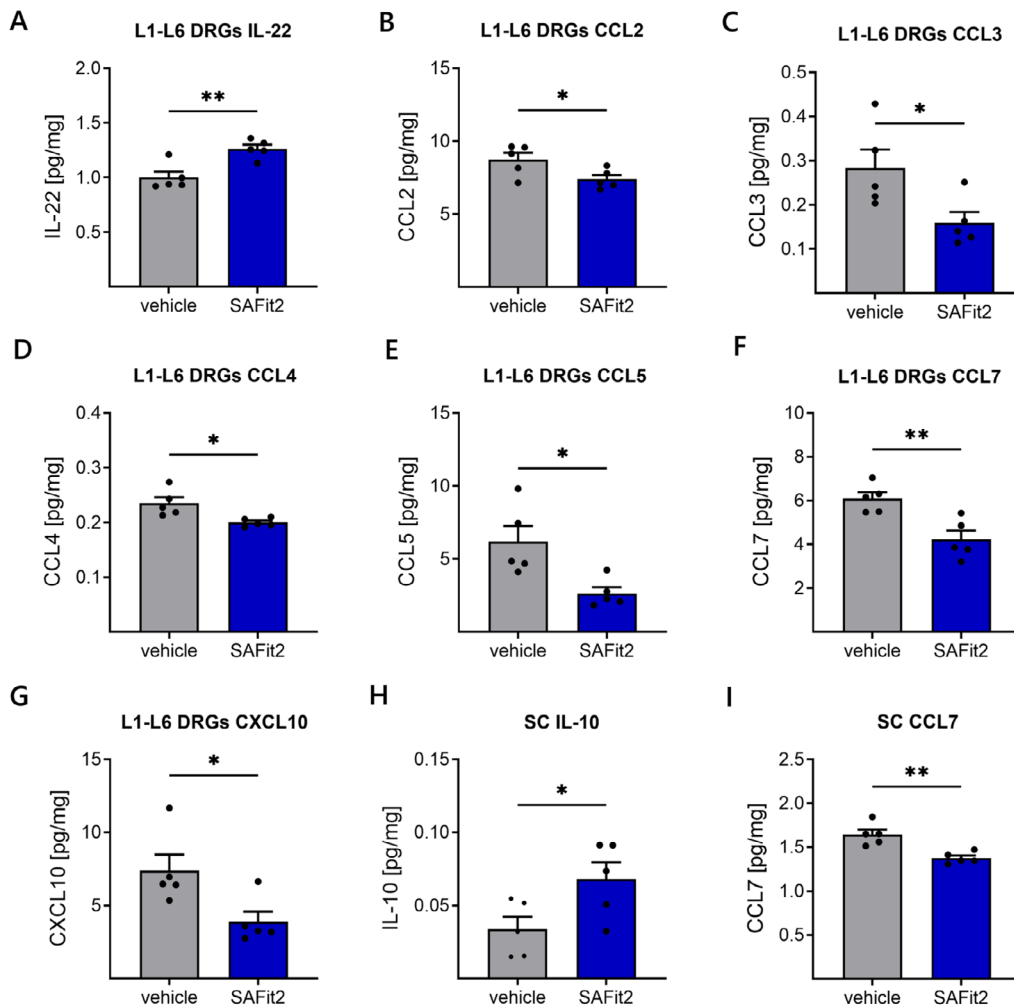


**Fig. 3** Increase of anti-inflammatory cytokines and decrease of pain-mediating chemokines in neuronal tissue of SAFit2-treated animals. After 12 days, DRGs and spinal cord samples were homogenized and analyzed using a multiplex immunoassay including a panel of 26 cytokines and chemokines. This figure displays a section of significantly altered cytokines and chemokines in the DRGs (**A–G**) and spinal cord (**H, I**) after vehicle and SAFit2 treatment. The data represent the mean ± SEM from 5 mice per group. The raw data were related to the total protein amount of the sample. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ Student's *t*-test with Welch's correction. *SAFit2* selective antagonist of FKBP51 by induced fit 2, *DRGs* dorsal root ganglia, *SC* spinal cord, *IL* interleukin, *CCL* C–C motif ligand, *CXCL 10* C-X-C ligand 10

investigated the effect of SAFit2 regarding spinal gliosis, since activated astrocytes and microglia are the predominant source of cytokines and chemokines in the spinal cord [30, 31]. To assess this, we performed immunohistochemical stainings of dorsal horn samples to assess the activation of astrocytes and the number of microglia (Fig. 4).

First, we analyzed the activation of astrocytes with the astrocyte activation marker glial fibrillary acidic protein (GFAP) in dorsal horn cryo-slices (Fig. 4A, C). In these stainings, we could observe a high number of GFAP-positive cells in the vehicle-treated paclitaxel group, indicating a strong activation of astrocytes after paclitaxel treatment (Fig. 4A). In contrast, the number of GFAP-positive cells was lower in the SAFit2-treated paclitaxel group. The control group, which consists of naïve animals, showed no or only a little GFAP-positive cells in comparison. In summary, the staining quantification confirmed these observations and revealed a significant reduction about 50% of astrocyte activation after SAFit2 treatment (Fig. 4D).

Next, we assessed the expression of the microglia marker ionized calcium-binding adaptor molecule 1 (IBA1) in the dorsal horn of the respective groups (Fig. 4B, D). The staining of the vehicle-treated paclitaxel group showed a strong IBA1 expression (Fig. 4B). However, the staining of the SAFit2-treated paclitaxel group showed less IBA1-positive cells. Stainings of the control group showed only weak signals, as expected. Interestingly, the quantification of IBA1-positive cells also revealed a significant reduction of approximately 50% after SAFit2 treatment. In conclusion, we observed that a SAFit2 treatment significantly reduced the number of activated astrocytes and microglia in the spinal cord, indicating that SAFit2 counteracts the paclitaxel-mediated gliosis.

### SAFit2 treatment leads to increased free fatty acid levels in neuronal tissue

Lipid mediators are important signaling mediators in the context of neuropathic pain as they can be released as paracrine signaling molecules to interact with neurons and glial cells as well as to modulate and resolve neuroinflammation [32]. Based on that, we hypothesized that SAFit2 might have an influence on lipid synthesis or release. For assessing this hypothesis, we first performed a high-resolution mass spectrometry lipidomic screen (LC–HRMS) to investigate lipid levels in nervous tissue (lumbar DRG and spinal cord samples) on a broad scale comparing vehicle and SAFit2-treated paclitaxel animals.
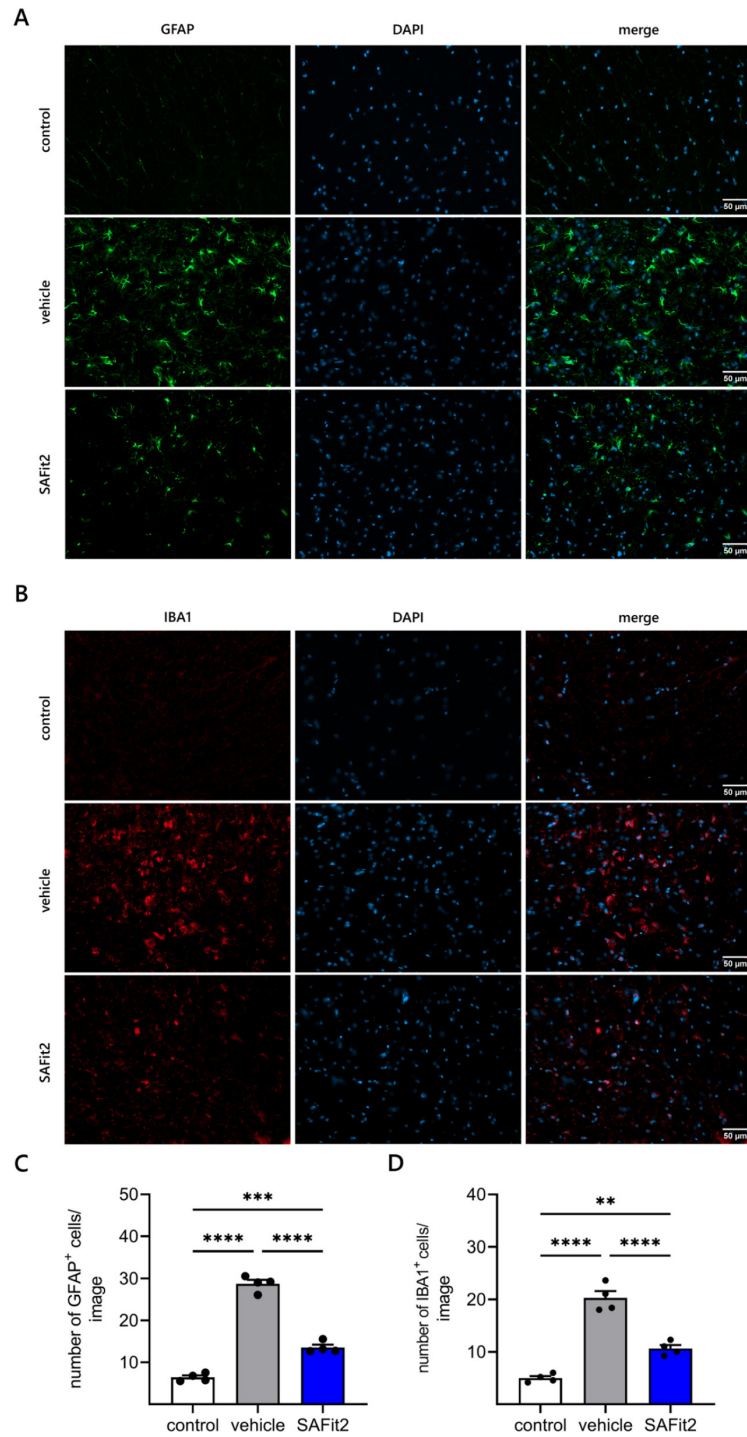
In this lipid screen, we detected 377 individual lipids that can be further subdivided into the following seventeen lipid classes: fatty acids, ceramides, glycosylceramides, diglycerides, carnitines, lysophosphatidylcholines, -ethanols, -glycerols, -inositols, -serines, phosphatidylcholines, -ethanols, -glycerols, -inositols, -serines, sphingomyelins and triglycerides (Fig. 5A). For further analysis, the values of SAFit2-treated animals were normalized to the values of vehicle-treated animals. Afterwards, the normalized values were compared class wise for DRG and spinal cord samples. Focusing on lipid classes that include potential lipid mediators, we observed a slight increase of lysophosphatidylinositols in the DRGs and ceramides as well as glycosylceramides in the spinal cord after SAFit2 treatment (Fig. 5B). However, we detected a strong increase of fatty acids in the spinal cord through the treatment of SAFit2 (Fig. 5B).

Since the class of fatty acids was strongly elevated after SAFit2 treatment and has a particular relevance in the context of neuropathic pain, we further determined differences between the groups for individual fatty acids. Thereby, we detected increased levels of all measurable fatty acids in the spinal cord. The most drastically changed fatty acids were arachidonic acid (20:4) and docosahexaenoic acid (22:6). We also observed a slight upregulation of myristic acid (14:0) and oleic acid (18:1) (Fig. 5C). In summary, we detected indeed an elevation of free fatty acids and especially of polyunsaturated fatty acids (PUFAs) after SAFit2 treatment, which can be rapidly metabolized into pain-relevant signaling mediators.
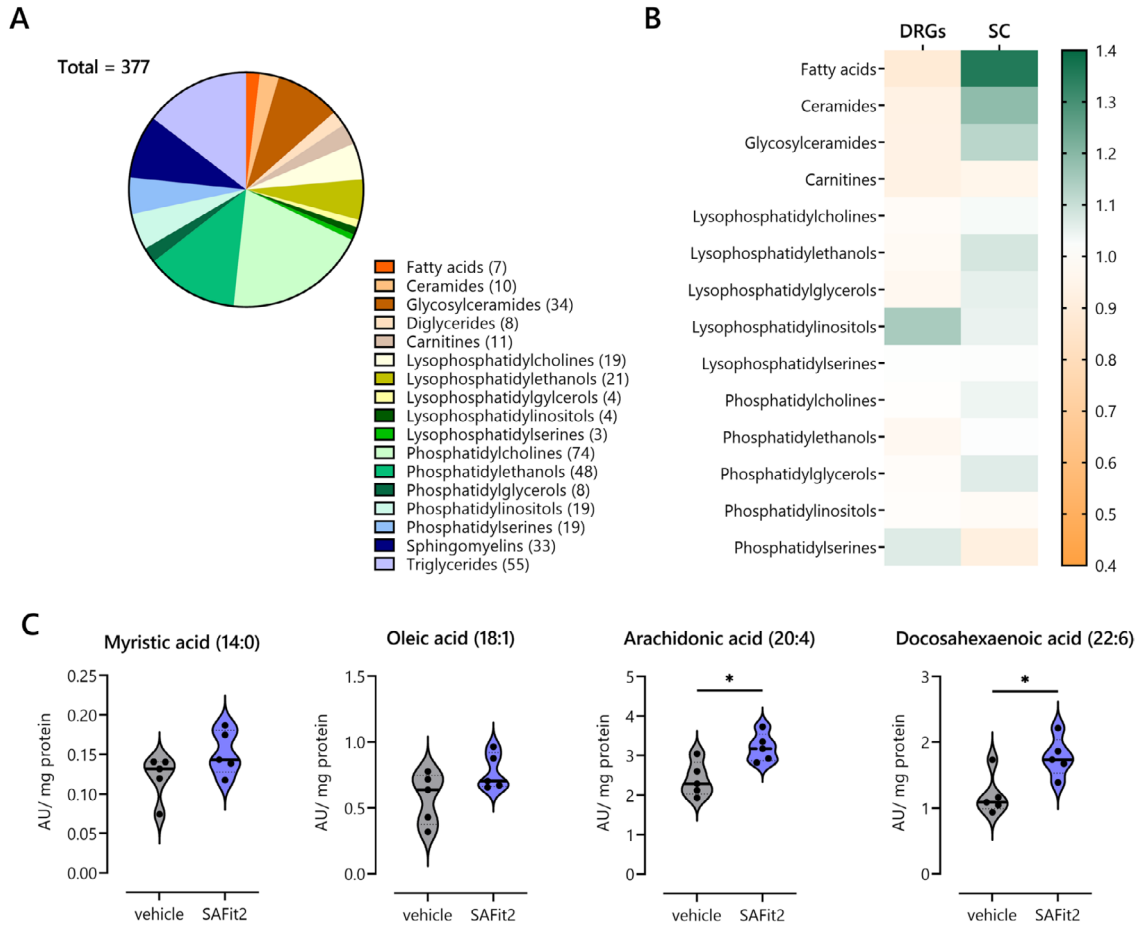
PUFAs can be absorbed from nutrition, but they can also be released from the lipid bilayer of cell membranes by the hydrolysis of an ester bond (Fig. 6A). In particular, the phospholipase 2 can hydrolase the ester bond from the second carbon of a phospholipid, whereby a fatty acid is released. This free fatty acid can consist of different chain length, however, arachidonic acid, linoleic acid, eicosapentaenoic acid and docosahexaenoic acid are the most abundant ones. Nevertheless, these PUFAs are usually rapidly metabolized into both pro- and anti-inflammatory mediators that can regulate and influence the transmission of neuropathic pain [16]. To analyze

(See figure on next page.)

**Fig. 4** Reduced activation of astrocytes and microglia in the spinal cord of SAFit2-treated animals. Immunohistochemistry staining of astrocyte activation marker GFAP (**A**) and microglia marker IBA1 (**B**) at 20X magnification (scale bar: 50 µm) of spinal cord (dorsal horn) slices. Samples of naïve animals were labeled as control. **C** Quantification of GFAP-positive cells. **D** Quantification of IBA1-positive cells. **C, D** Positive cells were counted in 15–20 images per animal and averaged. Data represent the mean ± SEM from 15–20 images of four mice per group. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ one-way ANOVA with Tukey's multiple comparison test. *SAFit2* selective antagonist of FKBP51 by induced fit 2, *GFAP* glial fibrillary acidic protein, *IBA1* ionized calcium-binding adaptor molecule 1

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**Fig. 4** (See legend on previous page.)

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**Fig. 5** LC–HRMS lipid analysis of DRGs and spinal cord from vehicle and SAFit2-treated paclitaxel mice. **A** Distribution of measured lipid classes of both tissues in LC–HRMS analysis. **B** Relative frequencies of lipid classes in lumbar DRGs and spinal cord from SAFit2-treated animals, which were normalized to those of vehicle-treated animals. **C** Violin plots comparing fatty acid levels of spinal cord samples from vehicle and SAFit2-treated animals. The data represent the mean $\pm$ SEM from five mice per group. * $p < 0.05$ Student's *t*-test with Welch's correction. *DRGs* dorsal root ganglia, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *LC–HRMS* liquid chromatography–high-resolution mass spectrometry

whether SAFit2 can enhance the release of PUFAs, we investigated the effect of SAFit2 on the phospholipase 2 activity in vitro. Interestingly, SAFit2 contributes to an enhanced activity of the phospholipase 2 isoforms cPLA$_2$, iPLA$_2$ and sPLA$_2$ in primary sensory neuron lysates as we observed a concentration dependent increase of hydrolyzed substrate due to increasing SAFit2 concentrations (Fig. 6B). In summary, we observed that SAFit2 increases PUFA levels in vivo and contributes to the phospholipase 2 mediated release of PUFAs from neuronal membranes.

**SAFit2 enhances pro-resolving mediators after paclitaxel treatment**

As a next step, we investigated the expression of phospholipases and fatty acid metabolizing enzymes as we have observed an increase of free fatty acids after SAFit2 treatment (Fig. 7A–G). Furthermore, we compared the expression of both vehicle and SAFit2-treated paclitaxel groups with the expression of naïve animals.

Consistent with our previous results, we observed an increased expression of the phospholipases cPLA$_2$ and iPLA$_2$ in SAFti2-treated paclitaxel animals compared to the expression of control animals (Fig. 7A, B). In addition, we measured a significant upregulation of the lipoxygenase 5 (ALOX5) in the DRGs of SAFit2-treated

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A



B
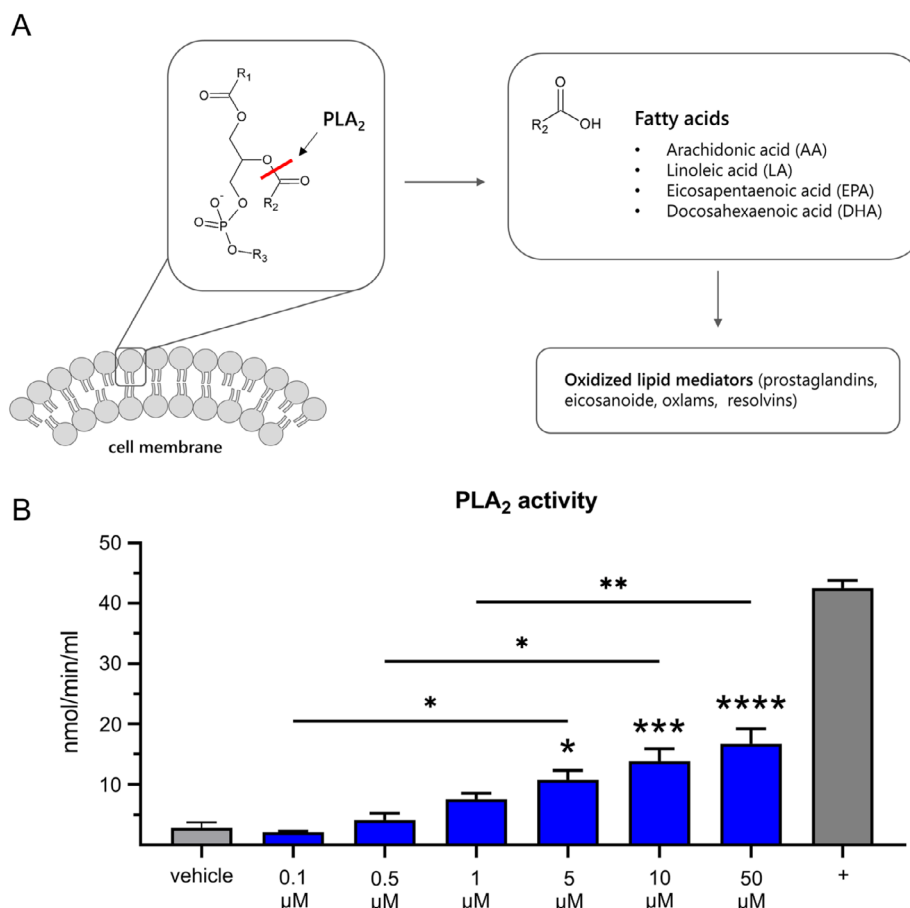


**Fig. 6** Influence of SAFit2 on phospholipase 2 activity in primary sensory neuron lysates. **A** Schematic illustration of the phospholipase 2 function and its products. Phospholipase 2 hydrolyzes the second ester bond of phospholipids that constitute the lipid bilayer of cell membranes. Thereby, polyunsaturated fatty acids were released, which were rapidly metabolized into oxylipins. **B** The influence of SAFit2 (0.1–50 µM) on the phospholipase 2 activity was assessed in lysed primary sensory neurons. Further, the basal phospholipase activity of the lysates was determined, the influence of the solvent DMSO (vehicle) verified and bee venom used as a positive control. Data represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ one-way ANOVA with Tukey's post-multiple comparison test. *PLA₂* phospholipase 2, *SAFit2* selective antagonist of FKBP51 by induced fit 2

paclitaxel animals (Fig. 7C). In contrast, the expression of this enzyme was significantly lower in the spinal cord of SAFit2-treated animals than in the vehicle-treated paclitaxel animals, yet the expression of both paclitaxel groups was not significantly altered from the control group. Nevertheless, the lipoxygenase 12 (ALOX12) was significantly increased in the DRGs of the SAFit2-treated group compared with both vehicle and control group (Fig. 7D). The expression of the lipoxygenase 15 (ALOX15) was not significantly altered in both tissues (Fig. 7E) and the expression of the cyclooxygenase 2 (COX2) was significantly reduced in the spinal cord for both paclitaxel groups compared to the vehicle group (Fig. 7F). However,

we observed a significant increase of the cytochrome-$P_{450}$-epoxygenase CYP2J6 in the DRGs after SAFit2 treatment (Fig. 7G). The expression of the cytochrome-$P_{450}$-epoxygenase CYP3A11 was lower than the detection limit in both tissues. In summary, we could observe significant differences in the expression of lipoxygenases and CYP2J6 comparing vehicle and SAFit2-treated paclitaxel animals.

Furthermore, we analyzed the expression of sphingosine kinases and ceramide synthases to detect any SAFit2-mediated alterations (Fig. 7F–K). However, we detected no significant changes in expression of vehicle and SAFit2-treated paclitaxel animals for the sphingosine

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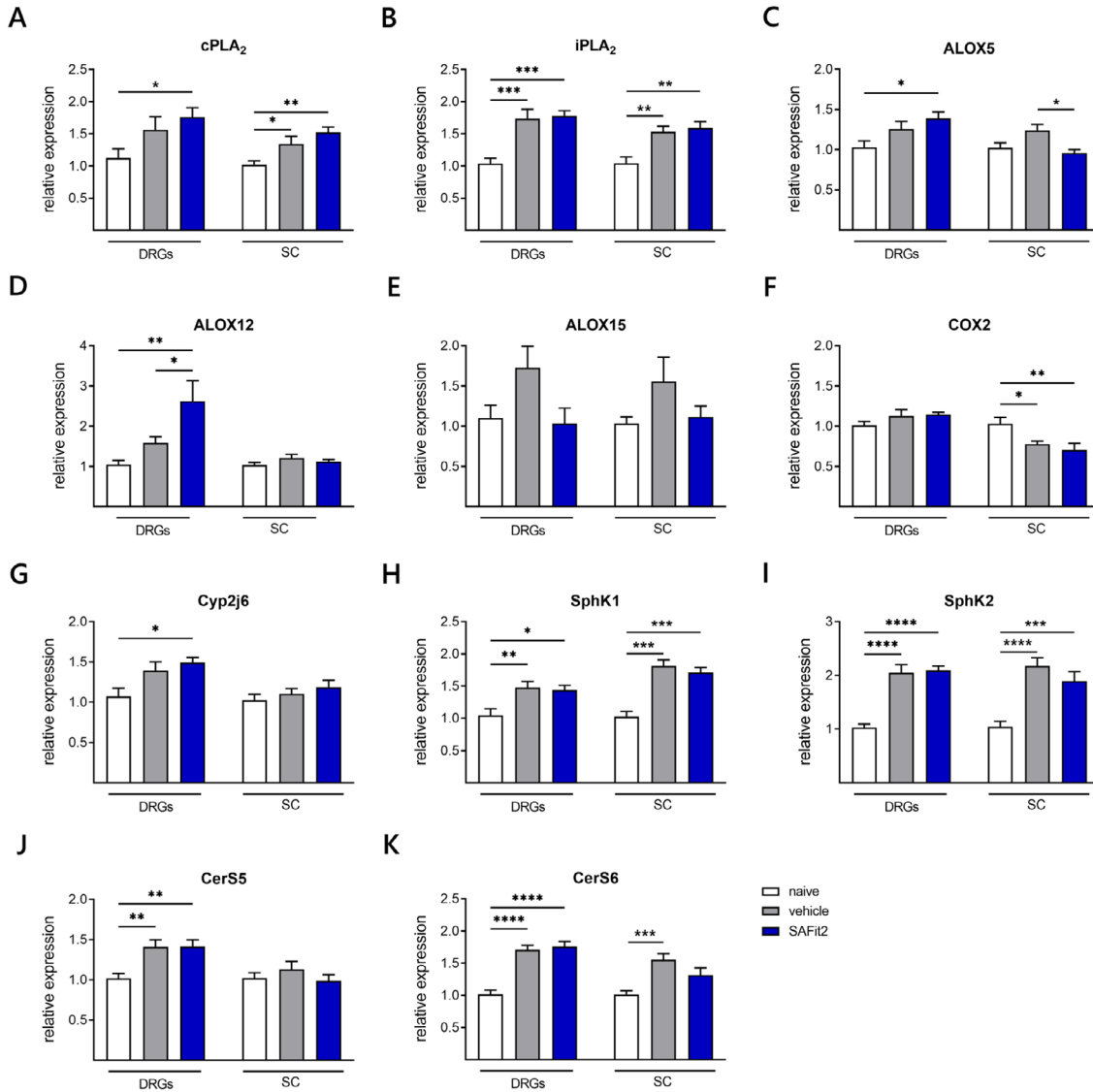


**Fig. 7** Gene expression of lipid synthesizing and metabolizing enzymes in neuronal tissue of SAFit2-treated paclitaxel mice. After twelve days, the expression of phospholipases (**A, B**), lipoxygenases (**C–F**), epoxygenase (**G**), sphingosine kinases (**H, I**) and ceramide synthases (**J, K**) was assessed via qPCR. The expression levels of vehicle and SAFit2-treated animals were normalized to the expression levels of naïve animals. The data represent the mean ± SEM from 3–4 mice per group, measured in technical triplicates, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with Tukey´s post hoc test was conducted per tissue. *DRGs* dorsal root ganglia, *SC* spinal cord, *SAFit2* selective antagonist of FKBP51 by induced fit 2, *cPLA₂* cytosolic phospholipase A2, *iPLA₂* calcium-independent phospholipase A2, *ALOX* arachidonate lipoxygenase, *CYP2J6* cytochrome P450 family 2 subfamily j polypeptide 6c, *COX2* cyclooxygenase 2, *SphK1/2* sphingosine kinase 1/ 2, *CerS* ceramide synthase

kinases one and two, though both groups were significantly increased compared to the control group (Fig. 7H, I). In addition, we observed no significant differences in the expression of ceramide synthase five and six for vehicle and SAFit2-treated paclitaxel mice (Fig. 7J, K). In summary, we detected a significant increase in the

expression of fatty acid oxidizing and metabolizing enzymes in the DRGs and spinal cord of SAFit2-treated animals.

Since we observed an increased PLA₂ activity, elevated free fatty acid levels and an increased expression of fatty acid metabolizing enzymes after SAFit2

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treatment, we further investigated the influence of SAFit2 on oxylipin levels. Oxylipins are mediators that are generated by the oxidation of free fatty acids and have been shown to play a crucial role as signaling molecules in the pathophysiological context of neuropathic pain [16, 33, 34]. To assess oxylipin levels of vehicle and SAFit2-treated paclitaxel mice as well as naïve mice, we performed a targeted LC–MS/MS analysis with DRG and spinal cord samples of the respective groups in which we were able to detect 27 oxylipins. Afterwards, the oxylipin levels of paclitaxel-treated animals were normalized to those of the control group and clustered in their precursor fatty acids (Fig. 8).

For the arachidonic acid metabolites, we detected a slight decrease of pro-inflammatory prostaglandins in the DRGs after SAFit2 treatment (Fig. 8A), which is shown representatively for prostaglandin D2 (Fig. 8B). Furthermore, we detected an increase of the anti-inflammatory and pro-resolving mediator 8,9-EpETrE in the DRGs. For the linoleic acid and alpha linoleic acid metabolites, we detected a significant decrease of the pro-inflammatory 12,13-DiHOME (Fig. 8C) and a significant increase of the anti-inflammatory 13-HOTrE after SAFit2 treatment (Fig. 8D). Consistently, we detected a significant increase of three docosahexaenoic acid metabolites 14-HDHA, 17-HDHA, 19.20-DiHDPA (Fig. 8E–G), which have also anti-inflammatory and pro-resolving properties. For the spinal cord samples, we could observe an increase of 12-HETE after SAFit2 treatment (Fig. 8A). However, 12-HETE was not significantly increased after SAFit2 treatment (Fig. 8H). Moreover, we measured a slight increase of 14-HDHA after SAFit2 treatment (Fig. 8I). In summary, we observed a decrease of pro-inflammatory and an increase of anti-inflammatory and pro-resolving oxylipins in the DRGs and spinal cord after the treatment with SAFit2.

In conclusion, we observed that SAFit2 enhances the $PLA_2$ mediated fatty acid release from neuronal membranes in paclitaxel-mediated pain states (Fig. 8J).

Thereby, predominantly the PUFAs arachidonic acid and docosahexaenoic acid were released and oxidized into oxylipins, which have previously been shown to influence and regulate neuropathic pain persistence and transmission. Interestingly, we mainly observed an increase of anti-inflammatory and pro-resolving fatty acid metabolites after SAFit2 treatment. Based on these results, we suggest that SAFit2 treatment leads to an increase of pro-resolving lipid mediators, which counteract neuroinflammation and spinal gliosis, resulting in an amelioration of paclitaxel-mediated neuropathic pain.

## Discussion

The need for CIPN therapeutics is increasing nowadays as available treatment strategies have either a low efficacy or severe side effects [35]. Interestingly, our data show that the novel and specific FKBP51 inhibitor SAFit2 can efficiently reduce mechanical hypersensitivity in paclitaxel-treated mice as it counteracts the paclitaxel-induced peripheral sensitization and neuroinflammation.

### SAFit2 reduces paclitaxel-induced peripheral sensitization

Firstly, we observed that SAFit2 has an influence on endogenous lipid levels as it decreases the levels of pro-inflammatory and transient receptor potential (TRP) channel sensitizing lipids mediators. More specifically, we detected that SAFit2 significantly reduces the levels of 12,13-DiHOME, which is a pro-inflammatory diol lipid that is metabolized from linoleic acid-derived epoxylipids. Furthermore, 12,13-DiHOME is known to mediate thermal hyperalgesia in mice as it sensitizes the pain-mediating TRPV1 channel and thereby increases the excitability of sensory neurons under inflammatory conditions [36]. In line with that, eicosanoids like the prostaglandin $E_2$ are also known to sensitize TRP channels and thereby enhance the excitability of sensory neurons [37, 38]. However, we detected a slight decrease of the prostaglandins $E_2$ and $D_2$ after SAFit2 treatment. A possible explanation for the decreased prostaglandin levels might

(See figure on next page.)

**Fig. 8** Alteration of oxylipins in DRGs and spinal cord of vehicle and SAFit2-treated paclitaxel mice. **A** Relative amount of oxylipins was measured in the DRGs and spinal cord of both groups and clustered in their precursor fatty acids. The raw data were normalized to the protein amount of the respective sample. **B–I** Violin plots display the relative lipid levels of those lipids, which were altered after SAFit2 treatment. The measured lipid levels of paclitaxel-treated animals were normalized to lipid levels of naïve animals. The analysis was conducted with six animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ student's $t$-test with Welch's correction. **J** Schematic summary of the effect of SAFit2 on lipid levels. SAFit2 seems to enhance the $PLA_2$ mediated fatty acid release from phospholipids. In particular, we detected an elevation of arachidonic acid and docosahexaenoic acid after SAFit2 treatment as well as an increase of their pro-resolving metabolites. Pro-inflammatory oxylipins were marked in red, anti-inflammatory/pro-resolving in green, the alteration after SAFit2 treatment is indicated by either a blue arrow for an increase or a blue tilde for no changes. Oxylipins indicated with dashed lines were below the detection limit. *DRG* dorsal root ganglia, *SC* spinal cord, *SAFit2*: selective antagonist of FKBP51 by induced fit 2, *AA* arachidonic acid, *LA* linoleic acid, *α-LA* alpha-linoleic acid, *DGLA* dihomo-γ-linolenic acid, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *PGE₂* prostaglandin E2, *PGD₂* prostaglandin D2, *TXB₂* thromboxane 2, *PGF2α* prostaglandin F2 alpha, *6-keto PGF₁ₐ* 6-keto prostaglandin F1 alpha, *HETE* hydroxyeicosatetraenoic acid, *DiHETrE* dihydroxyeicosatrienoic acid, *HHTrE* hydroxyheptadecatrienoic acid, *EpETrE* hydroxyepoxyeicosatrienoic acid, *HODE* hydroxyoctadecadienoic acid, *DiHOME* dihydroxyoctadecenoic acid, *HOTrE* hydroxyoctadecatrienoic acid, *HETrE* hydroxyeicosatrienoic acid. *HEPE* hydroxyeicosapentaenoic acid, *HDHA* hydroxydocosahexaenoic acid, *DiHDPA*: dihydroxydocosapentaenoic acid

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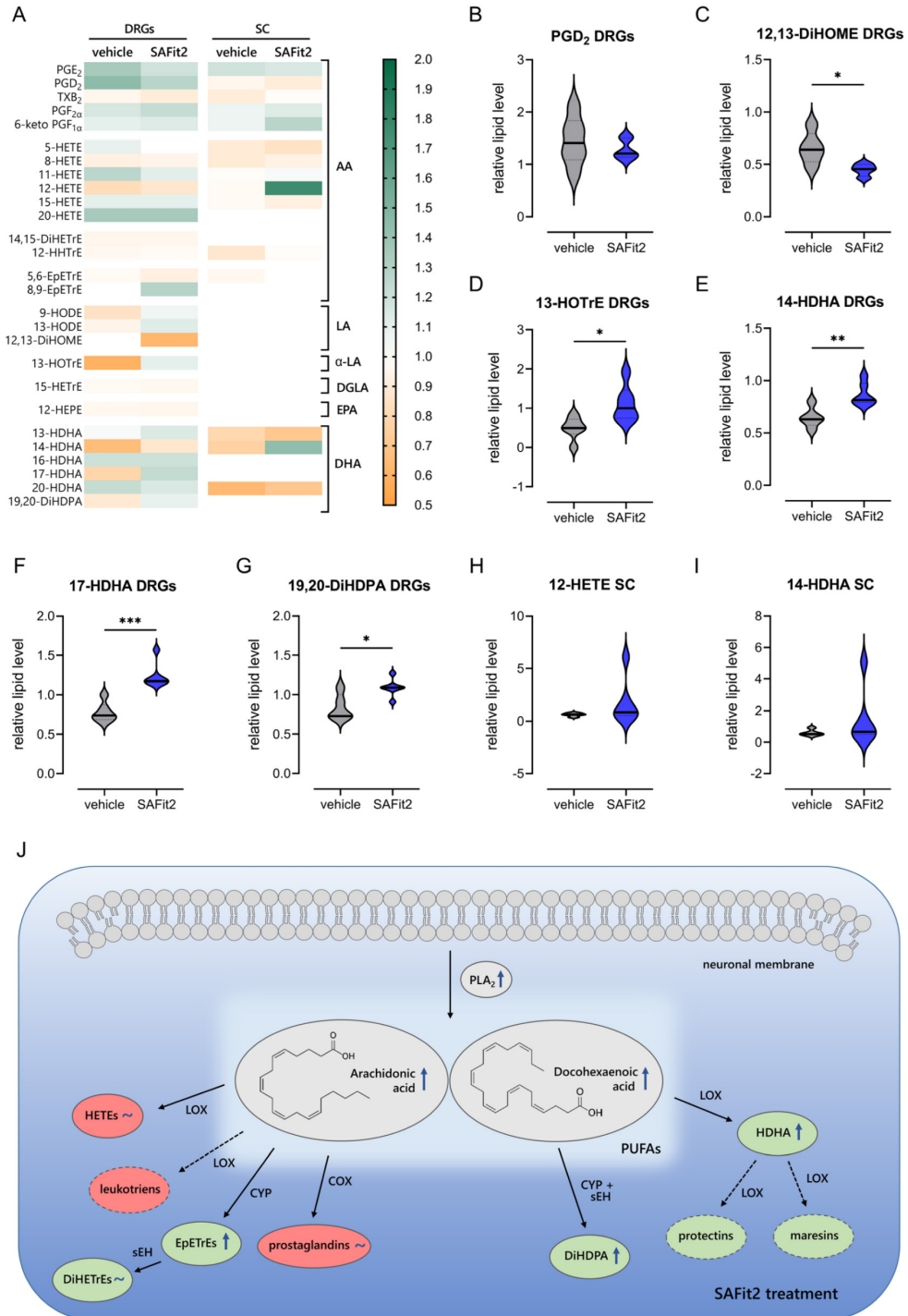


**Fig. 8** (See legend on previous page.)

be a reduced expression of COX2 after SAFit2 treatment, since prostaglandins are predominantly synthesized by the COX2 enzyme [39, 40]. Based on this observation, we assume that a SAFit2 treatment mediates a more anti-inflammatory lipid profile, by shifting oxylipin synthesis towards the LOX and CYP enzymes and not towards COX2, which might reveal one mechanism how SAFit2 is capable to counteract the enhanced excitability of sensory neurons after paclitaxel treatment.

### SAFit2 induces the resolution of paclitaxel-induced neuroinflammation

Peripheral sensitization can also be initiated and regulated by pain-mediating chemokines and pro-inflammatory mediators, that are released from activated glial cells [41]. In addition, it has been shown that paclitaxel-activated glial cells such as microglia and astrocytes can become neurotoxic [3]. Nevertheless, we observed that SAFit2 is capable to reduce gliosis after paclitaxel treatment, which leads to a reduction of pro-inflammatory and pain-mediating cytokines and chemokines in the spinal cord. In line with that, we detected a strong decrease of chemokines such as CCL2 and CCL7, which both are agonists of the C–C chemokine receptor type 2 that is expressed by both sensory neurons and glial cells [42]. The decrease of these chemokines has an essential influence on the pain perception of mice as CCL2 was shown to increase the frequency of excitatory post synaptic currents as well as to enhance the currents of AMPA and NMDA receptors, leading to an enhanced excitability of neurons and a sensitized pain state [43, 44]. In addition, both CCL7 and CCL2 have been shown to be direct activators of microglia [45, 46]. Another crucial pain-mediating chemokine, which is strongly downregulated after SAFit2 treatment, is CCL5, which was previously shown to induce hyperalgesia dose-dependently in mice [47–49]. Although some of the detected inflammatory mediators can be secreted from neurons (CCL2 and CXCL10) [32, 50], most of these mediators are released by immune cells, such as astrocytes (CCL2, CCL5, CCL7, CXCL10, IL-10), microglia (CCL5, CXCL10, IL-10) macrophages (CCL3, CCL4, IL-10) and T-cells (CCL4, IL-22) [51–57].

We did not investigate the levels of immune cells in neuronal tissue after SAFit2 treatment. However, the combined cytokine and chemokine data indicate a reduction of pro-inflammatory macrophages and an increase of anti-inflammatory macrophages and T-cells in DRGs after SAFit2 treatment. These data are also in line with our observations showing reduced numbers of activated astrocytes and microglia in the dorsal spinal cord after SAFit2 treatment. Mechanistically, we assume that SAFit2 on the one hand reduces the levels of these mediators as it counterbalances the enhanced NF-κB

activation [27] and on the other hand causes a more anti-inflammatory lipid profile. Thereby, SAFit2 is capable to decrease neuroinflammation and prevents gliosis and subsequently neurodegeneration in the spinal cord.

Furthermore, emerging evidence indicates that the resolution of neuroinflammation, which is mediated by pro-resolving mediators, becomes an essential issue in the relief of neuropathic pain [58–60]. In line with that, we detected an increase of anti-inflammatory cytokines like interleukin 10 and 22 after the treatment with SAFit2, indicating that SAFit2 even induces a resolution of neuroinflammation, which contributes to a relief of paclitaxel-mediated neuropathic pain.

### SAFit2 causes a shift towards an anti-inflammatory lipid profile in nervous tissue

Besides anti-inflammatory cytokines, PUFAs, such as DHA, have been shown to comprise anti-inflammatory and pro-resolving properties [61]. Interestingly, we observed an enhanced expression and activity of phospholipase 2 after SAFit2 treatment, which resulted in an increase of free polyunsaturated fatty acids and especially of the omega-3 PUFA DHA. These results further confirm our previously suggested SAFit2-mediated anti-inflammatory lipid profile, as DHA was shown to reduce neuropathic pain in a diabetic rat model as well as in a nerve injury rat and mouse model [62]. Moreover, studies reported that DHA has a protective effect on microglia and astrocytes as it counteracts oxidative stress induced calcium dysregulations and ER stress [63, 64].

Nevertheless, PUFAs such as DHA are known to be rapidly metabolized into oxylipins that play a crucial role in the regulation of neuropathic pain [16, 35]. In particular, the free fatty acid DHA gets metabolized by LOX and CYP enzymes into anti-inflammatory specialized pro-resolving mediators (SPMs) such as resolvins, protectins and maresins [65]. In line with that, we observed beside increased DHA levels also an enhanced expression of the respective LOX and CYP enzymes after SAFit2 treatment. Interestingly, these pro-resolving mediators and especially the class of resolvin mediators was shown to dampen inflammatory pain as they interfere in the mechanism of peripheral sensitization and reduce the excitability of sensory neurons [66]. Furthermore, the SPM neuroprotection D1 was able to reduce spinal gliosis and mechanical hypersensitivity in a neuropathic nerve injury model in vivo [67]. Unfortunately, we were not capable to detect SPMs in the DRG and spinal cord samples. However, we observed an increase of their anti-inflammatory precursors after SAFit2 treatment. More specifically, we detected an increase of hydroxydocosahexaenoic acids (HDHAs) such as 14-HDHA and 17-HDHA after SAFit2 treatment. Based on the elevation of DHA and

its metabolites after SAFit2 treatment, we conclude that SAFit2 enhances the levels of pro-resolving DHA-originated lipid mediators, which contribute to an anti-inflammatory lipid profile.

Apart from the DHA-originated mediators, we also detected an increase of the alpha linoleic acid metabolite 13-HOTrE. Although less is known about alpha linoleic acid-derived oxylipins in the literature, 13-HOTrE was associated with anti-inflammatory properties as it was shown to suppress the expression of the inflammatory cytokine IL-1β in human chondrocytes and to reduce glomerulomegaly in obese rats [68]. Moreover, we observed an increase in EpETrEs (e.g., 8,9-EpETrEs) after SAFit2 treatment, which are epoxyeicosatrienoic acids that are generated by the oxidation of arachidonic acid [69]. EpETrEs have also been shown to have anti-inflammatory properties as they inhibit the NF-κB translocation into the nucleus and thereby reduce the expression of, e.g., pro-inflammatory mediators or enzymes, that generate pro-inflammatory mediators, such as COX2 [70]. Based on these results, we assume that SAFit2 induces an anti-inflammatory lipid profile after paclitaxel treatment that reduces peripheral sensitization and consequently reduces paclitaxel-mediated mechanical hypersensitivity.

## Limitations and conclusion

A possible limitation of the study could be the lack of female mice. However, it has been previously shown that the involvement of FKBP51 in neuropathic pain is sex independent and its deficiency leads to an equal pain relief in both sexes [11]. A further limitation could also be the investigation of SAFit2-mediated effects on exclusively the peripheral nervous system and spinal cord regions as these compartments mediate and regulate the development and maintenance of neuropathic pain. Nevertheless, SAFit2 is also capable of affecting FKBP51 levels in the central nervous system as it passes the blood–brain barrier. However, the inhibition of FKBP51 in the brain displays not a disadvantage but rather seems to be beneficial as previous studies have shown that the inhibition of FKBP51 in the brain helps to prevent psychological disorders as well as stress-related and endocrinologic-mediated diseases such as depression, type two diabetes and obesity [71].

Indeed, SAFit2 has already been tested in several neuropathic models and showed promising effects in reducing mechanical and thermal hypersensitivity in vivo. The models themselves differ profoundly concerning the degree of neuroinflammation to pain hypersensitivity. While the nerve injury models SNI and CCI (spared nerve injury, chronic constriction injury) are usually connected with a strong spinal gliosis and related neuroinflammation, the chemotherapy-induced neuropathy models usually show a more neurotoxic and less inflammatory effect, although this depends on the cytostatic drug that is used [32, 72–74].

In line with these reports, we observed elevated levels of cytokines (especially in the dorsal spinal cord) and chemokines (especially in DRGs) in the SNI model, many of which were significantly reduced after SAFit2 treatment [27]. Similar results were observed in animals deficient of FKBP51 [10]. In the current manuscript, we do not see this strong and general increase in (neuro)inflammatory mediators, especially not in pro-inflammatory interleukins, which is in line with previous results [11]. In this model, we observe that SAFit2 rather causes a decrease of pro-inflammatory chemokines in DRGs and an increase of anti-inflammatory mediators (IL-10, IL-22). Moreover, we show, that SAFit2 treatment also causes a shift towards a pro-resolving lipidomic profile within DRGs and the dorsal spinal cord.

In conclusion, we show that the novel and specific FKBP51 inhibitor SAFit2 reduces gliosis in the spinal cord, which leads to a reduction of pain-mediating chemokines. Furthermore, we observed an increase of anti-inflammatory and pro-resolving cytokines and lipid mediators due to a SAFit2 treatment, which contribute to a resolution of neuroinflammation and peripheral sensitization after paclitaxel treatment. In summary, these effects can explain the SAFit2-mediated pain relief after paclitaxel treatment and highlight the role of SAFit2 as potential novel analgesic drug candidate.

**Abbreviations**

| | |
|---|---|
| AA | Arachidonic acid |
| CCL | C-C motif ligand |
| CIPN | Chemotherapy-induced peripheral neuropathy |
| CXCL | C-X-C motif ligand |
| DGLA | Dihomo γ linolenic acid |
| DHA | Docosahexaenoic acid |
| DiHDPA | Dihydroxydocosapentaenoic acid |
| DiHETrE | Dihydroxyeicosatrienoic acid |
| DiHOME | Dihydroxyoctadecenoic acid |
| DRGs | Dorsal root ganglia |
| EPA | Eicosapentaenoic acid |
| EpETrE | Epoxyeicosatrienoic acid |
| FKBP51 | FK506 binding protein 51 |
| GFAP | Glial fibrillary acidic protein |
| HDHA | Hydroxydocosahexaenoic acid |
| HEPE | Hydroxyeicosapentaenoic acid |
| HETE | Hydroxyeicosatetraenoic acid |
| HETrE | Hydroxyeicosatrienoic acid |
| HHTrE | Hydroxyheptadecatrienoic acid |
| HODE | Hydroxyoctadecadienoic acid |
| HOTrE | Hydroxyoctadecatrienoic acid |
| IBA1 | Ionized calcium-binding adaptor molecule 1 |
| IL | Interleukin |
| LA | Linoleic acid |
| LC–HRMS | Liquid chromatography–high-resolution mass spectrometry |
| PG | Prostaglandin |
| PlA$_2$ | Phospholipase A2 |
| PUFA | Polyunsaturated fatty acids |
| SAFit2 | Selective antagonist of FKBP51 by induced fit |

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| SC | Spinal cord |
|---|---|
| TRP | Transient receptor potential |
| TRPV1 | Transient receptor potential vanilloid 1 channel |
| $TXB_2$ | Thromboxane 2 |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-023-02835-5.

> **Additional file 1: Figure S1.** Enhanced FKBP51 expression in spinal cord and L4-L5 DRG slices of paclitaxel-treated animals. **Figure S2.** Pharmacokinetic study of SAFit2 comparing two formulations. **Figure S3.** Reduced ATF3 expression in L4 and L5 DRGs of SAFit2-treated animals. **Figure S4.** Reduced cFOS expression in spinal cord and L4-L5 DRG slices of SAFit2-treated animals. **Figure S5.** Cytokines and chemokines measured in the DRGs of paclitaxel-treated mice. **Figure S6.** Cytokines measured in the spinal cord of paclitaxel-treated mice. **Figure S7.** Chemokines measured in the spinal cord of paclitaxel-treated mice.

## Author contributions

FH, GG and MS conceptualized the project. SW performed the behavioral assays and collected the tissue. SW, LH, YS, DT, CN and MU performed measurements. TH synthesized the batch of SAFit2. NdB supervised the behavioral study and the pharmacokinetic study together with MVS. SW performed the data analysis and curation. SW and MS wrote the original draft. All authors read and approved the final manuscript.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional information file.

## Declarations

### Ethics approval and consent to participate

All experiments involving animals were approved by the local Ethics Committee for Animal Research (Darmstadt) under the permit numbers F152/1021, F152/1015 and FK1113. Furthermore, all animal experiments were performed according to the recommendations of the Preclinical Pain Research Consortium for Investigating Safety and Efficacy (PPRECISE) Working Group [75], and according to the updated ARRIVE guidelines for reporting animal research [76]. All efforts were made to minimize suffering.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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**Publisher's Note**

# Additional information

## SAFit2 ameliorates paclitaxel-induced neuropathic pain by reducing spinal gliosis and elevating pro-resolving lipid mediators

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Additional Figures: 7

A

control    paclitaxel

FKBP51 (FITC)

FKBP51 (Cy3)

B

control    paclitaxel
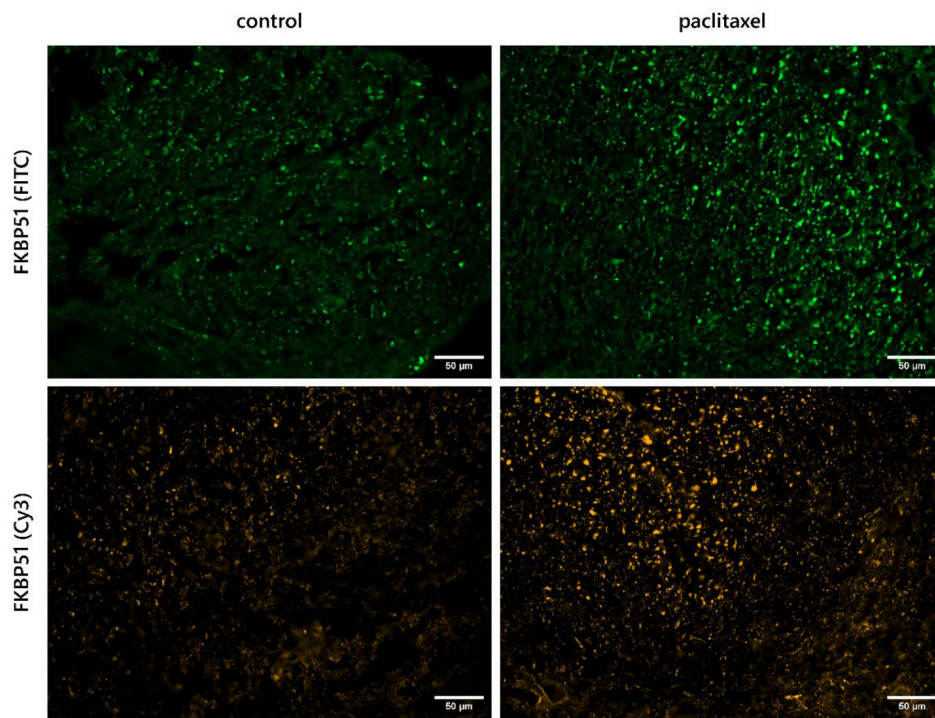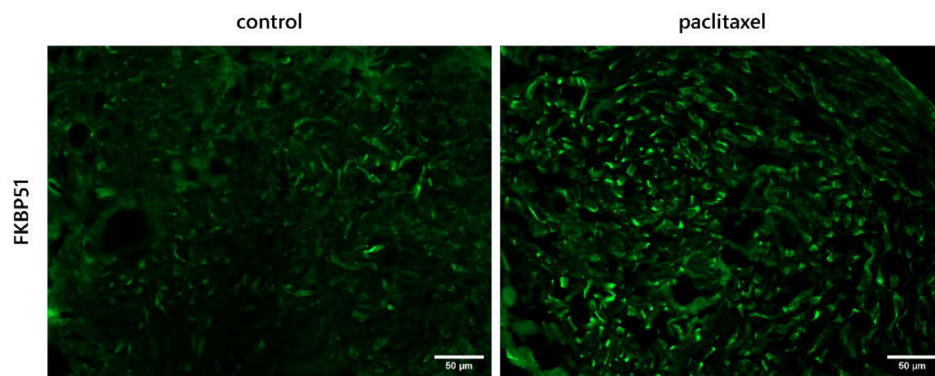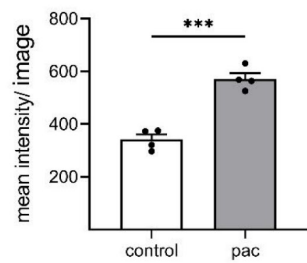
FKBP51

C                          D

**Figure S1: Enhanced FKBP51 expression in spinal cord and L4-L5 DRG slices of paclitaxel-treated animals.** Immunohistochemistry staining of the protein FKBP51. Representative images of FKBP51 stained spinal cord (dorsal horn) **(A)** and L4-L5 dorsal root ganglia (DRG) **(B)** slices at 20X magnification (scale bar: 50 μm). Samples of naïve animals were labeled as control. Quantification of the mean intensity per image for spinal cord samples **(C)** and DRG samples **(D)**. Data represent the mean ± SEM from 10 quantified images per mouse. Each condition comprises data from four mice. ** p < 0.01, *** p < 0.001, student's t-test with Welch's correction.

**A** SAFit2 concentration in plasma

**B** SAFit2 concentration in the brain (cerebellum)
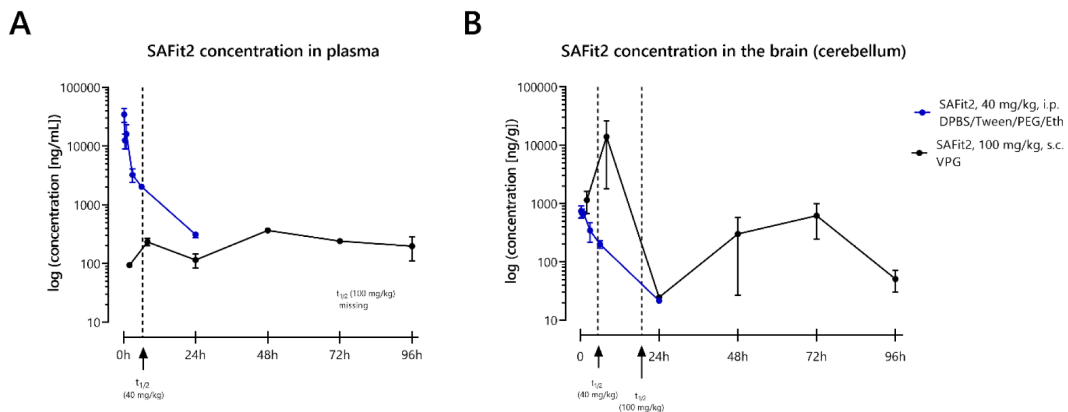
**C**

|  | plasma | | brain | |
|---|---|---|---|---|
|  | SAFit2, 40 mg/kg i.p. | SAFit2, 100 mg/kg VPG s.c. | SAFit2, 40 mg/kg i.p. | SAFit2, 100 mg/kg VPG s.c. |
| $T_{1/2}$ [h] | 6.4 | – | 5.4 | – |
| $T_{max}$ [h] | 0.25 | 48 | 0.25 | 8 |
| $C_{max}$ [ng/ml],[ng/g] | 34484 | 367 | 741 | 1801 |

**Figure S2: Pharmacokinetic study of SAFit2 comparing two formulations.** The animals received one dose of SAFit2 formulated either in PBS supplemented with 5% PEG400, 5% Tween and 0.7% ethanol or as slow-release formulation in vesicular phospholipid gel (VPG). To assess the concentrations of SAFit2 in plasma **(A)** and brain **(B)**, samples were collected from three mice for each indicated time point. **(C)** The table shows the calculated half-time as well as the maximum time and the maximum measured concentration of SAFit2.
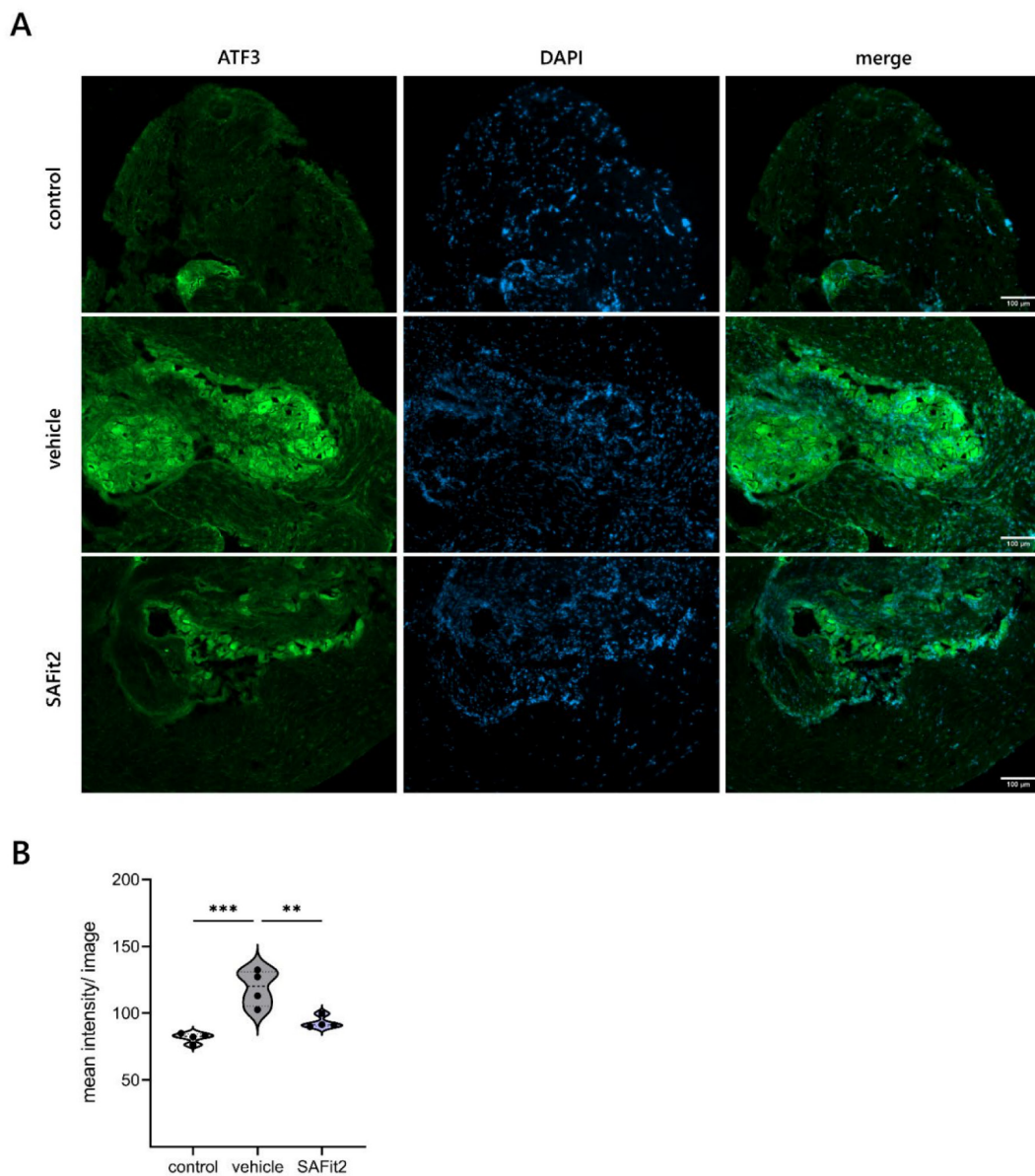
**Figure S3: Reduced ATF3 expression in L4 and L5 DRGs of SAFit2-treated animals.** Immunohistochemistry staining of the neuronal stress marker ATF3. **(A)** Representative images of ATF3 stained L4 and L5 dorsal root ganglia (DRGs) at 10X magnification (scale bar: 100 μm). Samples of naïve animals were labeled as control. **(B)** Quantification of the mean intensity per image. Data represent the mean ± SEM from 10 quantified images per mouse. Each condition comprises data from four mice. ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA with Tukey´s multiple comparison test.
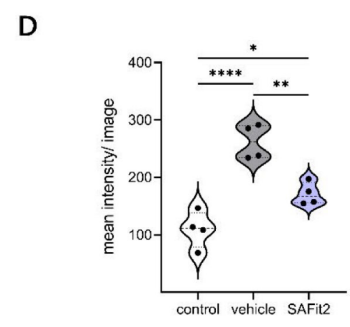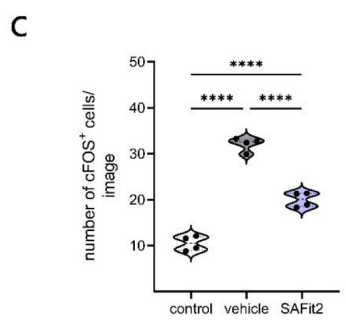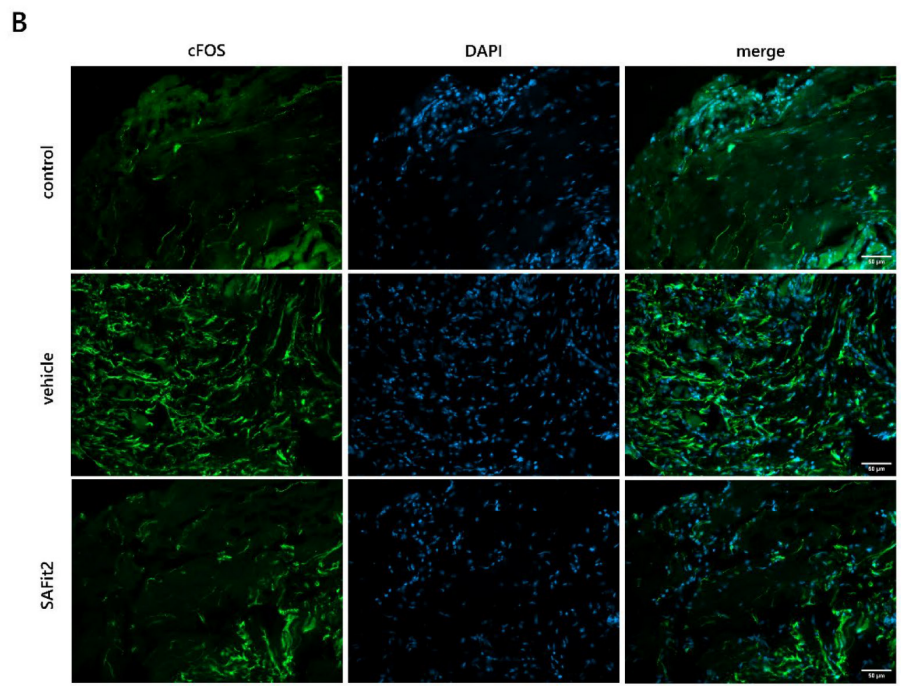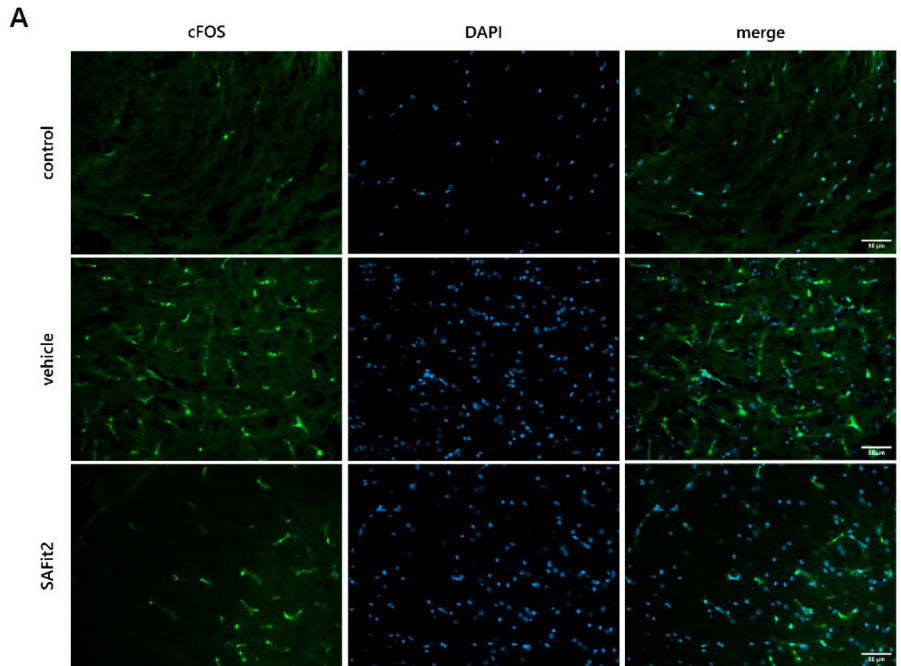
**Figure S4: Reduced cFOS expression in spinal cord and L4-L5 DRG slices of SAFit2-treated animals.** Immunohistochemistry staining of the neuronal activity marker cFOS. Representative images of cFOS stained spinal cord (dorsal horn **(A)** and L4-L5 dorsal root ganglia (DRGs) **(B)** slices at 20X magnification (scale bar: 50 µm). Samples of naïve animals were labeled as control. **(C)** Quantification of cFOS positive signals per image. **(D)** Quantification of the mean intensity per image. Data represent the mean ± SEM from 10 quantified images per mouse. Each condition comprises data from four mice. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ one-way ANOVA with Tukey's multiple comparison test.
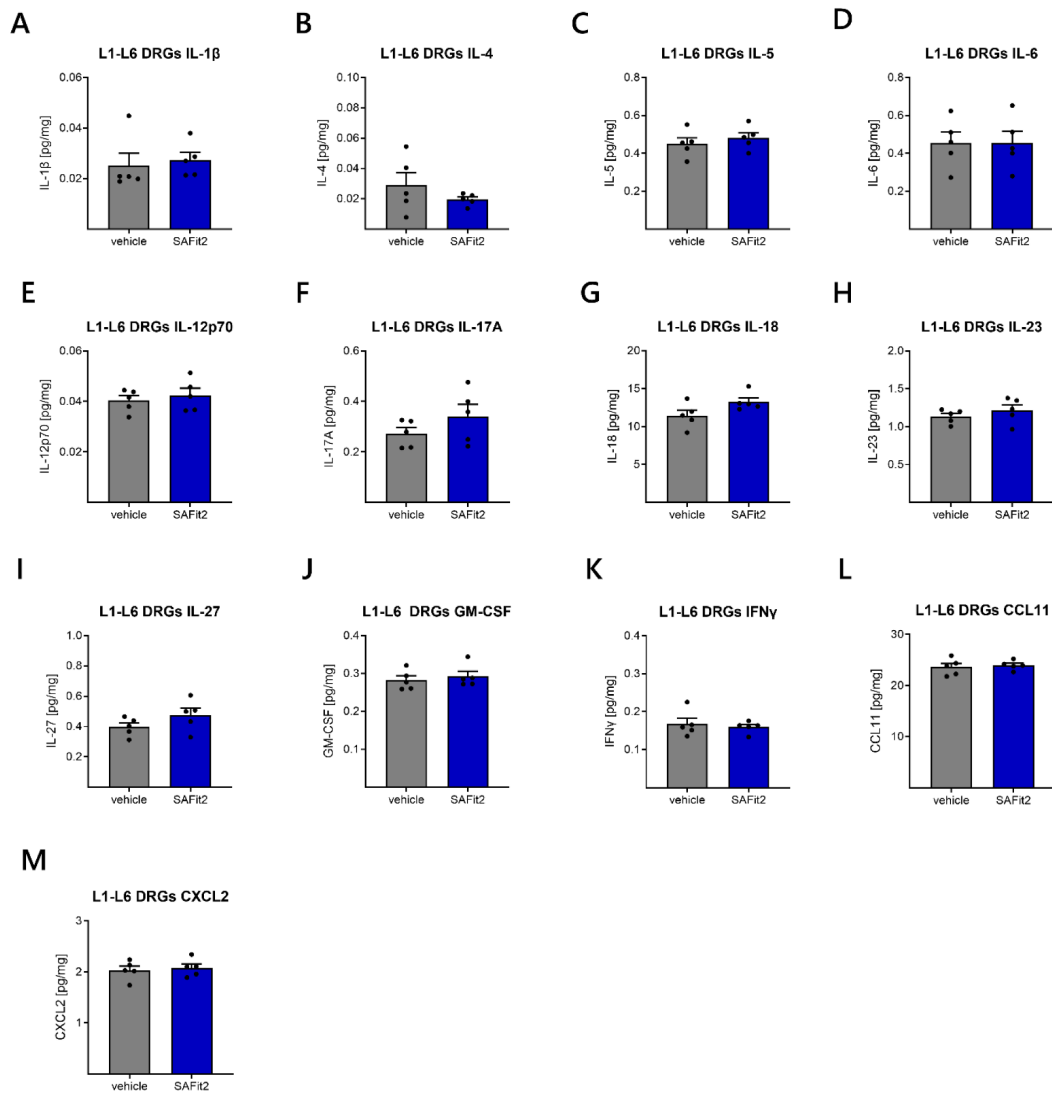
**Figure S5: Cytokines and chemokines measured in the DRGs of paclitaxel treated mice.** After 12 days, DRGs samples were homogenized and analyzed using a multiplex immunoassay including a panel of 26 cytokines and chemokines. The data represents the mean ± SEM from 5 mice per group. The raw data was related to the total protein amount of the sample.
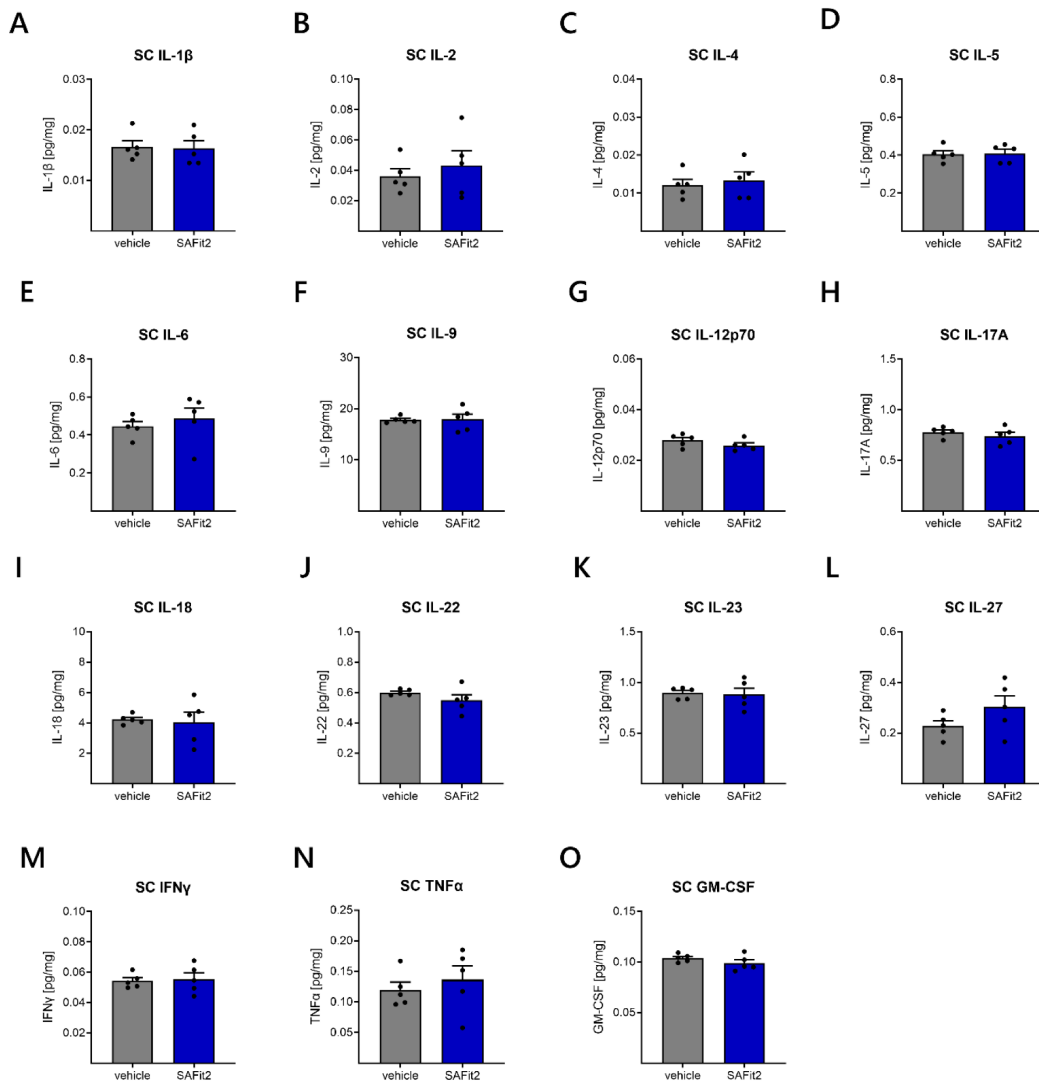
**Figure S6: Cytokines measured in the spinal cord of paclitaxel treated mice.** After 12 days, spinal cord samples were homogenized and analyzed using a multiplex immunoassay including a panel of 26 cytokines and chemokines. The data represents the mean ± SEM from 5 mice per group. The raw data was related to the total protein amount of the sample.
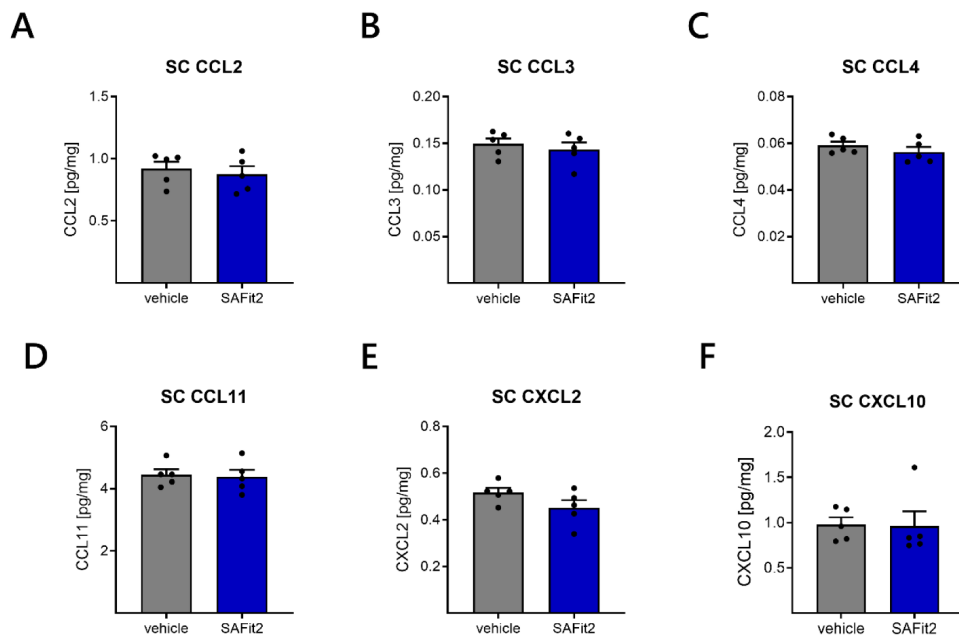
**Figure S7: Chemokines measured in the spinal cord of paclitaxel treated mice.** After 12 days, spinal cord samples were homogenized and analyzed using a multiplex immunoassay including a panel of 26 cytokines and chemokines. The data represents the mean ± SEM from 5 mice per group. The raw data was related to the total protein amount of the sample.

**Supplementary Methods section: DRG immunohistochemistry**

The L4 and L5 DRG samples were obtained as described in the "tissue isolation" method section in the main manuscript. Afterwards, the tissue samples were embedded in Tissue-Tek and frozen at -80 °C. For immunohistochemistry stainings, the frozen tissue samples were serially sliced into 12 µm tissue slices with a cryostat (Leica Biosystems). Next, the slices were stained according to the protocol, which is described in the "immunohistochemistry" method section in the main manuscript. The primary antibodies anti-ATF3 (sc-188, Santa Cruz) and anti-cFOS (9F3, Cell Signaling) were diluted 1:50 and 1:200, as recommended, respectively in 1% BSA solution and applied for an overnight incubation at 4 °C. The primary antibody anti-FKBP51 (sc-271547, Santa Cruz) was diluted 1:50 as recommended in 3% BSA solution to minimize cross-reactivity as the host species is mouse. The secondary antibody goat anti-rabbit Alexa Fluor 488 (ab150077, Abcam) was diluted 1:1000 in 1% BSA solution and applied for one hour at room temperature. The secondary antibodies goat anti-mouse Alexa Fluor 488 (CF488A, Biotium) and sheep anti-mouse Cy3 (C2181, Sigma Aldrich) were diluted in 3% BSA solution. For quantification purposes, 10 images were taken per animal per staining. One treatment group comprises samples from four animals as for the spinal cord samples.

# 5 General discussion

Within this thesis, the central aim was to elucidate whether SAFit2, a selective FKBP51 inhibitor, might serve as potential novel drug candidate for the treatment of nerve injury- and chemotherapy-induced, especially paclitaxel-induced, neuropathic pain. This guiding aim was based on previous data which showed that FKBP51 deficient mice experienced significantly less inflammatory and chronic pain (343, 344). Moreover, the lack of efficient neuropathic pain therapeutics highlights a relevance for novel targets, drug candidates, and treatment strategies. Based on this, we analyzed the impact of SAFit2 on different neuropathic pain states *in vivo* and on pain-associated mechanisms in primary cell cultures and with primary tissue samples *in vitro* (153, 370, 371). In line with this, we discovered that SAFit2 might constitute as novel treatment option for nerve injury- and chemotherapy-induced neuropathic pain as it efficiently relieved pain in both mouse models.

## 5.1 SAFit2-mediated effects in nerve injury- and chemotherapy-induced neuropathic pain in relation to the glucocorticoid receptor signaling

At first, the influence of SAFit2 on pain sensation was assessed in mouse models of nerve injury- and chemotherapy-induced neuropathic pain. Therefore, mice underwent either a surgery to cause a spared nerve injury or were repetitively injected with paclitaxel as cytostatic to induce the respective types of neuropathic pain. Afterwards, the mice were treated either with SAFit2 or the respective vehicle for a distinct period of time. Based on this, we observed an alleviation of mechanical hypersensitivity, which is defined as indication for persistent pain in rodents (373), after SAFit2 treatment in both neuropathic pain models (370, 371). Since SAFit2 gained a pain relief in two models that diverge in their initiation and vary in their underlying mechanisms, a SAFit2-mediated pain relief was suggested to be primarily mediated by an interference of SAFit2 into the disrupted FKBP51-GR interplay.

In line with this assumption, FKBP51-mediated dysregulations in the GR signaling were previously suggested to contribute to the development of neuropathic pain (343, 344). Associated to this, the expression of glucocorticoid receptors was increased in the spinal cord of nerve-injured mice and in the hippocampus of focal brain-injured mice (374, 375). Furthermore, previous studies associated the GR signaling with an upregulation of NMDA receptors, which play an important role in central sensitization mechanisms, in the spinal

cord of nerve-injured mice. Likewise, antagonizing GRs reduced the upregulation of NMDA receptors after nerve injury and antagonizing NMDA receptors reduced nerve injury-induced hypersensitivity in rodents (376, 377). Moreover, antagonizing GRs in an ankle joint inflammation model significantly reduced mechanical hypersensitivity in mice (343). However, targeting GRs in naïve mice led to the development of mechanical hypersensitivity. This indicates an anti-nociceptive function of the GR signaling at basal conditions and a pro-nociceptive role of the GR signaling in the development of inflammatory and neuropathic pain states (343). Based on this, the dysregulation of the GR signaling was suggested to mediate pro-nociceptive functions. Accordingly, previous studies indicated that an ablation of FKBP51, which functions as co-chaperone and downstream target of GR, established a constant anti-nociceptive property of the GR signaling (343, 344). According to these insights, counteracting the sustained upregulation of FKBP51 in neuropathic pain states would be beneficial for restoring GR properties and therefore ameliorating neuropathic pain. In line with this, SAFit2 mediated a pain relief in nerve injury- and paclitaxel-induced neuropathic pain states in mice (370, 371), indicating that SAFit2 efficiently interferes in the pathological and FKBP51-mediated dysregulation of the GR signaling (Figure 10).

A dysregulation of the GR signaling and the associated glucocorticoid resistance may also play an essential role in neuropathic pain underlying mechanisms which regulate the development and maintenance of neuropathic pain. More specifically, the GR signaling has been indicated to regulate the activation of the NF-κB signaling pathway and thereby to modulate the transcription of cytokines under physiological conditions (378). Based on this, high levels of glucocorticoids can facilitate a strong anti-inflammatory response. However, studies postulated that in chronic and excessive inflammatory states such as those in neuropathic pain, FKBP51-GR homeostasis is disrupted and GR insensitivity arises (379, 380). In addition, pro-inflammatory cytokines are suggested to mediate such dysregulations as they have been shown to decrease the expression of GRs and to block the GR translocation into the nucleus (381). In consequence, endogenous glucocorticoids fail to resolve neuroinflammation or inflammation in neuropathic and chronic inflammatory pain states.

However, this pathological glucocorticoid resistance might be resolved through an SAFit2 application in neuropathic pain states, since counteracting and preventing the pathologically increased FKBP51 levels, restored FKBP51-GR homeostasis and GR sensitivity *in vivo* (343). In addition, the anti-nociceptive and anti-inflammatory properties of the GR signaling could

be restored with SAFit2. In line with this hypothesis, we observed decreased pro-inflammatory cytokine levels in neuronal tissue of nerve-injured mice after SAFit2 treatment (Figure 10). Moreover, we observed a reduction in the NF-κB pathway activation in DRGs and spinal cord of nerve-injured mice after SAFit2 treatment (370). In accordance to this, a reconstituted GR signaling was shown to counteract an inflammation-driven and pathologically increased NF-κB activation (378). Beside the reduction of pro-inflammatory cytokines, paclitaxel-treated animals showed additionally an increase in anti-inflammatory cytokines like IL-10 and IL-22 after SAFit2 treatment. Likewise, the GR signaling is known to repress pro-inflammatory cytokines and to induce the expression of anti-inflammatory cytokines (382, 383), highlighting the complexity of the GR signaling. Nevertheless, these insights lead to the assumption that SAFit2 has the potential of restoring a physiological FKBP51-GR homeostasis in neuropathic pain states.

Interestingly, the comparison of cytokine levels reveals differences between the nerve injury- and the paclitaxel-induced neuropathic pain model after SAFit2 treatment (370, 371). Notably, SAFit2 reduced pro-inflammatory cytokine levels only in nerve-injured mice and not in paclitaxel-treated mice (Figure 10). In addition, SAFit2 increased anti-inflammatory cytokine levels only in paclitaxel-treated mice. These differences might be mediated by the different characteristics of the models. Paclitaxel-induced neuropathic pain encompasses a minor inflammatory component, whereas nerve injury-induced neuropathic pain is characterized by an inflamed injury site. This inflamed nerve injury in turn leads to a strong neuroinflammation, including an enhanced release of pro-inflammatory mediators such as cytokines (237, 261, 265, 274, 384-386).

In contrast to the model-dependent differences, the reduction of chemokine levels was of similar magnitude in DRGs and spinal cord of both models (370, 371). Chemokines are known to play a major role in the transmission of pain as they can directly act on sensory neurons and glial cells (275, 387). Furthermore, chemokines are known to activate glial cells and resident immune cells as well as to facilitate the migration and invasion of immune cells (388, 389). In particular, they serve as chemoattractants and enhance the expression of adhesion molecules in endothelia cells to enable a successful invasion of immune cells into tissue (390). Accordingly, we detected a reduced immune cell infiltration into DRGs and spinal cord of nerve-injured mice and a reduced glial cell activation in the spinal cord of paclitaxel-treated mice (Figure 10). However, we observed no alterations in the number of

immune cells at the site of injury, the sciatic nerve, in nerve-injured mice (370). This is of particular interest, as the application of glucocorticoids and the corresponding GR signaling are associated with systemic anti-inflammatory and immunosuppressive actions (390-392). In comparison, SAFit2- and glucocorticoid-mediated effects show major differences. Whereas glucocorticoids seem to overshoot the system and to switch a GR insensitivity towards a GR hyperactivation, SAFit2 seems to restore a physiological homeostasis of the GR signaling. This restored homeostasis was assumed after SAFit2 treatment since SAFit2 reduced the infiltration of immune cells into DRGs and spinal cord but did not impair the number of immune cells at the site of injury. Thereby, systemic neuroinflammation and sensitization processes were reduced without effecting the resolution of inflammation at the site of injury.
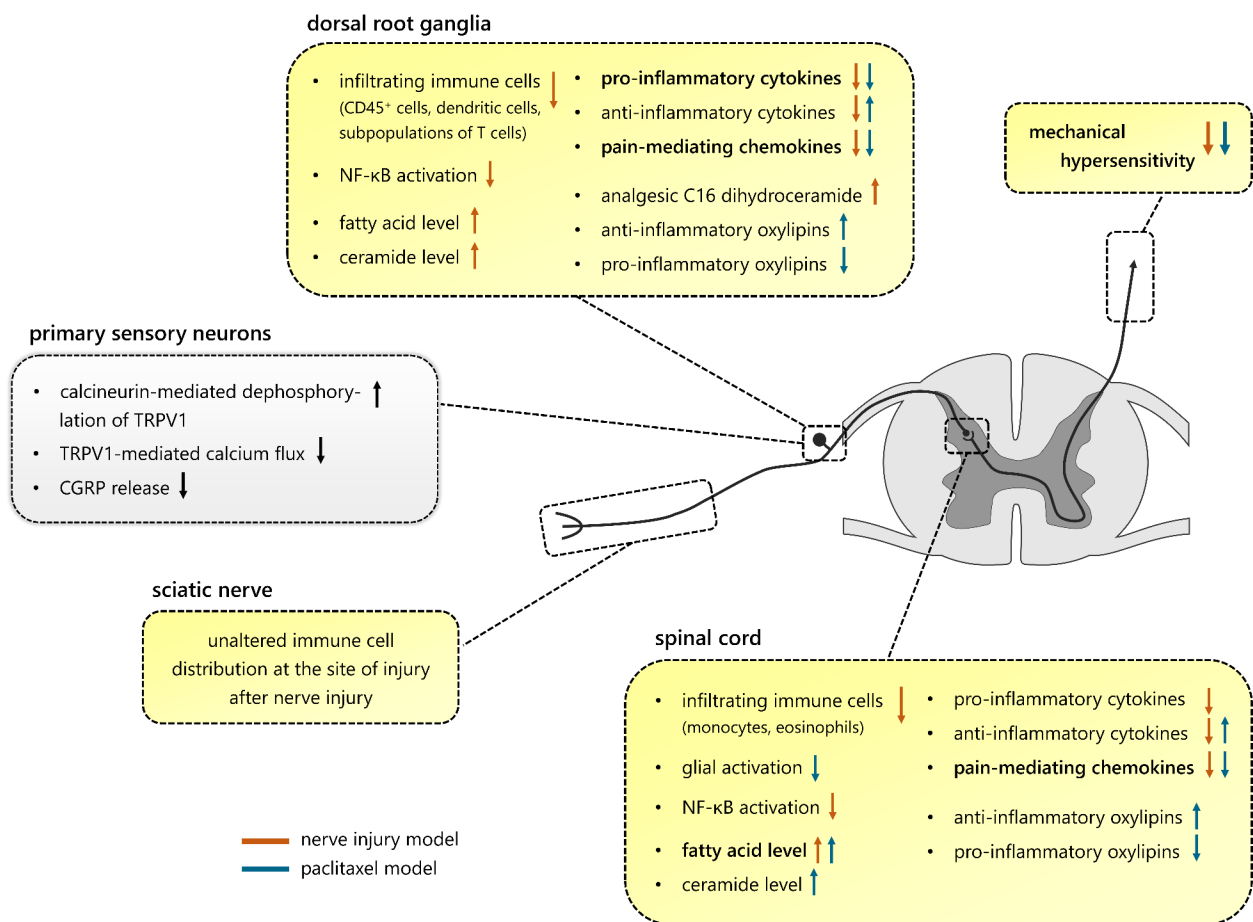


**Figure 10: Summary of SAFit2-mediated effects in nerve injury- and paclitaxel-induced neuropathic pain models**. SAFit2 significantly reduced mechanical hypersensitivity, a symptom of persistent pain, in both models. Furthermore, it significantly reduced levels of pro-inflammatory cytokines and pain-mediating chemokines in DRGs and spinal cord of nerve-injured mice. In contrast, paclitaxel-treated mice showed an increase of anti-inflammatory cytokines and a decrease of pain-mediating chemokines in DRGs and spinal cord

after SAFit2 treatment. Nevertheless, SAFit2 leads to changes in the number of activated immune cells in both models. Whereas SAFit2 decreases the invasion of immune cells into DRGs and spinal cord in the nerve injury model, SAFit2 reduces the activation of glial cells in the paclitaxel model. Notably, SAFit2 did not alter the number of immune cells at the site of injury, the sciatic nerve, in the nerve injury model. Moreover, SAFit2 counteracted an enhanced NF-κB pathway activation in DRGs and spinal cord of nerve-injured mice. Besides this, SAFit2 seems to increase the levels of free fatty acids as well as ceramides in both models. Furthermore, it restored the levels of an analgesic ceramide, dihydroceramide C16, after neve injury and of anti-inflammatory oxylipins after paclitaxel treatment. In primary sensory neurons, SAFit2 lead to the desensitization of the ion channel TRPV1 and a reduced release of the neuropeptide CGRP. Orange arrows represent changes in the nerve injury model, whereas blue arrows display changes in the paclitaxel model. In addition, light orange boxes display either *in vivo* or *ex vivo* data, whereas the grey box illustrates results from primary sensory neuron cultures. SAFit2-mediated effects that are similar in the nerve injury- and the chemotherapy-induced neuropathic pain are printed in bold. The illustration was created with images from motifolio.

The equilibrating property of SAFit2 displays a key benefit in the context of pain treatment, as the GR signaling displays a beneficial pathway for the regulation of anti-inflammatory and anti-nociceptive actions at basal levels (343). However, its homeostasis and regulation seem to be quickly impaired especially in the context of enhanced and chronic inflammation (343, 380, 393, 394). Furthermore, counteracting this disbalance either with GR antagonists or glucocorticoids, GR agonists, resulted in either an analgesic but not anti-inflammatory or strong anti-inflammatory but also immunosuppressive outcome (343, 377, 395-398). In line with this, many studies postulated diverging effects of pain sensation, when targeting the GR signaling with conventional therapeutics in complex mechanisms such as neuroinflammation (399). These contradicting outcomes especially involve mechanisms that include and affect neuronal and immunological components like the activation of astrocytes (400). Based on this, reconstituting the FKBP51-GR homeostasis with SAFit2 seems to be a promising approach to target complex neuroinflammatory mechanisms in pain.

Beside the GR signaling in chronic inflammatory states, glucocorticoids also induce contradictory effects in lipid metabolism. On the one hand, the acute increase of glucocorticoids lead to the release of stored energy like lipids, on the other hand, long term elevated glucocorticoid levels are associated with adipose tissue accumulation (401). However, the investigation of the GR signaling in the context of obesity and type II diabetes increases (402). In line with this, previous studies showed that FKBP51 deficient mice had lower lipid plasma levels, gained less body weight, and comprised less glucose intolerance

than wild type animals (339, 403). Nevertheless, the influence of the GR signaling on endogenous lipid mediators such as oxylipins is not well understood yet.

In this work, we firstly assessed lipid levels in nervous tissue after pharmacologically targeting FKBP51 (Figure 10). Thereby, we observed increased poly unsaturated fatty acid and ceramide levels in both models after SAFit2 treatment (369, 371). Although, the role of ceramides is poorly understood in the mediation of neuropathic pain, we identified one dihydroceramide which was significantly altered after SAFit2 treatment in the nerve injury model. Interestingly, we observed that the C16 dihydroceramide comprises analgesic properties as it desensitized the pain-mediating TRPV1 channel *in vitro* as well as reduced thermal hypersensitivity in a capsaicin mouse model (369). Based on this, we firstly revealed a lipid mediator that is restored by SAFit2 after nerve injury. Beside the C16 dihydroceramide, we also observed an increase in PUFAs in DRGs and spinal cord in both neuropathic pain models, highlighting especially the increase of docosahexaenoic acid (DHA) (369, 371). DHA is known as an anti-inflammatory PUFA which is significantly reduced after nerve injury and restored to a sham comparable level after SAFit2 treatment (369). In the paclitaxel model, DHA levels are even increased after SAFit2 treatment compared to the respective vehicle control. Moreover, we observed elevated DHA oxylipin levels, which are precursors for SPMs such as resolvins, after SAFit2 treatment in paclitaxel-treated mice (371). Based on these results among others, we assume that SAFit2 might cause a shift in lipid distribution towards an anti-inflammatory lipid profile and thereby contributes to the resolution of neuropathic pain.
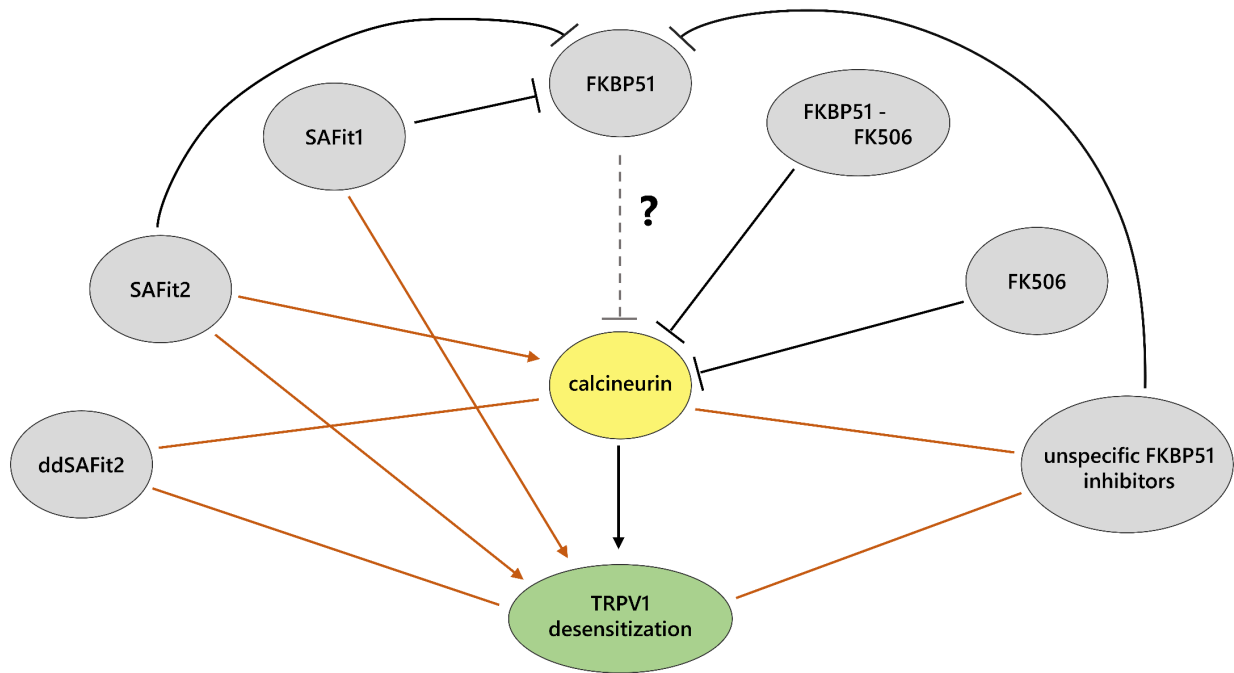
In summary, SAFit2 led to an amelioration of mechanical hypersensitivity in both nerve injury- and paclitaxel-induced neuropathic pain mouse models. Furthermore, it reduced signs of neuroinflammation like the reduction of pro-inflammatory mediators or the activation of glial cells. Lastly, SAFit2 increased anti-inflammatory lipid mediators in nerve injury- and paclitaxel-induced neuropathic pain mouse models, highlighting its comprehensive and multimodal regulation in the context of neuropathic pain.

## 5.2 The influence of SAFit2 on TRPV1 sensitization and the phosphatase calcineurin

Besides the promising *in vivo* effects of SAFit2, we observed *in vitro* that SAFit2 desensitizes the pain-mediating TRPV1 channel probably by decreasing its phosphorylation state (370). The dephosphorylation of TRPV1 is mainly mediated by the phosphatase calcineurin in sensory neurons (404, 405). In line with this, we observed that the calcineurin inhibitor cyclosporine reversed the SAFit2-mediated desensitization of TRPV1 in primary sensory neurons (370). Based on this, we investigated the influence of SAFit2 on calcineurin *in vitro*, detecting that SAFit2 enhanced the calcineurin-mediated phosphate release (370). Based on this insight, we suggest that SAFit2 desensitizes the pain-mediating TRPV1 channel in a calcineurin dependent manner. Although these findings would be beneficial in the context of pain and would contribute to a pain relieve *in vivo*, this data also raised the question whether an increased calcineurin-mediated dephosphorylation of TRPV1 might be an off-target effect of SAFit2.

To address this question, the molecular structure of SAFit compounds, which was developed on the molecular basis of FK506, has to be considered (367, 406). FK506 is predominantly known for its immunosuppressive function, mediating the inhibition of calcineurin (407, 408). However, the immunosuppressive capacity of SAFit compounds was neglected in different stimulation assays (367, 409). In those assays, SAFit compounds neither impaired T cell activation nor T cell cytokine secretion, highlighting that SAFit compounds have no immunosuppressive capacity (367). Nevertheless, elucidating whether the calcineurin-mediated TRPV1 desensitization is an on- or off-target effect of SAFit2, becomes a challenging issue, since the regulation of calcineurin is multifarious (Figure 11). Furthermore, the influence of uncomplexed FKBP51 on calcineurin is not fully discovered as only one group proposed the direct inhibition of calcineurin by FKBP51 (410). However, the inhibition of calcineurin by the complex of FKBP1-FK506 was confirmed in various studies (411-414). In line with that, the concept of scavenging either FKBP51 or minimizing the complex of FKBP51-FK506 with SAFit2 would support the assumption of a FKBP51 dependent effect, since SAFit2 directly competes with FK506 in the binding of FKBP51. This assumption was supported by the results which showed that a SAFit2-mediated desensitization of TRPV1 could be reversed by the inhibition of calcineurin in primary sensory neurons (370).

**Figure 11: The SAFit2-FKBP51 network depicted as influencing factors of calcineurin and TRPV1 desensitization**. The influence of FKBP51 on the activity of the phosphatase calcineurin is controversially discussed in literature. However, both the complex of FKBP51 and FK506 as well as FK506 alone have been shown to inhibit the activity of calcineurin, whereas SAFit2 or analogues either have no effect on the activity of calcineurin or increased the calcineurin-mediated phosphate release. Furthermore, calcineurin was shown to mediate the dephosphorylation and subsequent desensitization of TRPV1 in primary sensory neurons. In line with this, SAFit2 and analogues also showed a desensitization of TRPV1 in sensory neurons. However, unspecific FKBP51 inhibitors as well as ddSAFit2, a chiral and biological inactive analogue, did not. Blocking lines indicate an inhibitory effect, arrows an inducing effect and straight lines illustrate neither an induction nor inhibition. Black lines indicate results from literature, whereas orange lines indicate insights from this work.

To verify the effect of SAFit2 on TRPV1, the impact of unspecific FKBP51 inhibitors on TRPV1 was assessed. Interestingly, we observed that unspecific FKBP51 inhibitors, which additionally inhibit other FKBPs like FKBP12, neither desensitize nor sensitize the TRPV1 channel in primary sensory neurons (370). However, unspecific FKBP51 inhibitors should mediate the same effects as SAFit2, given that they were applied in sufficient high concentrations to efficiently inhibit FKBP51 beside other FKBPs. Accordingly, other FKBPs have been shown to inhibit calcineurin in an increased fold in their complexed forms compared to complexed FKBP51 (411). Likewise, independent of the concentration of unspecific FKBP51 inhibitors, they should gain the same effects on calcineurin as SAFit2. Based on this, a SAFit2-mediated desensitization of TRPV1 was suggested to be either an

FKBP51 independent effect or can be reasoned by an FKBP51 dependent structural effect of SAFit2, which cannot be achieved by other inhibitors.

Associated with this suggestion, we showed that SAFit2 analogues such as SAFit1, 19a and 19b, which specifically target FKBP51, induced a desensitization of TRPV1 in primary sensory neurons, but with varying potency (415). Based on these insights, a SAFit2-mediated desensitization of TRPV1 can be reasoned by a structural effect of SAFit2 and its analogues. In line with this, a chiral SAFit2 analogue, which is biologically inactive instead, neither desensitized the TRPV1 channel nor increased the calcineurin-mediated phosphate release *in vitro* (370). Furthermore, macrocyclic FKBP51 inhibitors did not show a desensitization at all (unpublished data). Taken together, future research has to be conducted to point out the particular molecular influence of SAFit2 on calcineurin and thereby on TRPV1 sensitization. In summary, the calcineurin-mediated desensitization of TRPV1 is suggested to be caused by a structural effect of SAFit2 and its analogues, derived from their initial lead structure, that has not been identified yet.

## 5.3 Risks and advantages of targeting FKBP51

The FKBP51-GR signaling is described as a fragile signaling pathway that is involved in the regulation of multifunctional processes (382, 416-418). Moreover, maladaptive dysregulations of the GR signaling were implicated in the context of pain (343, 344, 374, 377). However, targeting this pathway either with glucocorticoids or GR antagonists revealed controversial results (343, 393). Glucocorticoids have been shown to acutely prevent post-operative pain as they strongly counteract inflammation (419). Furthermore, they have been shown to be beneficial in some nerve injury animal models, but at the risk of developing GR insensitivity and possible side effects (396, 399, 402, 420, 421). However, glucocorticoid application in chronic pain states even exacerbated pain sensations in animals and patients (377, 395, 399). In line with this, the application of GR antagonists like mifepristone, which is also used to counteract adverse effects of glucocorticoids, was shown to be beneficial in the context of some chronic pain states (343, 422). These paradoxical effects of GR signaling regulators indicate that targeting FKBP51, as a dysregulated endogenous modulator in neuropathic pain states, can restore the beneficial functions of an FKBP51-GR homeostasis.

However, targeting FKBP51 can either additionally affect other processes or might be beneficial in other pathological disorders than in chronic neuroinflammation and neuropathic pain. To evaluate further outcomes of targeting FKBP51, the impact of FKBP51 deficiency has to be analyzed at first in healthy animals. FKBP51 deficient mice displayed no pathophysiological alterations or disorders, no impaired immunity or fertility (423). Furthermore, a long-term treatment with an FKBP51 inhibitor for 30 days, which reflects a model for chronic treatment in rodents, did not induce any pathological phenotype in mice and rats (406). However, both, chronic treatment and complete FKBP51 deficiency, resulted in a leaner phenotype and improved glucose tolerance, although the body temperature, food intake and activity of those animals was not altered (424). These changes can be associated with the tissue specific expression pattern of FKBP51. A comprehensive study in humans revealed that, although FKBP51 is expressed throughout the body, it is expressed the highest in adipocytes, skeletal muscle cells and immune cells beside the brain (340). Based on the high expression especially in adipocytes and muscles, it is conceivable that targeting FKBP51 might affect the energy metabolism of mice (323).

According to the strong expression of FKBP51 in adipocytes, FKBP51 was shown to be involved in both phases of adipogenesis, adipocyte differentiation and maturation (339, 425). Likewise, the deficiency of FKBP51 prevented the development of adipocytes and the accumulation of lipid droplets (426), whereas FKBP51 itself repressed the lipolysis of stored lipids in white adipose tissue (339). Lipolysis is initially mediated by the AKT pathway in muscles and adipocytes, in which the AKT expression is mainly restricted to the isoform AKT2 (340). Upon the activation of AKT through its phosphorylation, it further activates the kinase p38 which subsequently phosphorylates GRα and the peroxisome-activated receptor γ (PPARγ) (427). Upon the phosphorylation of these two factors, lipolysis is stimulated and adipogenesis repressed (340). However, FKBP51 was shown to scaffold the phosphatase PH domain leucine-rich repeat phosphatase (PHLPP) which dephosphorylates AKT and thereby represses the downstream events, resulting in a repression of lipolysis and an induction of adipogenesis (426, 427). In line with this, FKBP51 deficient mice are protected from gaining weight, obesity and hepatic steatosis in high fat diets (423). Furthermore, they showed less white adipose tissue, revealing FKBP51 as a potential target for obesity (423).

Besides obesity, diabetes displays also an issue in the context of both metabolism and pain. Furthermore, human studies identified a correlation between SNPs of *FKBP5* and type two

diabetes, pointing out FKBP51 as marker for insulin resistance (340, 428, 429). In fact, FKBP51 was shown to reduce glucose tolerance by interfering in the AKT signaling pathway. Upon the binding of insulin to muscle cells, the phosphoinositide 3-kinase (PI3K) signaling is activated. This leads to the phosphorylation of AKT by the pyruvate dehydrogenase kinase 1 (PDK1) and mammalian target of rapamycin (mTOR) complex 2 (430-433). Activated AKT in turn activates its downstream effector apoptosis signal-regulating kinase 1 (Ask1) and AS160 (AKT substrate of 160 kDa) through phosphorylation (339, 424). AS160 then promotes the translocation of the glucose transporter 4 (GLUT4) to the cell membrane (424). Based on this mechanism, more glucose can be taken up from skeletal muscle cells and glucose tolerance is increased. However, FKBP51 represses the activation of AKT and its downstream events, leading to the induction of glucose intolerance (429). In line with these insights, FKBP51 deficient mice as well as SAFit2-treated mice showed an improved glucose tolerance under both control and high fat diet conditions, pointing out the involvement of FKBP51 in body glucose homeostasis (424). Considering these insights along with the upregulation of FKBP51 in diabetes type II patients, FKBP51 might also serve as beneficial target in the context of diabetes and insulin intolerance (340, 406, 434).

Nevertheless, studies postulated that FKBP51 is involved in myoblast differentiation and myotube formation (339, 435). This is of particular interest, since deleting FKBP51 could lead to muscle atrophy. In line with this, a study postulated a delayed muscle regeneration in FKBP51 deficient mice (435). This data was supported by another study which showed an enhanced protein expression after FKBP51 overexpression in C2C12 cells (436). Moreover, long term treatment with glucocorticoids or the Cushing´s syndrome itself are associated with the occurrence of muscle atrophy (420). Based on this, FKBP51 is suggested to repress the activity of GRα through inhibiting the AKT pathway in those patients and thereby to counteract muscle atrophy (339, 426, 427). According to these recent findings, short-term targeting of FKBP51 could be beneficial to address chronic pain states. In contrast, long-term treatments should be applied with caution, since antagonizing FKBP51 could impair muscle growth and regeneration. However, it has to be taken into account that a complete deficiency of FKBP51 induces more severe effects than inhibiting FKBP51 to a certain amount. In conclusion, further studies need to be performed to further elucidate to which extend FKBP51 comprises a role in the context of muscle regeneration and growth and which impact a SAFit2 treatment would have.

Beside the strong expression of FKBP51 in adipocytes and muscles, it has also been shown to be highly expressed in the brain (340). Within the brain, FKBP51 is predominantly linked to many neurological disorders which are caused by a disruption of the GR signaling and will be discussed in a following chapter (321, 437). Beside neurological disorders, FKBP51 was associated to autophagy-mediated processes which play an important role in Huntington disease (438-440). Huntington disease is a rare condition that is predominantly described by the loss of neurons in the CNS and is mediated by a modification of the huntingtin (HTT) gene (441, 442). In addition, lowering the levels of the mutant HTT (mHTT) protein was shown to ameliorate Huntington disease (437). However, FKBP51 was suggested to mediate a conformational change in mHTT that prevents an autophagy-driven clearance of this protein (437). In line with this, lowering FKBP51 levels by pharmacologically targeting FKBP51, lowered the levels of the mutant HTT (mHTT) protein *in vitro* and *in vivo* (437). Associated to this, FKBP51 was shown to be involved in the complex regulation of autophagy in the hippocampus. Neither the complete deficiency nor an overexpression of FKBP51 led to a functional autophagy signaling (439). Exclusively moderate levels of FKBP51 were able to facilitate a functional autophagy signaling (439). In summary, an autophagy signaling can only be facilitated at basal levels of FKBP51 which seems to be restored via targeting FKBP51 with SAFit2. Based on this, targeting FKBP51 to restore autophagy reveals a novel and interesting treatment field since disrupted autophagy is linked to many disorders and diseases (443-445).

In addition to the suggested involvement of FKBP51 in autophagy, FKBP51 has been shown to comprise an essential role in the context of cancer. Furthermore, FKBP51 was postulated as biomarker for cancer by several studies (446-450), although the expression of FKBP51 is controversially discussed in several cancer types. Likewise, a hyperexpression of FKBP51 was recorded in many cancer types such as lymphomas, gliomas, melanoma, idiopathic myelofibrosis, skin melanoma, colon cancer, ovary cancer and prostate cancer (449-452). However, a downregulation of FKBP51 was identified in pancreatic cancer and breast cancer (453-455). The diverging expression levels of FKBP51 in different cancer types has been mainly suggested to depend on the originating cell type and the associated underlying mechanism (352, 449). Based on this, elucidating the underlying mechanisms of FKBP51 in the respective cancer types is crucial for targeting FKBP51 in the context of chemotherapy-

induced neuropathic pain. Therefore, suggested mechanisms are described in the following, starting with cancer types that are linked to an FKBP51 overexpression.

In the case of prostate cancer, the signaling of the androgen receptor becomes particularly important. Although FKBP51 represses the activity of GR and PR, it enhances the transcriptional activity of AR (325, 446). In fact, FKBP51 promotes the recruitment of the co-chaperone p23 to the HSP90 complex and thereby facilitates the expression of AR target genes, resulting in cell growth (456). Furthermore, FKBP51 itself displays a target gene of the androgen receptor, forming a short positive feedback loop (457). Based on this, FKBP51 stimulates tumor progression in prostate cancer which reveals FKBP51 as additional target beside common treatment strategies such as irradiation and chemotherapy (446, 456, 458). In line with FKBP51 as cancer target, studies reported a FKBP51-mediated resistance towards chemotherapeutics such as taxanes in ovarian cancer cells (352, 450, 453). Furthermore, silencing FKBP51 in those cells increased the sensitivity and efficiency of paclitaxel as cytostatic (459). These insights highlight a promising treatment strategy of targeting FKBP51 in paclitaxel-treated ovarian and prostate cancer patients with CIPN, enabling both an efficient cancer treatment and a reduction of CIPN.

Beside prostate and ovarian cancer, studies identified FKBP51 as a beneficial target in melanoma and lymphoblastic leukemia cancers based on other underlying mechanisms. Those studies identified that irradiating or chemically treating these cancer types, increased the activation of the NF-κB signaling pathway, while FKBP51 is overexpressed (446). However, counteracting the FKBP51 overexpression in turn reduced the activation of the NF-κB signaling and thereby increased their sensitivity towards irradiation and chemical treatment (355, 357, 364, 451, 460). Furthermore, a study revealed that silencing FKBP51 in melanomas reduced their growth, metastasis, and angiogenesis in a xenograft model (356). Another cancer type, in which the NF-κB signaling plays an essential role, is the ulcerative colitis-associated colorectal cancer as it is developed from a chronic inflammatory state (461). Based on this and in line with data from other NF-κB-related cancers, FKBP51 was postulated as biomarker for the development of colorectal cancer to predict the survival rate of patients (462). Thereby, an increased FKBP51 expression worsens the prognosis of patients (462). In summary, targeting FKBP51 seems to contribute to an efficient cancer treatment in NF-κB signaling related cancer types.

In contrast, FKBP51 was shown to play an essential role in breast cancer patients. Breast cancer patients show decreased FKBP51 levels in tumor tissue and 70% of breast cancer patients have an enhanced estrogen receptor alpha (ERα) expression and activity (463). Unfortunately, ERα interacts with the HSP90 complex and promotes cell proliferation upon transcriptional activity (464). FKBP52 was shown to enhance the ERα-HSP90 complex stability, whereas FKBP51 does not (454). Moreover, FKBP52 was shown to interact with the breast cancer susceptibility gene 1 (BRCA1) which monoubiquitinates ERα and thereby prevents its proteasomal degradation (454, 465). In addition, the deletion of FKBP51 was shown to increase the stability of ERα (454). Based on these insights, targeting FKBP51 in breast cancer patients with CIPN should be avoided until it is discovered whether targeting FKBP51 with SAFit2 or analogues affects the stability of ERα.

Another cancer type which displays low FKBP51 expression is pancreatic cancer (453). In this cancer type, FKBP51 expression was related to a decreased tumor progression, since FKBP51 represses the proliferation-related AKT pathway (466). However, AKT has different isoforms, whereas only AKT1 is related to the regulation of cell cycle and proliferation (340). Furthermore, it has been proven that SAFit2 and its analogues do not interfere in the FKBP51-mediated repression of AKT1 as SAFit2 binds to the binding pocket of FK1 in FKBP51 without sterically impairing its interaction with AKT1 (467). In line with this, targeting FKBP51 pharmacologically with SAFit2 for treating CIPN in AKT1-related cancer types should not enhance AKT-mediated tumor progression.

In summary, all these insights reveal a tissue specificity of FKBP51-mediated effects as its expression varies between tissues as well as the expression of its antagonistic homolog FKBP52 (340, 468). Furthermore, the interaction of FKBP51 with tissue specifically expressed AKT isoforms was controversially discussed in literature (308, 323, 339, 453, 467). Until now, FKBP51 has been proposed to interact with AKT1 and AKT2, but not with AKT3. AKT3 is predominantly expressed in the brain, whereas AKT2 is expressed mainly in muscles and fat, where it regulates glucose homeostasis, and AKT1 has postulated to be widely expressed and to regulate cell proliferation (340). Furthermore, SAFit2 and its analogues have been shown to not interfere in the FKBP51-mediated repression of AKT1, whereas they seem to prevent the FKBP51-mediated repression of AKT2, based on the recent data (340, 424, 467). However, the interaction of FKBP51 with AKT2 in the presence of FKBP51 inhibitors is controversially discussed and has to be investigated in further studies. In conclusion, FKBP51

seems to be a promising target in many pathologies such as pain, obesity, diabetes and cancer with FKBP51 overexpression, while targeting FKBP51 could be problematic in disease states like muscle atrophy and breast cancer. Furthermore, unexplored interaction partners of FKBP51 have to be further identified to reduce the risk of side effects.

## 5.4 SAFit2 as novel treatment option for pathological disorders especially neuropathic pain?

To date, SAFit2 represents the gold standard for pharmacologically targeting FKBP51 from over 400 other SAFit analogues, as it comprises the best overall profile concerning selectivity, affinity as well as pharmacokinetic and ADME (adsorption-distribution-metabolization-excretion) properties (406). Admittedly, other SAFit analogues showed singly improved parameters, but they did not perform comparably to SAFit2 in multiple parameters (406). SAFit2 comprises a binding affinity around 6 nM towards FKBP51, which is 10,000 times more selective than for its homologue FKBP52, making it a highly specific FKBP51 inhibitor (367). Nevertheless, SAFit2 additionally comprises affinity for FKBP12 and FKBP12.6, which is two times increased and four times lower than for FKBP51, respectively (469). FKBP12 is known to mediate immunosuppression in complex with FK506 through the inhibition of calcineurin (470). However, a study by Gaali et al. identified that SAFit1 does not repress T cell activation and cytokine production at 100 nM *in vitro,* which is a common effect of complexed FKBP12 (367, 471). Nonetheless, SAFit1 shows 10 times less cell permeability than SAFit2 in a Caco2 assay from Eurofins (469). Based on these insights, the influence of SAFit2 on FKBP12-mediated processes should be validated *in vitro*. Furthermore, evaluations of SAFit2-mediated effects should consider an FKBP12 involvement.

Besides the affinity for FKBP12, SAFit2 comprises an appreciable affinity for FKBP12.6. Although less is known about FKBP12.6, it was discussed to interact with the ryanodine receptor type two which is predominantly expressed in cardiac muscles (472). In addition, one study showed that FKBP12.6 dysfunctions can contribute to a disruption in the calcium-induced calcium release (CICR) system *in situ* (473, 474). The CICR system facilitates regular heart contractions, implicating that CICR system disruptions could lead to disruptions in calcium waves and arrhythmias (474). In contrast, an *in vivo* study showed that FKBP12.6 deficient mice did not develop any arrhythmias (475). However, chronic stress was shown to induce anxiogenic behavior in mice and to destabilize the binding of FKBP12.6 to ryanodine

receptor type two. This behavioral outcome could be prevented by the treatment with a tool compound, which stabilizes the interaction of FKBP12.6 and the ryanodine receptor (476). In summary, previous findings highlight a possible risk of SAFit2 treatment, since its impact on FKBP12.6 and FKBP12.6 interactions is not elucidated yet. Nevertheless, focusing on the cardiovascular impact, a cardiac toxicity test revealed that SAFit2 only marginally affects QT prolongation *in vitro*. In addition, only exceedingly high concentrations of SAFit2 around 10 µM, which are not reached *in vivo,* induced a tail current inhibition of 21.4% (406). Based on these insights and numerous *in vivo* studies, which also include chronic SAFit2 treatments, no SAFit2-mediated toxicity was reported (424, 477-484). However, for a further drug development, formal toxicity studies have to be performed.

In addition to SAFit2 specific targets, a broad screen, including 45 CNS-relevant drug targets, was performed to identify potential off-targets of SAFit2. The sigma two receptor was revealed as first off-target with an inhibitory constant of 226 nM (406). Sigma receptors are predominantly expressed in neuronal tissue und comprise remarkably similar binding pockets (485). Although their particular function is not discovered in detail yet, they display promising targets for the treatment of neurodegenerative disorders (486, 487). Furthermore, antagonists for both sigma receptors, one and two, are currently under investigation in clinical or preclinical studies (486). Based on this, inhibiting the sigma two receptor with SAFit2 seems not to raise any safety issues, but rather extend treatment possibilities for SAFit2. Secondly, the histamine receptor four was identified as off-target of SAFit2 with a low inhibition constant (Ki) of 3382 nM (406). The histamine receptor four is a GPCR that comprises a high affinity for histamine and was shown to mediate chemotaxis and migration in eosinophils and dendritic cells (488, 489). Moreover, the histamine receptor four is suggested as target for many allergy-driven diseases like asthma and pruritus but also for chronic pulmonary diseases, dermatitis and psoriasis (489). In conclusion, both off-targets of SAFit2 have been revealed as targets for other diseases and do not implicate a risk of developing any severe side effects (487, 490).

Another essential off-target and inhibitory function of SAFit2 is the inhibition of the CYP3A4, inhibiting already 58.1% of CYP3A4 at 1 µM (406). The CYP3A4 is the most abundant CYP enzyme with building up one third of the CYP enzymes in the liver. Likewise, CYP3A4 displays the most clinically relevant drug metabolizing enzyme (491). The inhibition of CYP3A4 reduces the metabolism and clearance of numerous drugs which leads either rapidly to

toxicity and overdosing or to an impaired efficiency of prodrugs (492). Therefore, the co-administration of SAFit2 with any other drug may lead to pharmacokinetic interactions due to the strong inhibitory effect of SAFit2 on CYP3A4. Regarding the treatment of paclitaxel-induced neuropathic pain, it has to be taken into account that paclitaxel itself comprises cell and neurotoxic properties that could be enhanced by co-treating with SAFit2, since paclitaxel is partially metabolized via the CYP3A4 (493). Based on this, a reduction of CYP3A4 inhibition should be achieved for further optimized clinical lead compounds. Associated to drug metabolization, it is yet unexplored how SAFit2 is metabolized and whether SAFit2 metabolites might have toxic properties (406). However, referring to the previously discussed SAFit2 toxicity, SAFit2 metabolites are suggested to be non-toxic, since chronic treatment with SAFit2 over 30 days and very high doses of 100 mg/kg (unpublished data) did not reveal evidence for any toxicity (406, 424). Nevertheless, the maximal tolerated dosage of SAFit2 is unknown.

Beside metabolization and toxicity, the distribution of SAFit2 was elucidated in pharmacokinetic studies which comprised different formulations and dosages of SAFit2 (367, 371). These revealed a half-life time of SAFit2 of around nine hours in plasma levels and a high volume of distribution with standard formulation (367, 406). A high volume of distribution describes the property of a drug to enter extravascular compartments, meaning that a higher dose is required to achieve high plasma levels (494). In line with this, a moderate brain permeability of SAFit2 was discovered (367). However, SAFit2 concentrations in other tissues such as muscles and fat remain unknown and should be investigated in the future based on the high distribution volume of SAFit2. Nevertheless, a study could show that treating animals with 20 mg/kg two times daily resulted in stable plasma levels between 1500-2000 ng/ml (424). However, to gain stable plasma concentrations repetitive injections of SAFit2 are still necessary, since it is not orally administrable until now (406). Based on this, the bioavailability of future analogues and lead compounds should be improved.

Independent from the improvable pharmacological profile of SAFit2, it has been shown to readjust the balance between FKBP51 and FKBP52 in a neuronal cell line and in primary hippocampal neurons, leading to an enhanced neurite outgrowth (367, 476, 478). Interestingly, FKBP51-deficient neurons did not show an enhanced neurite outgrowth, indicating that especially the balance between FKBP51 and FKBP52 facilitates this mechanism in neurons (476). In line with this, various studies identified FKBP51 and

especially FKBP51 dysregulations, which can be either induced by chronic stress or caused by SNPs, as driver of several disorders (329, 335, 336, 363, 466, 495). However, SAFit2 has been shown to successfully address these dysregulations, since SAFit2 readjusted and enhanced the glucocorticoid receptor sensitivity to enable a negative feedback loop towards the HPA axis and thereby reduced corticosterone levels at peak times of secretion (476).

Associated to these counteracting properties of SAFit2, administration of SAFit2 has been shown to prevent stress-induced behavior such as social avoidance and anxiety like behavior in mice and rats (477, 496). These behaviors are highly predominant in psychiatric disorders that are stress induced like depression, post-traumatic stress disorder, and schizophrenia (477, 497, 498). Beside this anxiety like behaviors, SAFit2 was shown to reduce stress-related alcohol consumption and preference in rodents (481, 499). Furthermore, SAFit2 has been shown to limit cocaine re-abuse in male rats, indicating SAFit2 as approach for treating substance abuse disorders (500). This property might also be beneficial in the context of pain, since opioids are less used for pain treatments due to the high risk of abuses, although they are efficiently analgesic (297, 501).

In the context of cancer, SAFit2 was additionally shown to improve cancer treatments as it counteracts the pathological outcomes of FKBP51 overexpression in several cancer types (406, 502). Likewise, overexpression of FKBP51 was shown to enhance proliferation, mediate irradiation and chemotherapy resistance and very recently to protect tumors from their immune environment (354, 446, 503-505). In line with this, SAFit2 was shown to counteract immune evasion of glioblastomas by reducing the expression of programmed cell death ligand-1 (PD-L1) (482). Based on this, SAFit2 reduced tumor growth and activated apoptosis in glioblastomas *in vitro* as well as in a glioblastoma xenograft model *in vivo* (482), revealing SAFit2 as beneficial treatment in the management of cancer. In summary, SAFit2 seems to be a very promising drug precursor for the treatment of many diseases such as stress-related disorders, psychiatric disorders, substance abuse and cancer.

In comparison to first-line treatments for neuropathic pain such as amitriptyline and pregabalin, SAFit2 comprises a broad spectrum of neuroinflammatory effects. Moreover, SAFit2 mediates a quite efficient pain relief in nerve injury-induced and paclitaxel-induced neuropathic pain in mice, whereas human data is not existing yet (370, 371). In contrast, amitriptyline achieved no pain relief in chemotherapy-induced neuropathic pain states in

humans (506). Nevertheless, amitriptyline revealed overall a relatively low NNT (number needed to treat) around three to four, indicating that three patients have to be treated with amitriptyline to gain a 50% pain relief (507). However, the distinct mechanism of action of amitriptyline is relatively unknown, since the antidepressant drug is used off-label. Likewise, amitriptyline is not suggested to relieve pain via its unselective serotonin and noradrenalin reuptake inhibiting function, but rather through off-target effects including the inhibition of voltage-dependent sodium channels and the activation of potassium channels. Furthermore, amitriptyline comprises several other interaction partners like muscarine, histaminergic and dopaminergic receptors, indicating a high unspecificity of amitriptyline (492). In contrast, pregabalin was developed as voltage-dependent calcium channel blocker for the treatment of neuropathic pain, inhibiting the release of neurotransmitters like glutamate, noradrenalin, and substance P (508). However, pregabalin comprises a NNT around seven to eight, besides several side effects like amitriptyline as well and an increasing risk of abuses (507, 509, 510). Based on this, even recommended first-line treatments for neuropathic pain display either a lack of specificity or a low efficacy and comprise a lot of severe side effects. Hence, SAFit2 displays promising properties as drug precursor for neuropathic pain, although it has to be further optimized in pharmacological parameters for a clinical usage.

## 5.5 Future perspectives and conclusion

Beside the beneficial effects of SAFit2 in psychiatric disorders, substance abuse, cancer and neuropathic pain, the influence of SAFit2 or analogues has not been investigated in neurological disorders such as Parkinson disease and Alzheimer disease (352, 363, 437, 511). However, those diseases have been associated with FKBP51 and FKBPs in general, highlighting further approaches of targeting FKBP51 with either SAFit2 or future improved analogues. Furthermore, SAFit2 efficiently restored glucocorticoid sensitivity *in vivo*, indicating SAFit2 as potential co-treatment for glucocorticoids to prevent the development of a glucocorticoid resistance (353, 476). Glucocorticoids are given in various diverging inflammatory diseases such as multiples sclerosis, rheumatoid arthritis or asthma (512). Notably, the inflammatory component of respective diseases points out another treatment option for SAFit2, since increased FKBP51 levels have been shown to activate the NF-κB signaling pathway, leading to the expression of numerous pro-inflammatory cytokines (308, 353). However, SAFit2 was shown to counteract this enhanced activity and to reduce pro-

inflammatory cytokine expression, beside restoring glucocorticoid sensitivity (370). Based on this, SAFit2 might be a potential drug candidate for the treatment of various diseases comprising an inflammatory component independently from their occasion.

Altogether, the involvement of FKBP51 has been identified in numerous diseases. In line with this, the upregulation of FKBP51 was described in different pathological pain forms, especially in neuropathic pain states. Neuropathic pain occurs after nerve lesions and is only barely treatable due to inadequate treatment options, highlighting the need of novel targets and potential drug candidates for neuropathic pain. In this study, it was observed that SAFit2, the recent gold standard for targeting FKBP51, ameliorates nerve injury- and paclitaxel-induced neuropathic pain in mice. Furthermore, it was observed that SAFit2 counteracted neuroinflammation in both mouse models and increased pro-resolving lipid mediator levels in peripheral nervous tissue. In summary, targeting FKBP51 with SAFit2 or future improved analogues seems to be a promising approach for efficiently relieving neuropathic pain after nerve injury and paclitaxel treatment. Furthermore, the influence of SAFit2 on other neuropathic pain types seems to be promising, but has to be investigated separately, since the underlying mechanisms of different neuropathic pain forms are divergent. However, selectivity, pharmacological profile and bioavailability of SAFit2 have to be further optimized in order to develop a clinical lead compound.

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## List of figures

respectively. Furthermore, several phosphorylation sites, consisting of serine and threonine, are located along both terminal ends that can in turn be phosphorylated by protein kinase A and C or CaMKII. In addition, each terminal end provides a calmodulin (CaM) binding site. The TRPV1 channel can be activated by noxious heat stimuli, protons that act on glutamic acid residues (indicated at the extracellular loops) and capsaicin that was proposed to bind close to the fourth transmembrane domain. The illustration was created with images from motifolio.

**Figure 4: Structure of the murine TRPA1 channel.** The TRPA1 channel is composed of four subunits that build a tetramer (bottom left). One subunit comprises six transmembrane domains from which domains five and six build up the conducting pore with the respective domains of the other subunits (top right). The amino terminal of the murine TRPA1 channel consists of 14 ankyrin repeat domains and several phosphorylation sites. Furthermore, the amino terminal comprises a suggested binding region for electrophilic agonists. In contrast menthol was predicted to bind near the fifth transmembrane domain. The carboxy terminal comprises predominantly a large voltage sensitive domain and provides a calmodulin (CaM) binding site. The illustration was created with images from motifolio.

**Figure 5: PUFA release by the phospholipase $A_2$ from cellular membrane.** Polyunsaturated fatty acids (PUFAs) can be released by the phospholipase A2 from the cellular membrane. Thereby, phospholipase A2 hydrolyses the second esther bond of glycerophospholipids, liberating a lysophospholipid and a PUFA. Furthermore, PUFAs can be converted into other PUFAs upon elongation and desaturation processes. However, the depicted direction of PUFA conversion serves only for explanatory purposes and does not implicate that e.g. arachidonic and docosahexaenoic acid cannot be incorporated into cell membranes. The molecular structures were drawn with ChemSketch and the lipid bilayer was modified and originates from motifolio.

**Figure 6: Enzymatic reactions catalyzed by LOX, CYP and COX enzymes.** The iron containing lipoxygenases (LOX enzymes) generate hydroperoxyl fatty acids under incorporation of one oxygen molecule. Cytochrome-$P_{450}$-epoxygenases (CYP enzymes) catalyze epoxygenase and hydroxylase activities in dependency of iron ions, attaching either an epoxide group, preferentially to the double bond closest to the amino group,

inflammatory cytokines and a decrease of pain-mediating chemokines in DRGs and spinal cord after SAFit2 treatment. Nevertheless, SAFit2 leads to changes in the number of activated immune cells in both models. Whereas SAFit2 decreases the invasion of immune cells into DRGs and spinal cord in the nerve injury model, SAFit2 reduces the activation of glial cells in the paclitaxel model. Notably, SAFit2 did not alter the number of immune cells at the site of injury, the sciatic nerve, in the nerve injury model. Moreover, SAFit2 counteracted an enhanced NF-κB pathway activation in DRGs and spinal cord of nerve-injured mice. Besides this, SAFit2 seems to increase the levels of free fatty acids as well as ceramides in both models. Furthermore, it restored the levels of an analgesic ceramide, dihydroceramide C16, after neve injury and of anti-inflammatory oxylipins after paclitaxel treatment. In primary sensory neurons, SAFit2 lead to the desensitization of the ion channel TRPV1 and a reduced release of the neuropeptide CGRP. Orange arrows represent changes in the nerve injury model, whereas blue arrows display changes in the paclitaxel model. In addition, light orange boxes display either *in vivo* or *ex vivo* data, whereas the grey box illustrates results from primary sensory neuron cultures. SAFit2-mediated effects that are similar in the nerve injury- and the chemotherapy-induced neuropathic pain are printed in bold. The illustration was created with images from motifolio.

**Figure 11: The SAFit2-FKBP51 network depicted as influencing factors of calcineurin and TRPV1 desensitization**. The influence of FKBP51 on the activity of the phosphatase calcineurin is controversially discussed in literature. However, both the complex of FKBP51 and FK506 as well as FK506 alone have been shown to inhibit the activity of calcineurin, whereas SAFit2 or analogues either have no effect on the activity of calcineurin or increased the calcineurin-mediated phosphate release. Furthermore, calcineurin was shown to mediate the dephosphorylation and subsequent desensitization of TRPV1 in primary sensory neurons. In line with this, SAFit2 and analogues also showed a desensitization of TRPV1 in sensory neurons. However, unspecific FKBP51 inhibitors as well as ddSAFit2, a chiral and biological inactive analogue, did not. Blocking lines indicate an inhibitory effect, arrows an inducing effect and straight lines illustrate neither an induction nor inhibition. Black lines indicate results from literature, whereas orange lines indicate insights from this work.

# Abbreviation list

| | |
|---|---|
| AA | arachidonic acid |
| AMPA | amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| AP-1 | activator protein 1 |
| AR | androgen receptor |
| AS160 | AKT substrate of 160 kDa |
| Ask1 | apoptosis signal-regulating kinase 1 |
| ATP | adenosine triphosphate |
| BDNF | bone derived neurotrophic factor |
| CaM | calmodulin |
| cAMP | cyclic adenosine monophosphate |
| CCL | C-C motif ligand |
| CCR | C-C motif chemokine receptor |
| CGRP | calcitonin gene-related peptide |
| CICR | calcium-induced calcium release |
| CIPN | chemotherapy-induced peripheral neuropathy |
| CNS | central nervous system |
| COX | cyclooxygenase |
| cPLA$_2$ | cytosolic PLA$_2$ |
| CREB | cAMP response element-binding protein |
| CSF1 | colony-stimulating factor 1 |
| CXCL | chemokine C-X-C motif ligand |
| CYP | cytochrome-P$_{450}$-epoxygenases |
| DGLA | dihomo-gamma-linolenic acid |
| DHA | docosahexaenoic acid |
| DiHDPAs | dihydroxydocosapentaenoic acids |
| DiHOME | dihydroxy-octadecenoic acid |
| DRG | dorsal root ganglia |
| EDPs | epoxydocosapentaenoic acids |
| EEQs | epoxyeicosatetraenoic acids |
| EETs | expoxyeicosatrienoic acids |
| EPA | eicosapentaenoic acid |
| EpOMEs | epoxyoctadecenoid acids |
| ER | estrogen receptor |

| | |
|---|---|
| ERα | estrogen receptor alpha |
| FKBP51 | FK506 binding protein 51 |
| GFAP | glial fibrillary acidic protein |
| GLUT4 | glucose transporter 4 |
| GPCRs | G protein coupled receptors |
| GR | glucocorticoid receptor |
| HEPEs | hydroxyeicosapentaenoic acids |
| HETE | hydroxyeicosatetraenoic acids |
| HETrEs | hydroxyeicosatrienoic acids |
| HODEs | hydroxyoctadecadienoic acids |
| HOTrEs | hydroxyoctadecatrienoic acids |
| HPA | hypothalamic–pituitary–adrenal |
| HpEPEs | hydroperoxyeicosapenataenoic acids |
| HpETEs | hydroperoxyeicosatetraenoic acids |
| HSP90 | heat shock protein 90 |
| HTT | huntingtin gene |
| IASP | international association for the study of pain |
| IBA1 | ionized calcium-binding adaptor molecule-1 |
| IL | interleukin |
| $iPLA_2$ | calcium-independent $PLA_2$ |
| LA | linoleic acid |
| LOX | lipoxygenase |
| Lys | lysine |
| MAPK | mitogen-activated protein kinase |
| mHTT | mutant huntingtin gene |
| mPTP | mitochondrial permeability transition pore |
| MR | mineralcorticoid receptor |
| mTOR | mammalian target of rapamycin |
| MyD88 | myeloid differentiation primary response 88 |
| NF-κB | nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| NGF | nerve growth factor |
| NMDA | N-methyl-D-aspartate |
| NNT | number needed to treat |
| NSAID | non-steroidal anti-inflammatory drug |
| PDK1 | pyruvate dehydrogenase kinase 1 |

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|---|---|
| PD-L1 | programmed cell death ligand-1 |
| Phe | phenylalanine |
| PHLPP | PH domain leucine-rich repeat phosphatase |
| PI3K | phosphoinositide 3-kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLA$_2$ | phospholipase A II |
| PNS | peripheral nervous system |
| PP2B | serine/threonine-protein phosphatase 2B |
| PPARγ | peroxisome-activated receptor γ |
| PPIase | prolyl-peptidyl-isomerase |
| PR | progesterone receptor |
| PUFA | polyunsaturated fatty acids |
| ROS | reactive oxygen species |
| S1P | sphingosine-1-phosphate |
| SAFit | selective antagonist for FKBP51 by induced fit |
| SC | spinal cord |
| sEH | soluble epoxide hydrolase |
| SHR | steroid hormone receptors |
| SNP | single nucleotide polymorphism |
| SNRIs | serotonin-noradrenaline reuptake inhibitors |
| sPLA$_2$ | secretory PLA$_2$ |
| SPM | specialized pro-resolving mediator |
| TLR4 | toll like receptor 4 |
| TNFα | tumor necrosis factor α |
| TRIF | TIR domain-containing adaptor inducing IFN-β |
| TRP | transient receptor potential |
| TRP domain | tetratricopeptide repeat domain |
| TRPA | transient receptor potential ankyrin |
| TRPM | transient receptor potential melastin |
| TRPML | transient receptor potential mucolipin |
| TRPP | transient receptor potential polycistin |
| TRPV | transient receptor potential vanilloid |
| α-LA | alpha linoleic acid |

## Declaration - Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht. Die eingereichte elektronische Version stimmt mit der schriftlichen Version überein.

Darmstadt, den .........................

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Saskia Wedel

## Acknowledgement

# Curriculum Vitae

## Personal Information

### Saskia Wedel

◎ Frankfurt am Main
📅 24.12.1997

## Professional Experience

**April 2021 – October 2023**

**PhD candidate** at the Institute of Clinical Pharmacology
- **Research topic**: "Investigation of SAFit2 as a potential treatment option for nerve injury- and chemotherapy-induced neuropathic pain"

**February 2021 – March 2021**

**Research Assistant** at the department of R&D at R-Biopharm AG
- Production and management of in-process controls
- Planning, implementation, and evaluation of stability and release tests

**December 2020 – January 2021**

**Tutor** at the Technical University of Darmstadt
- Conducting seminars for students

**September 2017 – January 2020**

**Student employee** at R-Biopharm AG
- Generation and distribution as well as quality and stability testing of in-process controls

## Education

**October 2018 – January 2021**

**Master Technical Biology** at the Technical University of Darmstadt
- Course of specialization: stem cell biology, neurobiology, radiation biology and immunology

  **Research internship** at the Paul-Ehrlich-Institute (12 weeks)
  - **Research topic**: "Investigation of the immune metabolic phenotype of dendritic cells stimulated with the allergen product Pollinex Quattro®"

  **Master thesis** at the research group Neurophysiology and Neurosensory Systems headed by Prof. Dr. Laube
  - **Research topic**: "Investigating low dose irradiation promoted differentiation of murine neural stem cells including a characterization of respective regulatory signaling pathways"

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|---|---|
| **October 2015 – October 2018** | **Bachelor Biology** at the Technical University of Darmstadt<br>• Course of specialization: developmental biology, microbiology, animal physiology and radiation biology<br><br>**Research internship** at R-Biopharm AG (6 weeks)<br>• Generation and testing of in-process controls<br><br>**Bachelor thesis** at the at the research group Stem Cell and Developmental Biology headed by Prof. Dr. Nuber<br>• **Research topic**: "Generation of neural cell type-specific lentiviral cre expression vectors for controlled gene inactivation" |

## Trainings

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|---|---|
| **April 2021 – October 2023** | **Graduate school** (SFB 1039)<br>• PhD speaker of the graduate school, archived poster price 2021<br>• Poster an programming workshops |
| **May 2021 – October 2023** | **GRADE program** at the Goethe University<br>• GxP workshops<br>• Presentation and scientific writing workshops |
| **July 2021 – October 2023** | **Ingenium program** at the Technical University of Darmstadt<br>• Leadership workshops<br>• Project management workshops<br>• Agile project management workshops (certified scrum master) |

## Grants

| | |
|---|---|
| **2023 – 2024** | **e-Fellows scholarship** |
| **2022 – 2023** | **Mentee** in Mentoring Hessen |
| **2019 – 2020** | **Deutschlandstipendium** |

## Conferences

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|---|---|
| **September 2023** | **International symposium on lipid signaling** in Frankfurt, Germany<br>• Poster presentation |
| **September 2023** | **International congress on neuropathic pain** (NeuPSIG) in Lisbon, Portugal<br>• Poster presentation |

## Publication list

**WEDEL, S.**, HAHNEFELD, L., SCHREIBER, Y., NAMENDORF, C., HEYMANN, T., UHR, M., SCHMIDT, M. V., DE BRUIN, N., HAUSCH, F., GEISSLINGER, G. & SISIGNANO, M. 2023. SAFit2 ameliorates paclitaxel-induced neuropathic pain by reducing spinal gliosis and elevating pro-resolving lipid mediators. *Journal of Neuroinflammation*, 20, 149; https://doi.org/10.1186/s12974-023-02835-5

KAUP, F.H., MEYNERS, C., SUGIARTO, W.O., **WEDEL, S.**, SPRINGER, M., WALZ, C., GEIGER, T.M., SCHMIDT, M., SISIGNANO, M. & HAUSCH, F. 2023. Structure-Based Discovery of a New Selectivity-Enabling Motif for the FK506-Binding Protein 51. *J. Med. Chem.*,66, 5965-5980; https://doi.org/10.1021/acs.jmedchem.3c00249

**WEDEL, S.**, HAHNEFELD, L., ALNOURI, M. W., OFFERMANNS, S., HAUSCH, F., GEISSLINGER, G. & SISIGNANO, M. 2022a. The FKBP51 Inhibitor SAFit2 Restores the Pain-Relieving C16 Dihydroceramide after Nerve Injury. *Int J Mol Sci,* 23, 14274; https://doi.org/10.3390/ijms232214274

**WEDEL, S.**, MATHOOR, P., RAUH, O., HEYMANN, T., CIOTU, C. I., FUHRMANN, D. C., FISCHER, M. J. M., WEIGERT, A., DE BRUIN, N., HAUSCH, F., GEISSLINGER, G. & SISIGNANO, M. 2022b. SAFit2 reduces neuroinflammation and ameliorates nerve injury-induced neuropathic pain. *Journal of Neuroinflammation,* 19, 254; https://doi.org/10.1186/s12974-022-02615-7

**WEDEL, S.**, OSTHUES, T., ZIMMER, B., ANGIONI, C., GEISSLINGER, G. & SISIGNANO, M. 2022c. Oxidized linoleic acid metabolites maintain mechanical and thermal hypersensitivity during sub-chronic inflammatory pain. *Biochem Pharmacol,* 198**,** 114953; https://doi.org/10.1016/j.bcp.2022.114953

ZIMMERMANN, J., GORETZKI, A., MEIER, C., WOLFHEIMER, S., LIN, Y. J., RAINER, H., KRAUSE, M., **WEDEL, S.,** SPIES, G., FÜHRER, F., VIETHS, S., SCHEURER, S. & SCHÜLKE, S. 2022. Modulation of dendritic cell metabolism by an MPLA-adjuvanted allergen product for specific immunotherapy. Front Immunol, 13,916491; https://doi.org/10.3389/fimmu.2022.916491