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The role of sphingosine 1-phsophate in mouse hypersensitivity to noxious thermal stimuli

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Abstract

Pain, as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Def. IASP, Task force on taxonomy), arises as a consequence of noxious stimulation and information processing in the central nervous system. Unmyelinated and thinly myelinated nociceptors detect noxious stimuli, transduce and transform them and finally transfer nociceptive information to the spinal cord. These nociceptors can be sensitized by proinflammatory cytokines like TNFα and others inducing hypersensitivity to mechanical and thermal stimuli. The bioactive sphingolipid sphingosine-1-phosphate (S1P) has attract attention because of his intracellular and extracellular effects on many cellular processes. Active S1P is generated by phosphorylation of sphingosine by sphingosine kinases 1 and 2 (SphK1/2). S1P regulates capsaicin-sensitive small diameter neurons via activation of its G-protein coupled receptor $S1P_1$. In this study, we investigated the importance of SphK1 for nociception *in vivo* by comparing mechanical and thermal sensitivity of $SphK1^{-/-}$ mice and C57BL/6J wt littermates in the complete Freund's adjuvant inflammation model. We further investigated the role of a presumable S1P agonist using an *in vitro* skin-nerve preparation.

Our results revealed a significantly increased CFA-induced paw swelling in $SphK1^{-/-}$ mice as compared to wt littermates. No differences in mechanical or thermal hypersensitivity of $SphK1^{-/-}$ were observed. We suggest that alterations of endothelial cell barrier function may be responsible for differences of CFA-induced oedema formation. Since S1P levels are vigorously controlled in vivo, upregulation of SphK2-gene may compensate for the lack of SphK1 in $SphK1^{-/-}$ mice and normal S1P levels in $SphK1^{-/-}$ in inflamed tissue.

We further observed that FTY720 did not induce a thermal hypersensitivity via direct nociceptor sensitisation. *In vivo*, FTY720 is phoshorylated in the liver and because of the lack of phosphorylation and absence of the active metabolite FTY720-P *in vitro* it is therefore not surprising that local injection of the precurser FTY720 does not induce nociceptor sensitisation *in vitro*.

Zusammenfassung

Schmerz, „ein unangenehmes Sinnes- oder Gefühlserlebnis, das mit tatsächlicher oder potenzieller Gewebeschädigung einhergeht oder von betroffenen Personen so beschrieben wird, als wäre eine solche Gewebeschädigung die Ursache“ (Def.: IASP, task force of taxonomy), entsteht als Konsequenz von Schmerzwahrnehmung und Informationsverarbeitung im Zentralnervensystem. Unmyelinisierte und dünn myelinisierte freie Nervenendigungen nehmen Schmerzreize wahr, transduzieren und transformieren sie und leiten die nozizeptiven Informationen letztendlich an das Rückenmark weiter. Diese Nozizeptoren können durch proinflammatorische Zytokine wie TNFα für thermische und mechanische Reize sensibilisiert werden. Das bioaktive Sphingolipid Sphingosin-1-Phosphat erregte Aufmerksamkeit dadurch, dass es sowohl intrazelluläre als auch extrazelluläre Effekte auf verschiedene Zellprozesse hat. Das aktive S1P wird durch die Phosphorylierung von Sphingosin durch die Sphingosin Kinasen 1 und 2 (SphK1/2) gebildet. S1P reguliert über Aktivierung seines G-Protein gebundenen Rezeptors $S1P_1$ Capsaicin-sensitive afferente Fasern. In dieser Studie erforschten wir *in vivo* die Rolle von SphK1 in der Nozizeption, indem wir mechanische und thermische Sensibilisierung von $SphK1^{-/-}$ Mäusen und C57BL/6J Wildtyp Littermates in einem „Complete Freund’s Adjuvant“ Entzündungsmodell verglichen. Des Weiteren erforschten wir die Rolle eines mutmaßlichen S1P Agonisten an einem *in vitro* Nerven-Haut-Präparat.

Unsere Ergebnisse zeigen, dass $SphK^{-/-}$-Mäuse eine stärkere Schwellung auf eine CFA-Injektion entwickeln als Wildtyp-Mäuse. Es wurden ansonsten keine Unterschiede in der mechanischen und thermischen Hitzwahrnehmung innerhalb von 72 Stunden festgestellt. Wir vermuten, dass Veränderungen der endothelialen Zellbarriere verantwortlich sind für die Unterschiede in der durch CFA ausgelösten Ödembildung. Da die S1P Spiegel *in vivo* stark reguliert werden, könnte eine Hochregulierung des SphK2 Genes den Mangel an SphK1 in $SphK1^{-/-}$ Mäusen kompensieren und so auch für normale S1P Spiegel in dem entzündeten Gewebe sorgen.

Wir beobachteten außerdem, dass FTY720 keine Veränderung der Hitzesensibilisierung an Nozizeptoren auslöst. *In Vivo* wird FTY720 in der Leber phosphoryliert und damit aktiviert. Es ist deswegen nicht überraschend, dass ein lokales Verabreichen des Vorläufers FTY720 *in vitro* keine Sensibilisierung von Nozizeptoren auslöst, da der Mangel an Phosphorylierung von FTY720 eine Abwesenheit des aktiven FTY720-P bedeutet.

Abbreviations

A(HT)M	High-Threshold Mechanosensitive Aδ-fibre
A(LT)M	Low-Threshold Mechanosensitive Aδ-fibre
ABC-Transporter	ATP-Binding Cassette Transporter
AC	Adenylyl Cyclase
AM RA	Rapid Adapting Mechanosensitive Aδ-fibre
AM SA	Slow Adapting Mechanosensitive Aδ-fibre
AM	Mechanosensitive Aδ-fibre
AMC	Mechano-cold sensitive Aδ-fibre
AMH	Mechano-heat sensitive Aδ-fibre
AMP, cAMP	Adenosine Monophosphate, cyclic Adenosine Monophosphate
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASIC	Acid-sensing ion channel
C(HT)	High-threshold Mechanosensitive C-fibre
C(LT)M	Low-threshold Mechanosensitive C-fibre
CC	Cold-sensitive C-fibre
CCK	Cholecystokinin
CFA	Complete Freund's Adjuvant
CGRP	Calcitonin gene-related peptide
CH	Heat-sensitive C-fibre
CH_iM_i	Mechano-insensitive and heat-insensitive C-fibre
CMC	Mechano-cold sensitive nociceptor
CMH	Mechano-heat sensitive nociceptor
CNS	Central nervous system
COX, COX-1/2/3	Cyclooxygenases 1/2/3
CRPS	Complex regional pain syndrome
DAG	Diacylglycerol
DHS	DL-threo-dihydrosphingosine
DMS	Dimethylsphingosine
DNIC	Diffuse noxiuos inhibitory control
DRG	Dorsal root ganglion
EDG	Endothelial differentiation gene
EGF	Epidermal growth factor

ERK	Extracellular signal-regulated Kinase
GABA	γ-Amino-butyric acid
GPCR	G-protein coupled receptors
IASP	International Association of Study of Pain
IFN-γ	Interferon γ
IGF	Insulin-like growth factor
IL1β	Interleukin 1β,
IL6	Interleukin 6
IP_3	Inositoltriphosphate-3
LPA	Lysophosphatidic acid
LPP	Lipid phosphate phosphatase
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte-chemoattractant protein 1
NGF	Nerve growth factor
NMDA	N-Methyl-D-aspartate
NNDS	N,N-Dimethylsphingosine
NOS	Nitric oxide synthase
NRS	Numeric rating scale
PAG	Periaqueductal grey
PDGF	Platelet-derived growth factor
PGH_2 , ,	Prostaglandin H_2,
PGE_2	Prostaglandin E_2,
PGD_2	Prostaglandin D_2,
PGF_2	Prostaglandin F_2
PGI_2	Prostacyclin
PI_3K	Phosphatidyl-inositol-triphosphate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RVM	Rostral ventromedial medulla
S1P	Sphingosine 1-phosphate
$S1P_{1-5}$	S1P Receptor 1-5
SEM	Standard error of the mean

SIF	Synthetic interstitial fluid
SphK1/2	Sphingosine kinase 1/2
TNFα	Tumour necrosis factor-α
TrkA	Tyrosine kinase receptor A
TRPV1	Transient receptor potential, vanilloid subfamily, member 1
TTX-R	Tetrodotoxin-resistant sodium channel
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WDR	Wide Dynamic Range Neurons

1 Introduction

1.1 Pain processing in physiological and pathological conditions

Pain is an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Def. IASP, Task force on taxonomy). As reported by Breivik and coworkers, between 12 % and 30 % of the population of the European countries are suffering from chronic pain defined as pain of more than six months duration and a pain intensity of 5 or ore on a 10-point Numeric Rating Scale (NRS) with 1 = no pain to 10 = the worst pain imaginable (Breivik et al., 2006). From this population nearly 60% report on pain lasting for 2 up to 15 years (Breivik et al, 2006). Pain arises as a consequence of noxious stimulation and information processing in the spinal cord, thalamic and cortical structures. The sensation of pain includes sensory-discriminative, affective as well as motor and autonomic components (Casey, 2000). The nociceptive afferent pain pathway starts at specialised nociceptive primary afferents that innervate skin and most other tissues which were first discovered by Charles Sherrington (Sherrington, 1906). Noxious stimuli are detected by free nerve endings (transduction), transformed into a series of action potentials which then travel along unmyelinated or thinly myelinated axons towards the spinal cord. The cell bodies of nociceptive primary afferents are localised in the dorsal root ganglia (DRG) or trigeminal ganglia (TG) and their central processes terminate in superficial layers of the spinal cord dorsal horn (Millan, 1999) or, in case of the trigeminal nociceptors, to the trigeminal sensory nuclei in the brainstem (Millan, 1999). Primary afferent axons can be classified according to diameter, nerve conduction velocity and myelination. Nociceptive primary afferent axons are found among Aδ- and C-fibres. Aδ-fibres are thinly myelinated, with diameters between 2 to 6 μm and conduction velocity of 4-20 m/s (Adriaensen et al., 1983; for review see Lumpkin and Caterina, 2007; Zimmermann et al., 2009). Two main classes of Aδ-fibres can be discriminated, both of them promote the perception of acute pain and trigger the withdrawal reflex (for review see Zimmermann et al., 2009): slow-adapting mechano-sensitive fibres with mechanical von Frey thresholds between 1-128 mN and are sensitive to heat or intense cold stimuli with are termed mechano-heat sensitive Aδ-fibres (AMH) or mechano-cold sensitive Aδ-fibres (AMC). The second are mechanoreceptors, which only respond to mechanical stimuli (mechano-sensitive Aδ-fibres [AM]) and can be further discriminated into slowly adapting (AM-SA) and rapidly adapting (AM-RA) fibres with low (A-LTM; von Frey threshold <1-5.7mN) or high (A-HTM; von Frey threshold ~5.7-

128mN) threshold (for review see Zimmermann et al., 2009). Nearly 85% of all Aδ-fibres found in skin of primates are AMH units (Xu et al., 2010). In the muscle, activation of Aδ-fibres can produce an aching sensation without differentiation between the pain qualities and with less quality of localisation (Millan, 1999). Their modalities range from innocuous mechanical, thermal and chemical stimuli as well as noxious stimuli like painful pressure or ischemic and hypoxic pain (for review see Julius et al., 2001; Zimmermann et al., 2009; Djouhri and Lawson, 2004). C-fibres are unmyelinated, slowly adapting fibres with diameters between 0.4 to 1.2 μm and conduction velocities of 0.5 to 2.0 m/s. They convey a more diffuse, dull and delayed pain sensation. Depending on the stimuli they react on, they can be classified into different types of C-fibres, including mechano-heat sensitive nociceptors (CMH) which make up one third of the C-fibre population in most species (Schmidt et al., 1995), mechano-sensitive receptors of low threshold (C[LT]M; Von Frey threshold <1-5.7mN) and high threshold (C[HT]M; Von Frey threshold ~5.7-128mN), as well as thermo-sensitive receptors just reacting of cooling (Cold Receptors, CC) and heating (CH). Approximately two thirds react to multiple types of stimuli including noxious heat, cold, pressure or chemicals and are therefore termed polymodal (Reeh, 1988; Kress et al., 1992; Koltzenburg et al., 1997; for review see Lumpkin and Caterina, 2007). Also polymodal mechano-cold sensitive nociceptors (CMC) and C-fibres that are insensitive to mechanical and heat stimuli (CH_iM_i), are members of the C- fibre class. They express transducer ion channels and metabotropic receptors for chemical mediators like substance P, acetylcholine, histamine, prostaglandins, serotonin and proteolytic enzymes. (for review see Zimmermann et al., 2009; Schmidt et al., 1995; Julius et al., 2001; Garry et al., 2004). The cranial primary afferent fibres reach the brain stem through the trigeminal ganglion and nucleus, while the thoracolumbar and sacral primary afferent fibres are connected to the spinal cord via the dorsal root and dorsal root ganglia. Their primary axons end in the dorsal horn within the grey matter, a cluster of neuron cell bodies and glia cells, where the axons form synapses with second-order neurons mainly in the superficial Rexed laminae I-II or in the deeper Rexed laminae V, VI, VII and X ((Handwerker et al., 1975b; Handwerker et al., 1975a), Craig, 1991; Craig et al., 2001; Zhang et al., 2000; Millan, 1999). The second-order neurons can be discriminated into nociceptive specific neurons, which receive their information just by Aδ- and C-Fibres and respond to intense mechanical, heat and chemical stimuli, into non-nociceptive neurons, which react on peripheral stimuli like weak stimuli transmitted by Aβ- and Aδ-fibres and into wide dynamic range (WDR) neurons. The WDR-neurons, mainly located in lamina V, respond to low- and high-intensity peripheral stimulation of Aβ-, Aδ-

and C-fibres (Schaible and Grubb, 1993; Willis, 1985; Handwerker et al., 1975). In contrast to nociceptive-specific neurons their firing frequency increases linearly or exponentially with increasing stimulus intensity (Dubner et al., 1989). One WDR neuron can receive afferent input from different tissues like skin and muscles, i.e. that their receptive field varies explicitly (Gillette et al., 1993; Gebhart, 1995; Ness and Gebhart, 1990). WDR-neurons receive input from large-diameter Aα- and Aβ fibres as well as from small-diameter Aδ- and C fibres (Millan, 1999). After the modification of the incoming sensitive information in the gray matter the output from the dorsal horn to higher centres in the brain is carried by spinal projection neurons along ascending pathways in the contralateral ventrolateral ascending fibre tract of the spinal cord; the main afferent pain pathway is the spinothalamic tract. The central relay station between the ascending spinal input and higher cortical areas is the thalamus, which receives, modulates/filters and transmits ascending spinal information to various parts of the cortex including the primary sensory cortex S1, S2, cingulate cortex and insular cortex (Brooks and Tracey, 2005; Tracey, 2005).

1.2 Inflammatory pain and his mediators

Tissue injury and inflammation initiate a sequence of physiological defense processes that finally limit the damage inducing factor and initiate repair mechanisms to restablish normal tissue function. The inflamed tissue is characterized by five main signs which are swelling (tumor), temperature increase (calor), flare/reddening (rubor), loss of function (functio laesa) and pain (dolor). The inflammatory reaction triggers a change of the extracellular milieu with hyperkalaemia and tissue acidification and the release of mediators from damaged tissue components or invading immune cells including classical algogens like histamine, bradykinin, serotonin, ATP, nitric oxide and other proinflammatory mediators like cytokines and bioactive lipids. These substances act on nociceptive nerve terminals via ionotropic receptors like the acid-sensing ion channels (ASICs) for H^+ or P_2X purinoreceptors for ATP, or the activate metabotropic receptors like Bradykinin B_2-receptors, which frequently sensitise the nociceptor for other stimuli via activation of second messenger cascades (Kress and Reeh, 1996). Later phases of the inflammatory reaction are characterized by activation of novel inflammatory pathways, like the cyclooxygenase (COX2)/prostaglandin pathway and the recruitment of immune cells and sequential release of cytokines, that contribute to a pathological pain sensitivity (Cunha et al., 2005; Sommer and Kress, 2004). Release of pro-inflammatory cytokines like tumour necrosis factor-α (TNFα), interleukin 1β (IL1β),

Interleukin 6 (IL6), nerve growths factor (NGF) and prostaglandins induce thermal and mechanical hyperalgesia via sensitisation of nociceptors (Verri et al., 2006; Oprée and Kress, 2000). They can induce expression and protein biosynthesis, e.g. of COX2 and at the same time regulate transducer channels like the nociceptor specific heat sensor transient receptor potential vanilloid subfamily 1 (TRPV1) or voltage-gated sodium channels by activation of second messenger cascades like cAMP/protein kinase A (PKA) or diacyl-glycerol/protein kinase C (PKC). The TRPV1 transducer channel is generally accepted as one of the main targets of different cytokines and pro-inflammatory mediators under conditions associated with pathological pain (Tominaga et al., 1998) It is one member of the thermoTRP family of ion channels that are activated by changes of temperature (Jordt et al., 2003; Talavera et al., 2008). TRPV1 reacts on noxious heat with a threshold temperature above 42°C and responds to capsaicin, the active pungent substance in red hot chilli peppers (8-methyl-N-vanillyl-6-nonenamide) (Caterina et al., 1997). Especially nociceptive Aδ- and C-fibres express this receptor, that is inactive at a normal body temperature. Phosphorylation in particular at serine and threonine sites lowers the threshold and facilitates the activity. Specific consensus sites for PKA, PKC and tyrosine kinases have been identified which are critical sites regulating the channels kinetic properties (for review see Tominaga and Tominaga, 2005). A number of receptor mediated intracellular pathways initiated by inflammatory mediators converge on TRPV1 regulation in nociceptors (for review see Tominaga and Tominaga, 2005). Therefore it is not surprising, that TRPV1 has been found essential for thermal hypersensitivity associated with inflammation (Davis et al., 2000; Caterina et al., 2000).

1.3 Sphingosine1-phosphate synthesis (S1P) and possible sources of S1P

Sphingolipids have attracted great attention in the last few years. They are known as important structural components of the cell membrane, are involved in formation of membrane crafts and caveolae, and as important signalling molecules. In particular, sphingosine 1-phosphate (S1P) is involved in many cellular functions including proliferation, cell differentiation, apoptosis, lymphocyte trafficking, angiogenesis and inflammation (Hla et al., 2008; Alvarez et al., 2007). S1P is generated from sphingomyelin that is degraded by sphingomyelinase to form ceramide. This in turn is cleaved by ceramidase and sphingosine is generated. Two isoforms of sphingosine kinases (SphKs) 1 or 2 are know which phosphorylate sphingosine. S1P levels are tightly regulated and S1P is either degraded to hexadecanal and phosphoetanolamine by S1P lyase or inactivated by dephosphorylation by

unspecific lipid phosphate phosphatase (LPP) or S1P phosphatase (Veldhoven and Mannaerts, 1991; Veldhoven and Mannaerts, 1993; Pyne and Pyne, 2000). SphK1 and SphK2 are located in different cell compartments, expressed in different tissues and are differently regulated. SphK1 occurs in blood, spleen, lung and kidney (Liu et al., 2000; Billich et al., 2003; Kihara et al., 2006) whereas Sphk2 is expressed in liver, heart, brain and kidney (Liu et al., 2000; Billich et al., 2003; Kihara et al., 2006). SphK2 is mainly found in cytosol and nucleus, whereas SphK1 is translocated from cytosol to the plasma membrane and can be secreted into the extracellular fluid (Marsolais and Rosen, 2009). A wide range of agonists can activate SphK1, e.g. lysophosphatidic acid (LPA), NGF, IL-1β, TNFα , platelet-derived growth factor (PDGF) or Interferon γ (IFN-γ), and via phosphorylation or translocation to the plasma membrane induce different biological responses. Acetylcholine, for example, activates SphK1 and thereby increases intracellular S1P levels, which in turn leads to intracellular Ca^{2+}-mobilization (van Koppen et al., 2001). The regulation of SphK2 is less clear, but it seems, that it can be stimulated by epidermal growth factor (EGF) and activates extracellular signal-regulated kinase 1 (ERK1) (Alvarez et al., 2007). Sphingosine kinases are expressed ubiquitously and nearly each cell can produce S1P (Hla et al., 2008). In mammals, S1P is enriched in blood plasma and lymph fluid (Pappu et al., 2007). While the S1P concentration in tissue interstitial fluid ranges just in nM concentration, the blood plasma contains S1P in µM concentration and lymph S1P concentration is ~¼ of the plasma level (Lee et al., 2007; Venkataraman, 2008). HDL and Albumin bind S1P in blood and therefore may decrease the concentration of active free S1P (Okajima et al., 2002). The sources of S1P seem differ between lymph fluid and blood. While the origin of lymphatic S1P is unknown, red blood cells, platelets and endothelial cells produce S1P, and in case of endothelial cells, they liberate it in response to physiological shear-stress (Hla et al., 2008). The mechanism of S1P extrusion into the extracellular environment is not fully understood, but it seems to involve transmembrane proteins from the family of ATP binding cassette transporter (ABC-transporter) (Hla et al., 2008).

1.4 S1P as an extra- and intracellular mediator and its G-protein coupled receptors

Although intracellular S1P effects have been reported (Zhang, et al., 1991; Olivera and Spiegel, 1993; Pyne et al., 1996; Su et al., 1994; Van Brocklyn et al., 1998), extracellular effects of S1P largely depend on metabotropic G-protein coupled receptors (GPCRs). Five members of the GPCR family of endothelial differentiation genes (EDGs) are known to

specifically bind S1P (Spiegel and Milstien, 2003; Brinkmann, 2007). The S1P-receptors $S1P_{1-5}$ couple to different G-proteins. $S1P_1$ couples exclusively to G_i, $S1P_2$ and $S1P_3$ to G_i, G_q and $G_{12/13}$ and $S1P_4$ and $S1P_5$ utilize G_i and $G_{12/13}$ (Spiegel and Milstien, 2002; Hla et al., 2001). Accordingly, various second messenger pathways will be activated and specific cellular responses initiated. While $G_{12/13}$ activates the small GTPase Rho (Spiegel and Milstien, 2003), G_i inhibits adenylyl cyclase (AC), and (via beta/gamma subunits) interacts with Phospholipase C (PLC) or extracellular signal-regulated kinases (ERKs), that can be activated by $S1P_{1-4}$ (Siehler and Menning, 2001) or inhibited by $S1P_5$ (Malek et al., 2000).

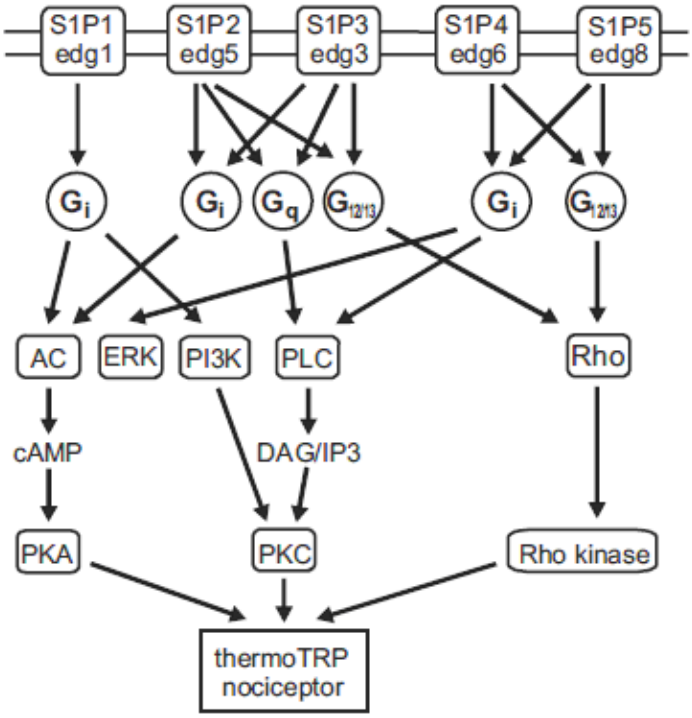

Fig. 1: Sphingosine-1-phosphate (**S1P**) is a ligand for five G-protein-coupled receptors of the endothelial differentiation gene (edg) family. Stimulation of the G-protein coupled receptor hydrolyses trimeric G-proteins, like $\mathbf{G_i}$, $\mathbf{G_q}$ or $\mathbf{G_{12/13}}$ which in turn activate or inhibit downstream signalling pathways: **AC**: adenylyl cyclase; **cAMP**: cyclic AMP; **ERK**: extracellular signal-regulated kinase; **PI3K**: phosphatidylinositol 3-kinase; **PLC**: phospholipase C; **Rho**: small GTPase of the Rho family; **PLC**: phospholipase C; (modified from Spiegel and Milstien, 2003)).

S1P receptors are found in many tissues and are involved in multiple physiological processes, like vasculogenesis and angiogenesis, IgE mediated mast cell degranulation, regulation of steroid hormone synthesis, chemotaxis and lymphocyte trafficking. (Alvarez et al., 2007). For most, the detailed mechanisms of S1P action are still unclear. Vasculogenesis and

Angiogenesis, for example, involve the $S1P_1$ receptor and SphK1, both upregulated by vascular endothelial growth factor (VEGF), whereas inhibition of SphK1 activity reduces adhesion molecule expression (Kim et al., 2001; Liu et al., 2000; Igarashi et al., 2003; Shu et al., 2002). Even growth factor receptors can cross-talk with S1P signalling. The NGF-TrkA pathway transactivates the $S1P_1$ and $S1P_2$ receptors by translocation of SphK to the cell membrane (Toman et al., 2004). Insulin-like growth factor (IGF) can increase intra- and extracellular levels of S1P and induces internalization of the $S1P_1$ receptor. (Alvarez et al., 2007). Sphingosine kinases and the paracrine or autocrine action of S1P are also involved in many different reactions of the immune system including adaptive and innate immunity (Kee et al., 2005; Baumruker and Prieschl, 2002). In lymphatic tissue S1P receptors are important for lymphocyte egress from the lymphoid organs, e.g. $S1P_1$ receptor activation leads to lymphocyte migration, tissue homing and recirculation (Jolly et al., 2002; Mori et al., 2007; Brinkmann et al., 2001). SphK activation in endothelial cells modulates expression of cell adhesion molecules, thereby increases the recruitment of leukocytes like monocytes and macrophages (Kee et al., 2005; Baumruker and Prieschl, 2002). The activation of mast cells via their IgE-receptor (FcεRI) leads to an increase of S1P, that triggers intracellular Ca^{2+} mobilization, mast cell degranulation and expression of pro-inflammatory cytokines likes TNFα and IL-5 (Kee et al., 2005). TNFα causes many processes and in sensory neurons, TNFα-induced calcium transients at least partially involve S1P (Pollock et al., 2002). The increase of intracellular S1P concentration leads to a receptor-independent Ca^{2+} mobilization via an unknown pathway (Pollock et al., 2002).

1.5 S1P in nociception

Pronociceptive and antinociceptive effects have been reported for S1P, depending on its site of action. Peripheral nociceptive stimulation reportedly lowers the S1P concentration of the cerebrospinal fluid in rats (Coste et al., 2008). Inhibition of S1P synthesis, for example by intrathecally administration of the SphK inhibitors N,N-dimethylsphingosine (NNDS) or DL-threo-dihydrosphingosine (DHS), results in an decrease of pain thresholds in the hot plate test in rats (Coste et al., 2008) and global SphK2 knock-out mice show a lower pain threshold in the hot plate test in comparison to wt litter mates (Coste et al., 2008). In excitatory spinal neurons of the laminae II and III S1P dose-dependently inhibits adenylyl cyclases through activation of an inhibitory G_i-protein (Coste et al., 2008). The following decrease of cAMP leads to a decrease of NMDA-receptor phosphorylation (Coste et al., 2008). On the other

hand, S1P enhances excitability and action potential frequency of capsaicin-sensitive small-diameter sensory neurons via an intracellular site of action, but does not change membrane potential or firing threshold (Zhang et al., 2006). This enhanced excitability is also initiated by ceramide and sphingosine, and dimethylsphingosine (DMS), a competitive inhibitor of SphK, blocks the increased firing rate by ceramide and sphingosine (Zhang et al., 2006). Sensory neurons express S1P receptors (Chi and Nicol, 2010; Mair et al., 2011). More recently, sensitization of small-diameter sensory neuron by S1P was associated with a $S1P_1$ dependent regulation of voltage-gated Na^+ inward current (I_{Na}) and K^+ outward current (I_K) (Zhang et al., 2006a). S1P decreases the peak of I_k by ~30% and increases the I_{Na} of TTX-resistent Na^+-channels (Zhang et al., 2006a). Thermal hypersensitivity is induced by S1P via activation of $S1P_1$ in nociceptors and a similar hypersensitivity to heat stimulation is also reported for SEW2871, a selective agonist at the $S1P_1$ receptor (Doyle 2010, Mair et al., 2011). The pathway is partially involved in the generation of thermal hypersensitivity following experimental inflammation since mice lacking $S1P_1$ specifically in nociceptive primary afferents are partially protected from CFA-induced thermal hypersensitivity (Mair et al., 2011).

2 Aims

We investigated the role of SphK1 in inflammatory thermal hypersensitivity and the role of S1P in nociceptor sensitization to experimental heat stimuli.

3 Materials and Methods

Two different models were used to test the role of SphK1 and S1P-receptors *in vivo* and *in vitro*. Thermal and mechanical withdrawal reflexes were investigated before and after experimental inflammation and the precursor of the S1P agonist FTY720 was used in a skin-nerve preparation model.

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3.1 Behavioural analysis of mechanical and heat sensitivity in sphingosine kinase 1 knock-out mice and wild type litter mates

Male C57BL/6J wild-type mice (older than 6 weeks) from an inbred colony were used in the experiments. SphK1 null mice (Allende et al., 2004) were a generous gift of Dr. R. Proia (NIDDK, US). Mice were single housed with free access to mouse chow and water. The room was temperature and humidity controlled on a 12 h light/dark cycle. All procedures were approved by the national Ethical Committee on Animal Care and Use (BMWF, Vienna, Austria) and in compliance with international laws and policies. Mice were allowed to accommodate to the behaviour testing room for four days prior to the actual experiment. Standard testing procedures were used to quantify changes in thermal and mechanical sensitivity. The area tested was the plantar side of the hind paw. Baseline measurements of heat paw withdrawal latencies and mechanical paw withdrawal thresholds were taken on two days before injection. For mechanical testing mice were placed in a plastic chamber (10.5 x 10.5 x 14 cm) with a metal grid floor and were allowed to habituate for at least one hour. Mechanical sensitivity at the plantar side was determined with a set of calibrated von Frey monofilaments with bending forces between 2.8 and 45.3 mN.

mN	2.8	4	5.7	8	11.4	16	22.6	32	45.3
G	0.285	0.408	0.581	0.815	1.162	1.6	2.3	3.3	4.6

Table 1: Range of von Frey filament used in mechanical test; Force is presented in milli Newton (mN) and gram (g).

First, the von Frey hair with a medial force of 11.4 mN was applied perpendicularly to the plantar surface of the hind paw and held for 3 seconds. Positive responses were measured

when the paw was withdrawn and a flinching movement was noticed immediately after removal of the von Frey hair. Other paw movements were rate as unclear response and were followed by repeating the stimulus. In case of positive reaction a filament with lower force was used, in case of negative response a filament with higher force was used. The withdrawal threshold was determined by increasing and decreasing stimulus intensity on the basis of the up–down method (Dixon, 1980; Chaplan et al., 1994). Trial was repeated six times from the first change, independent if the change was a positive-negative or negative-positive response. Afterwards the raw data were extracted using UDMAP V 3.0 program. Positive responses were marked with "x" and negative with "o".

Heat sensitivity was assessed using the Hargreaves test (Hargreaves et al., 1988). Mice were placed in a plastic chamber (10 x 9.5 x 12.x) and allowed to habituate for at least one hour. A radiant heat source which delivered an increasing heat stimulus was focused on the plantar surface of the hind paw; the time from the initiation of the heat stimulus until paw withdrawal (paw withdrawal latency) was measured automatically (Ugo Basile, Italy). Each paw was tested three times and mean withdrawal latency was calculated. The interval between two trials on the same paw was at least one minute.

Complete Freund's Adjuvant (CFA) or vehicle was injected subcutaneously in a total volume of 30 µl, 15 µl on each, dorsal and plantar side of the paw. The experimenter was initially unaware of the nature of the treatment, however, after 24 h inflammation became obvious by the increasing paw swelling. Sensitivity tests were obtained after 6h, 24h, 48 h and 72 h, then the paw swelling was measured in medio-lateral and in dorso-plantar diameter.

3.2 Electrophysiology of primary afferent nociceptors in a skin-nerve preparation in vitro

An *in vitro* skin-nerve preparation was used to investigate the properties of cutaneous afferent nerve fibres (Koltzenburg et al., 1999; Kress et al., 1992). C57BL/6J mice were killed in a CO_2 inhalation chamber. After cutaneous incision, the saphenous nerve was dissected from blood vessels and connective tissue. The proximal part of the nerve was cut close to the lumbar spinal cord. Subsequently, the hairy skin of the paw was dissected subcutaneously with functionally intact innervating saphenous nerve. The preparation was transferred to an artificial bath solution (synthetic interstitial fluid (SIF), Bretag, 1969) consisting of : 108 mM NaCl, 3.48 mM KCl, 3.5 mM $MgSO_4$, 26 mM $NaHCO_3$, 1.7 mM NaH_2PO_4, 1.5 mM $CaCl_2$,

9.6 mM Na-Gluconat, 5.55 mM Glucose, 7.6 mM Saccharose with a pH of 7.40 ± 0.5. The preparation was mounted with the corium-side up in an organ bath chamber and perfused with carbogen-saturated (95% O2, 5% CO2) SIF at 31°C ± 1°C. The proximal part of the nerve was pulled through a pinhole into a separate recording chamber filled with liquid paraffin. It was placed on a mirror and standard teased fibre technique was used to isolated fine nerve strands which were put on a gold-wire electrode (0.2 mm) for extracellular recording. The receptive field of single units was identified by mechanical probing of the skin with a blunt glass rod. The fibres were classified as unmyelinated fibres by conduction velocity (< 1.4m /s) calculated from the latency of the action potential after electrical stimulation at the receptive field and the distance between stimulation and recording electrodes, as well as by oscilloscopic comparison of the distinct fibre shapes (Oscilloscope GOULD OS 4020, Oscilloscope TEKTRONIX TPS 2024). Heat sensitivity was tested by focusing feedback controlled radiant heat source (PhysioGirl, Hofmann Electronics, Erlangen) to the receptive field which was separated from the bath by a self-sealing steel cylinder. A ramp-shaped heat stimulus with linear rise of the intracutaneous temperature from 31°C to a maximum value of 48°C within 21 s was applied. The temperature on the corium side was feed-back controlled with an external thermo sensor. A fibre was considered heat-sensitive if three or more action potentials were evoked during the stimulus. Heat stimuli were applied every 5 minutes before, during and after the restricted receptive area was perfused by conditioning stimulus solution containing 1µM of FTY720 (Mo Bi Tec Molecular Biotechnology, Göttingen, Germany). Action potentials were recorded, amplified (5000-fold), filtered (low pass 1 KHz, high pass 100 Hz), visualized on the above-mentioned oscilloscopes and stored on a PC-type computer with Spike/Spidi software package (Forster et al., 1990). Skin flaps were discarded after treatment with FTY720 to avoid contamination artefacts.

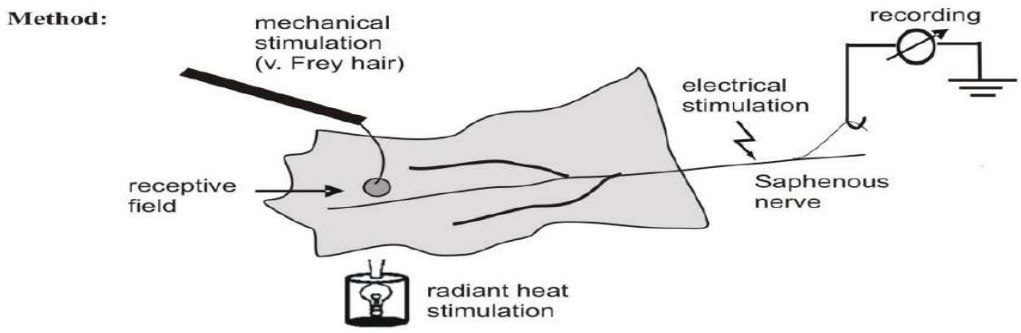

Fig. 2: Schematic illustration of in vitro single fibre recording from the saphenous nerve using the skin-nerve preparation. Application of mechanical, electrical and thermal stimuli on distinct receptive fields was performed (from Andratsch et al., 2008).

3.3 Statistical analysis

For statistical analysis SigmaStat 3.0 (SPSS Inc., Chicago, IL) was used. Data are presented as mean ± standard error of the mean (S.E.M.). For interindividual comparison of different populations the Mann-Whitney U-Test was calculated. Intraindividual comparisons before and after treatment were performed using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $p<0.05$. Graphs were created with Origin Pro 8 (Origin Lab Corporation, Northampton, MA) and GIMP Version 2.6 (GIMP Development Team, Compay, Boston, MA)

4 Results

4.1 CFA induced inflammation in SphK1 null mutant and in C57BL/6J wt mice

Complete Freund's Adjuvant, a mixture of mineral oil and heat-inactivated mycobacterium tuberculosis, induces an inflammatory response after subcutaneous injection. Thereby the inflammation produces thermal and mechanical hyperalgesia. Since S1P acts as immune modulator, we first addressed the question whether the degree of inflammation would be different between wt and SphK1$^{-/-}$ mice. We measured the dorso-plantar and the medio-lateral paw diameter before and after CFA injection at the ipsi- and contralateral paw with a calliper.

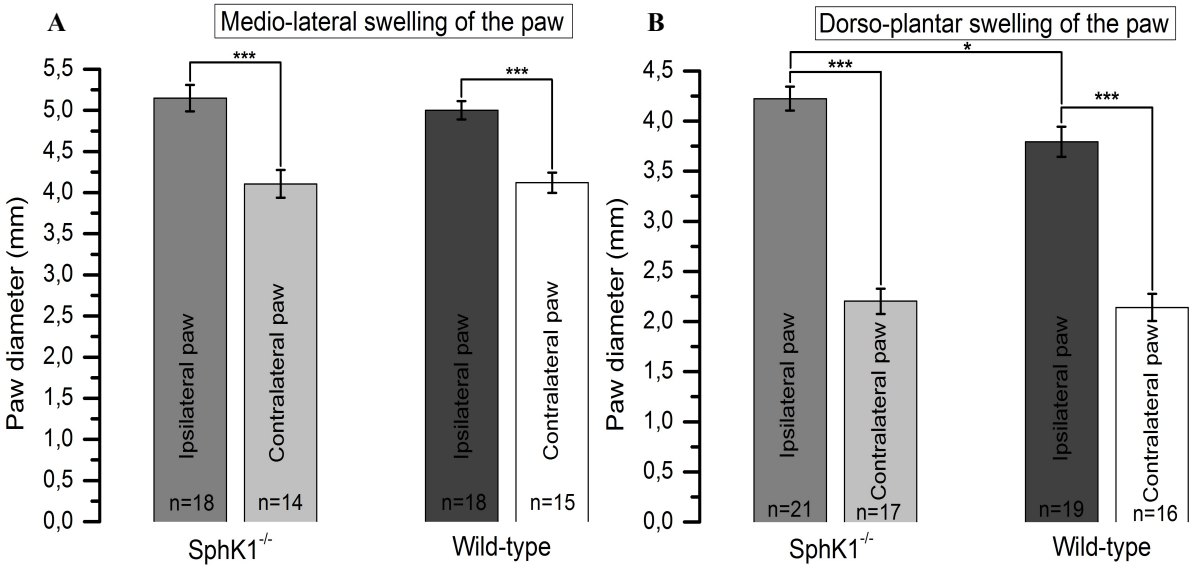

Fig. 3: Comparison of CFA-induced paw swelling in wt as well as in SphK1$^{-/-}$ mice. A highly significant increase of paw diameter was observed in both mouse strains, in medio-lateral diameter (**A**), as well as in dorso-plantar diameter (**B**) (***p< 0.001, Mann-Whitney U- Test). A significant difference between both strains was observed in dorso-plantar diameter (4.22mm ±0.12mm in SphK1-KO to 3.79mm ±0.15mm in WT, *p<0.05, Mann-Whitney U- Test).

CFA induced paw swelling in wt as well as in SphK1$^{-/-}$ mice (**Fig.3**). Both mouse strains showed a highly significant increase of paw diameter at the injected site as compared to contralateral paw. While the contralateral hind paws showed no diameter differences (wt: ↔4.12mm ±0.12mm; ↕2.14mm ±0.14mm vs. SphK1$^{-/-}$: ↔4.11mm ±0.17mm; ↕2.20mm ±0.13mm), a significant difference between the SphK1$^{-/-}$ and the wt littermates was observerved (SphK1$^{-/-}$: ↕4.22mm ±0.12mm vs. wt: ↕3.79mm ±0, p<0.05, Mann-Whitney U-Test)

4.2 Similar mechanical hypersensitivity of SphK1$^{-/-}$ and C57BL/6J wt mice after CFA-injection

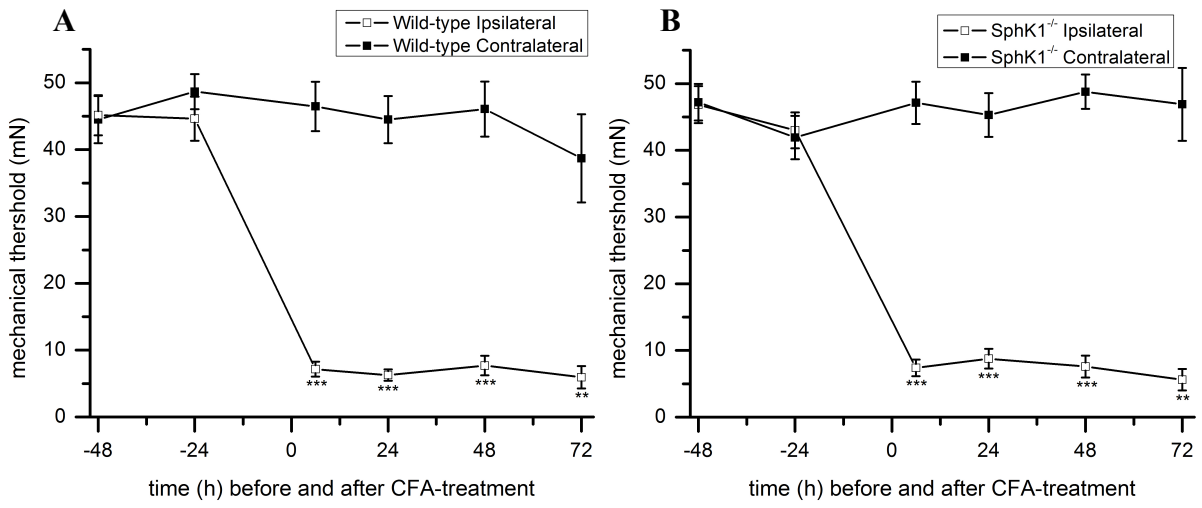

Fig. 4: Subcutaneous CFA-injection led to a decrease of mechanical pain thresholds in wt as well as in SphK1$^{-/-}$ mice. 6h after CFA-injection the mechanical threshold of wt mice dropped from 45.15mN ±3.0mN to 7.15mN ±1.11mN. Within 72h no recovery of was observed (**A**, n= 15, ***p<0.001, Mann-Whitney Rank Sum Test). The SphK1$^{-/-}$ mouse strain showed a similar behaviour (from 46.48mN ±2.8mN to 7.38mN ±1.25mN (**B**, n=14, ***p<0.001, Mann-Whitney Rank Sum Test) within the three days.

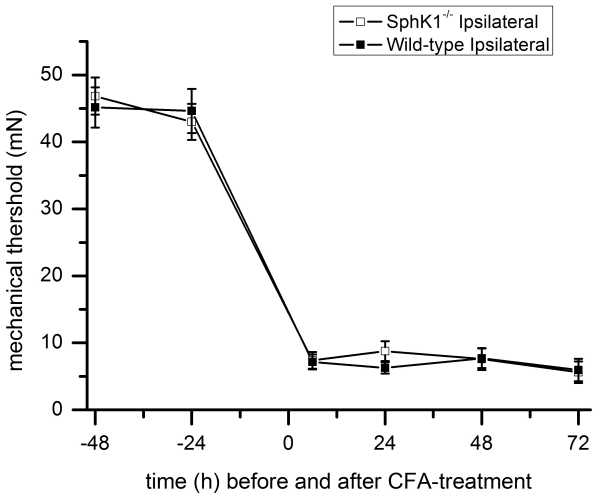

Fig. 5: SphK1$^{-/-}$ and C57BL/6J wt mice showed a similar mechanical hypersensitivity after CFA-injection. Within 72h no differences were observed (n=15, Mann-Whitney Rank Sum Test).

The SphK1$^{-/-}$ and C57BL/6J wt mice started with similar basal mechanical thresholds (44.89mN±2.2 vs. 44.92mN ±1.9mN, n=20, Mann-Whitney Rank Sum Test). As expected, mice developed signs of mechanical hypersensitivity at the injected side. Within 6 h after injection of 30 µl CFA mice exhibited a mechanical hypersensitivity that did not recover

within 72h (Fig.4). The contralateral paws of SphK1$^{-/-}$ and C57BL/6J mice did not show any signs of inflammation or mechanical hyperalgesia (**Fig.4**). No differences in mechanical pain thresholds between the mouse strains could be measured within 72h (**Fig.5**).

4.3 Similar thermal hypersensitivity of SphK1$^{-/-}$ and C57BL/6J wt mice after CFA-injection

In SphK1$^{-/-}$ and C57BL/6J wt mice, intracutaneous injection of CFA resulted in a transient and significant drop in paw withdrawal latencies. The basal thermal sensitivity between the two groups was similar (6.95 sec ±0.68sec in wt vs. 6.16 sec ±0.43 sec in SphK1$^{-/-}$, n=18, Mann-Whitney Rank Sum Test). The inflammatory hyperalgesia fully developed within 6 h and remained at constant levels for up to 48 h (**Fig.6**). 72h after CFA-injection no significant differences between ipsilateral and contralateral paw was observed.

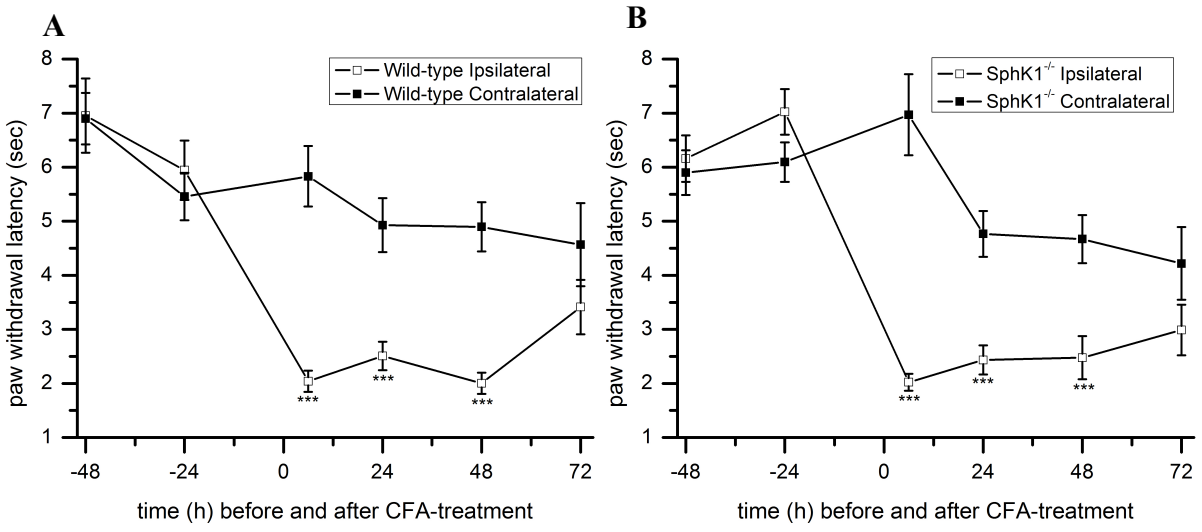

Fig.6: Changes in heat sensitivity following unilateral hindpaw injection of CFA in SphK$^{-/-}$ and C57BL/6J wt mice. Within 6h the PWL dropped from 6.95 sec ±0.68sec to 2.04 sec ±0.19 sec in wt mice (**A**, n= 18) respectively from 6.16 sec ±0.43 sec to 2.02 sec ±0.15 sec in SphK$^{-/-}$ (**B**, n= 15;) (***p>0.001, Mann-Whitney Rank Sum Test). 72h after injection no significant difference between ipsilateral and contralateral paw in both strains was measured (n=6, Mann-Whitney Rank Sum Test).

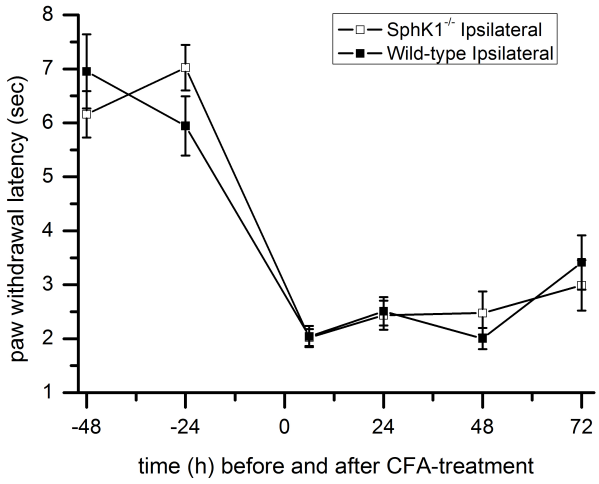

Fig.7: Comparison between SphK1 knock-out mice and C57BL/6J wt mice in paw withdrawal latency. After CFA-injection the mouse strains showed no different behaviour concerning of thermal hypersensitivity (n = 15, Mann-Whitney Rank Sum Test). Even the recovery 72h after injection was equal in both strains (n=6, Mann-Whitney Rank Sum Test).

A comparison between the two mouse groups showed no significant differences in paw withdrawal latency within 72h (**Fig.7**), n=18, Mann-Whitney Rank Sum Test, n.s.). The recovery of thermal hypersensitivity raised equally in both mouse strains (3.41 sec ±0.50 sec in wt vs. 2.99 sec ±0.46 sec, n=6, Mann-Whitney Rank Sum Test, n.s.). In summary no differences between SphK1$^{-/-}$ mice and C57BL/6J wt mice regarding thermal and mechanical hypersensitivity was observed.

4.4 FTY720 does not alter nociceptor heat sensitivity *in vitro*

After localisation of the cutaneous receptive field, basic electrophysiological properties of unmyelinated fibres were determined. Two consecutive heat stimuli were applied to the receptive field at 5 min intervals. This procedure does according to previous studies not affect the nociceptive response to noxious heat. The immune modulator FTY720 (Fingolimod) which acts as a ligand at all S1P receptor but $S1P_2$, was applied to the receptive field of identified CMH-fibres as a conditioning stimulus. Eight CMH fibres responded to heat with an average response frequency of 1.39 ± 0.29 imp/s. FTY720 (1µM, total application time 10 min) induced small increase in discharge rate in 4 of 8 heat-sensitive fibres, 1 fibre discharged constantly and 3 of 8 fibres reacted with an decrease in heat response rate 5 min after application was started. The mean rate of discharge did not change significantly (1.27 ± 0.29 imp/s). After 10 minutes 3 of 8 fibres reacted with an increase in discharging rate, 4 of 8

fibres (50%) decreased their rate of discharge and 1 of 8 fibres discharged constantly. The mean discharge rate was not significantly changed (1.21 ± 0.32 imp/s).

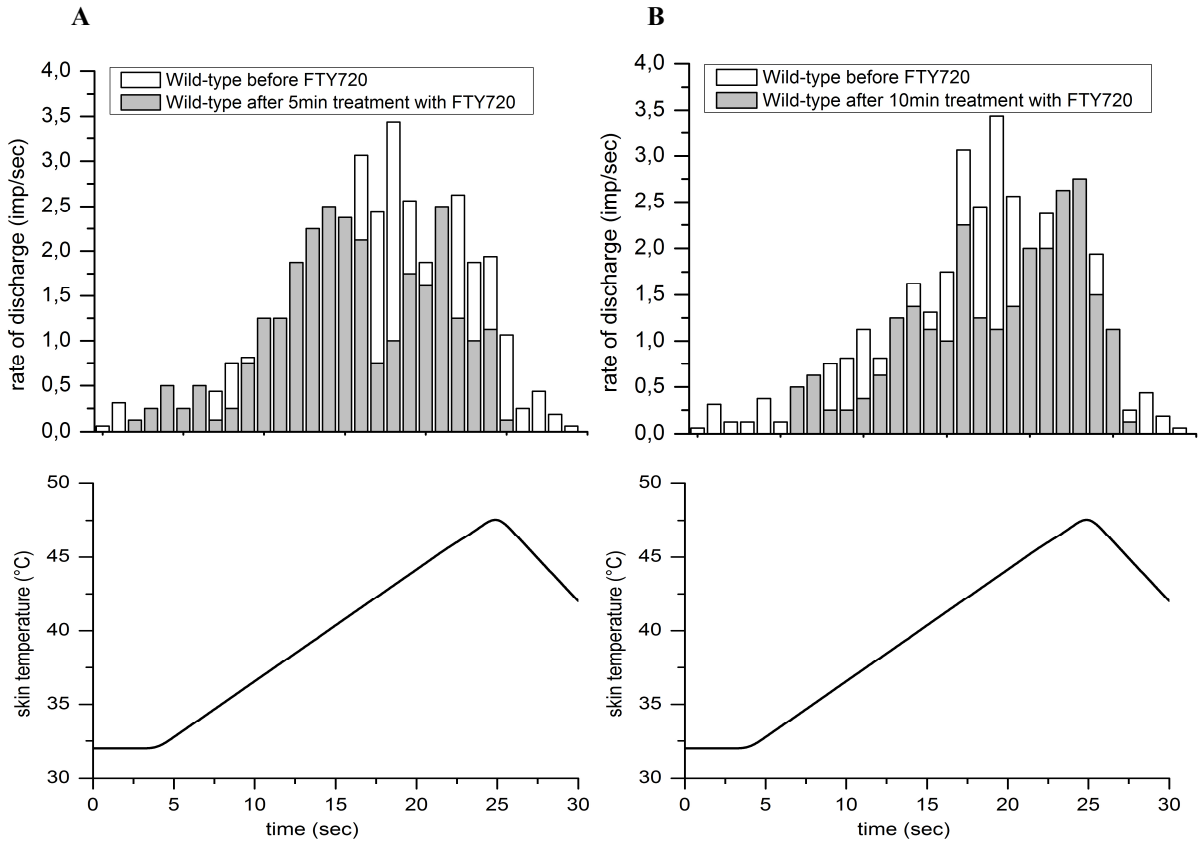

Fig 6: Heat response of nociceptive C-fibres from C57BL/6J wild-type mice. Application of FTY720 (1µg/ml) does not change activation threshold or impulse rate after 5 minutes (**A**, n=8, Wilcoxon Signed Rank Test, n.s.). Even after 10 minutes FTY720 (1µg/ml) does not change heat activation threshold (**B**, n=8, Wilcoxon Signed Rank Test, n.s.).

5 Discussion

Our data revealed an unexpected difference in CFA-induced inflammatory swelling of the hind paw in $SphK^{-/-}$ mice in which swelling was slightly but significantly increased compared to wt littermates. In contrast to our hypothesis, no differences in inflammatory thermal and mechanical hypersensitivity were observed between the two mouse strains within 72h after CFA injection.

A variety of cellular processes involving activation of S1P kinases and autocrine or paracrine S1P effects have been reported and may contribute to plasma extravasation and inflammation induced swelling (for review see Spiegel and Milstien, 2003; Alvarez et al., 2007). S1P and its receptors play a central role in endothelial cell barrier regulation. S1P induces reorganization of the endothelial cytoskeleton (Dudek et al.., 2004) and the distribution, assembly, and stabilization of adherent junction (Schaphorst et al, 2001) leading to a reduction in endothelial permeability to fluid (McVerry and Garcia, 2004). Alterations of endothelial cell barrier function followed by S1P and FTY 720 have been described (Sanchez et al., 2003; McVerry and Garcia, 2004; McVerry and Garcia, 2005) and reduced levels of S1P might explain the difference of inflammatory oedema of $SphK1^{-/-}$ mice.

Furthermore, differences in the cell composition within the inflamed tissue could be responsible for the increased swelling. Sphingosine kinases influence many different immune cells like lymphocyte and macrophages (Kee et al., 2005) and S1P and his receptor $S1P_1$ promote the lymphocyte egress from thymus, spleen and lymph nodes (Pappu et al., 2007). Direct activation of $S1P_1$ with the lyase-insensitive agonist FTY720 inhibits this egress by an unknown pathway (Allende et al., 2004; Rosen and Goetzl, 2005). In addition to an increased plasma extravasation, an increase egress of vascular immune cells into the inflamed tissue could be responsible for the increased tissue volume in $SphK1^{-/-}$ after CFA injection It would be useful to determine the cell composition as well as the concentration of pro-inflammatory cytokines and of S1P of the inflamed tissue to rule out a possibily the increased immune cell invasion into the tissue under inflammatory conditions in $SphK1^{-/-}$ mice.

In recent studies, it has been shown, that S1P sensitizes capsaicin-sensitive small-diameter primary afferent neurons and increases the action potential rate without modifying firing threshold or membrane potential (Mair et al., 2010; Zhang et al., 2006a; Zhang et al., 2006b),

and that the S1P-induced hyperalgesia involves $S1P_1$ (Doyle et al., 2010; Doyle et al. 2011). In contrast to a number of resports suggesting pro-algesic S1P effects, antinociceptive effects of S1P and sphingosine kinases in particular on spinal cord nociceptive transmision have been proposed (Coste et al., 2008). Our data revealed no differences in the degree of thermal and mechanical hypersensitivity in $SphK1^{-/-}$ and wt littermates induced by experimental inflammation within 72h. Also other inflammatory models using $SphK1^{-/-}$ mice did not show differences in acute or chronic inflammatory response (Allende et al., 2004, Michaud et al., 2006). Supported by the observation, that $SphK1^{-/-}$ and $SphK2^{-/-}$ knock-out mice do not show phenotypic abnormalities (Allende et al. 2004; Kharel et al., 2005; Mizugishi et al., 2007; Lynch and MacDonald, 2008), it is assumed that loss of one SphK-gene leads to an upregulation of the corresponding other sphingosine kinase and functional compensation of the null mutation. Nevertheless increasing SphK2 mRNA expression levels could not be proven yet (Allende et al., 2004; Kharel et al., 2005; Michaud et al., 2006). To avoid the compensatory upregulation of SphK2, it would be necessary to use $S1P_1{}^{-/-}$ mice to investigate the role of S1P in inflammatory hyperalgesia. Unfortunately $S1P_1$ receptor knock-out mice were created but died in utero due to vascular leakage as a consequence of deficient angiogenesis and vasculogenesis (Liu et al., 2000). Therefore, conditional KO mice are being generated and a first study of our group shows employing mice with a conditional deletion of $S1P_1$ receptor in nociceptive primary afferent neurons indeed suggests that $S1P_1$ receptor is critical for inflammatory thermal hypersensitivity (Mair et al., 2011).

FTY720 (Fingolimod), a ligand at $S1P_1$ and $S1P_{3-5}$ receptors, is a novel therapeutic drug, that is in clinical trial for different autoimmune diseases. Because of its ability to activate and quickly internalize its receptor, it acts as a functional antagonist on the S1P receptors (Brinkmann, 2009). Because of his high affinity binding to $S1P_1$ we wanted to see, if it provokes an increase in action potential firing rate in an in-vitro skin nerve preparation. Our data suggest that FTY720 does not induce a thermal hypersensitivity, *in vitro*. This is in contrast to S1P and the selective S1P1 agonist SEW2871 which have recently been shown to induce thermal hypersensitivity via direct nociceptor sensitisation (Mair et al., 2011; Doyle et al., 2010). Also, tumour necrosis factor α sensitises nociceptors to heat and some of its effects are mimicked by S1P (Constantin et al., 2008; Pollock et al., 2002). In addition, activation of $S1P_1$ induces an increase of excitability in primary afferent neurons by modification of different Na^+- and K^+ channels, e.g. TTX-R $NaV_{1.8}$. It has also been published, that S1P sensitises $TRPV_1$ -channels by phosphorylation. (Mair et al., 2010; Chi and Nicol, 2010;

Zhang et al., 2006a). In our experiments the non-phosphorylated FTY720 did not induce any signs of hyperexcitability or sensitivity increase in primary afferent neurons. It is known, that FTY720 needs to be phosphorylated by SphK2 to become biologically active as FTY720-P (Brinkmann et al., 2002; Mandala et al., 2002). This process in particular is known to be active in the liver (Billich et al., 2003). SphK2 is located in the cytosol and the nucleus (Venkataraman, 2006). It is unlikely, that extracellularly applied FTY720 is phosphorylated in vitro, however, as an inactive metabolite it does not bind to S1P receptors (Marsolais and Rosen, 2009; Melendez, 2008). Further experiments will be necessary to study FTY720-P or selective $S1P_1$ receptor agonists like the synthetic chemical modulators AUY954 or CYM-5442 (Marsolais and Rosen, 2009; Lynch and MacDonald, 2008) in this preparation.

Taken together, our data show that $SphK1^{-/-}$ mice do not exhibit any differences in pain like behaviour under inflammatory conditions although the degree of paw swelling is significantly increased. Furthermore, FTY720 did not induce nociceptor sensitisation in vitro. Nevertheless they do not exclude a pivotal role of S1P and S1P receptor pathways in inflammatory conditions. Further experiments are in progress to further elucidate the importance of S1P for the generation of pain associated with inflammatory disease.

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Curriculum Vitae

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