CHARLES UNIVERSITY
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
Department of Pharmacology and Toxicology

UNIVERSITÉ DE LIMOGES
FACULTÉ DE PHARMACIE
Working group "Maintenance myélinique et neuropathies périphériques"

Diploma thesis

Transcriptomic analysis of cutaneous inflammatory biomarkers in a mouse model of small fiber neuropathy

Supervisors: Dr. Claire Demiot, Dr. Aurore Danigo
Assoc. Prof. Přemysl Mladěnka, Ph.D

Hradec Králové 2018 Simona Benčová

UNIVERZITA KARLOVA
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
Katedra farmakologie a toxikologie

UNIVERSITÉ DE LIMOGES
FACULTÉ DE PHARMACIE
Pracovná skupina "Maintenance myélinique et neuropathies périphériques"

Diplomová práca

Transkriptomická analýza kožných zápalových biomarkerov u myší s neuropatiou malých nervových vlákien

Školitelia: Dr. Claire Demiot, Dr. Aurore Danigo
Doc. PharmDr. Přemysl Mladěnka, Ph.D.

Hradec Králové 2018

Simona Benčová

I hereby declare that this thesis is my original author work. All literature and other sources, which I used, are properly cited.

Prehlasujem, že táto práca je mojim pôvodným autorským dielom. Literatúra a ďalšie zdroje, z ktorých som pri spracovaní čerpala, sú uvedené v zozname použitej literatúry a v práci riadne citované. Práca nebola použitá k získaniu iného či rovnakého titulu.

V Hradci Králové, ……...

Simona Benčová

ACKNOWLEDGMENT

I would like to thank to my supervisors Dr.Claire Demiot for accepting me in the laboratory enabling me to participate on the experiments and Dr. Aurore Danigo for her great willingness to answer all my questions with a big patience and help with this work.

Many thanks to Dr. Sylvie Bourtoumieu who showed me each method and for her advices in work.

I would like to thank also all participants of the working group “Maintenance myélinique et neuropathies périphériques” for their friendly atmosphere in the laboratory.

Great thanks to my supervisor in the Czech Republic, Assoc. Prof. Přemysl Mladěnka, Pharm.D., Ph.D. for his useful advices and consultations.

Last, I am very grateful to my parents who always supported me in my studies.

Abstract

Charles University
Faculty of Pharmacy in Hradec Králové
Department of Pharmacology and Toxicology
Student: Simona Benčová
Supervisors: Dr. Claire Demiot, Dr. Aurore Danigo
Assoc. Prof. Přemysl Mladěnka, Ph.D

Title of diploma thesis: **Transcriptomic analysis of cutaneous inflammatory biomarkers in a mouse model of small fiber neuropathy.**

Peripheral neuropathy is an expanding public health problem conditioned by various diseases and associated with several adverse effects such as the occurrence of chronic pain or increased risk of pressure ulcers (PUs). The aim of this study is to explore, whether the inflammatory state of the skin is modified during peripheral neuropathy and in the course of the formation of a pressure ulcer. The transcriptomic analysis was performed with two different models of mice: PU model and uninjured model, to determine genes that differ in expression and in particular, those involved in inflammation. Small fiber neuropathy was induced in young mice by intraperitoneal injection of resiniferatoxin (50 µg/kg, i.p.) - transient receptor potential vanilloid 1 (TRPV1) agonist. PUs were induced by applying two magnetic plates on the dorsal skin. Gene expression was obtained based on RNA microarray and the results were subsequently verified by qPCR. The transcriptomic analysis of PU showed upregulation of several interleukins (IL1f5, IL1f6, IL11, IL17d, IL20, IL34) and marked downregulation of IL16 between RTX and control mice. The data were however not confirmed by quantitative PCR. The transcriptomic analysis of uninjured skin showed upregulation of IL24, IL33, IL6 as well as downregulation of IL15 and IL34. In addition to interleukins, changes in expression were visible also in chemokines and CD molecules. Our preliminary results indicate that the inflammatory state of the skin is dysregulated by RTX-induced neuropathy, and deregulation of inflammation is undoubtedly associated with the increased risk of pressure sores.

Abstrakt

Univerzita Karlova
Frmaceuticka fakulta v Hradci Králové
Katedra farmakológie a toxikológie
Študent: Simona Benčová
Školitelia: Dr.Claire Demiot, Dr. Aurore Danigo
Doc. PharmDr. Přemysl Mladěnka, Ph.D

Názov diplomovej práce: **Transkriptomická analýza kožných zápalových biomarkerov u myší s neuropatiou malých nervových vlákien.**

Periférna neuropatia je rozšírujúci sa problém zdravia verejnosti ako následok rôznych ochorení, spojený s niekoľkými nepriaznivými účinkami, ako napríklad výskyt chronickej bolesti či zvýšené riziko vzniku tlakového vredu. Cieľom tejto štúdie je preskúmať, či je zápalový stav kože modifikovaný počas periférnej neuropatie a v priebehu tvorby tlakového vredu. Na dvoch rôznych modeloch myší (myši s tlakovým vredom a myši s nepoškodenou kožou), bola uskutočnená transkriptomická analýza, aby sa určili gény, ktoré sa líšia v expresii a zároveň tie, ktoré sa podieľajú na zápale. Neuropatia malých nervových vlákien bola navodená u mladých myší intraperitoneálnou injekciou resiniferatoxínu (50 μg/kg, i.p), agonistu na vaniloidnom receptore TRPV1. Preležaniny boli spôsobené aplikáciou dvoch magnetických platničiek na koži na chrbte. Na základe RNA mikroarray bola získaná génová expresia a výsledky boli následne overené kvantitatívnou PCR. Transcriptomická analýza kože s tlakovým vredom ukázala upreguláciu niekoľkých interleukínov (IL1f5, IL1f6, IL11, IL17d, IL20, IL34) a výraznú downreguláciu IL16 pri porovnaní RTX a kontrolnych myší. Tieto výsledky avšak neboli potvrdené kvantitatívnou PCR. Transcriptomická analýza nepoškodenej kože ukázala upreguláciu IL24, IL33, IL6, ako aj downreguláciu IL15 a IL34. Okrem interleukínov boli zmeny v expresii viditeľné aj u chemokínov a CD molekúl. Naše predbežné výsledky naznačujú, že zápalový stav kože je dysregulovaný RTX-indukovanou neuropatiou a deregulácia zápalu je nepochybne spojená so zvýšeným rizikom tlakových vredov.

CONTENT

1 Introduction

The nociception impairment in diabetic patients is considered one of the main predisposing factors for developing pressure ulcers (PUs). However, diabetes is associated with intrinsic vascular changes that may contribute to the development of PUs along with nerve function impairment. We aimed to determine the consequence of a specific sensory small fiber neuropathy in mice by use of cutaneous inflammatory biomarkers in cases of healthy skin or pressure-induced ulcer. Small fiber neuropathy (SFN) was induced in mice by a single systemic injection of resiniferatoxin (RTX), a capsaicin analog that specifically affects sensory myelinated and unmyelinated fibers (Aδ and C). Seven days after RTX administration, PUs were induced by applying two magnetic plates on the dorsal skin. RTX induced a significant hypoalgesia associated with nerve substance P and calcitonin gene related peptide (CGRP) depletion without nerve degeneration. RTX-induced SFN also induced larger stage 2 ulcers 24 hours after the release of pressure. A specific functional alteration of small nerve fibers, associated with a decrease of CGRP and SP expressions, increases pressure-induced ulcer development. Thus, in addition to impairing nociception, alteration of skin nerve fibers by CGRP/substance P depletion may impede the normal protective response of the skin to ischemia. The finding that PU formation is enhanced by SFN, in the absence of microangiopathy is highly reminiscent of the human hereditary sensory and autonomic neuropathies. These observations may provide some clues about the pathogenesis of skin lesions in these patients. CGRP and substance P are the most common and best-studied neuropeptides involved in neurogenic inflammation. “Neurogenic inflammation” refers to inflammatory changes (vasodilatation, plasma extravasation, hypersensitivity) resulting from the release of substances from sensory nerve terminals during the injury (Richardson and Vasko, 2002). We suppose that vascular changes, induced by CGRP (hyperaemia) (Aubdool and Brain, 2011) and substance P (plasma extravasation) (Weidner et al., 2000), which occur after the pressure release, are essential to protect the skin against pressure-induced ulcer. Both CGRP and SP enhance inflammatory cell infiltration by locally increasing blood flow and stimulating mast cell degranulation (Steinhoff et al., 2003). Our data show that depletion of CGRP and SP in cutaneous small nerve fibers leads to an increase of necrosis and a reduced recruitment of inflammatory cells in ulcer tissue. Thus, normal cutaneous neurogenic inflammation seems crucial to protect skin against necrosis in PU formation. In this context, we performed a transcriptomic analyse of healthy skin back and pressure-induced ulcer from control and RTX mice.

2 Theoretical part

2.1 Skin

Human skin is the largest body organ and it acts as an effective barrier that protects the body against the pathogens and xenobiotics. It has further roles in thermoregulation, metabolism and sense perception. The skin has polymodal nervous system which can recognize different stimuli such as pain, touch, pressure, vibration and temperature by means of receptors. It consists of three layers: epidermis and dermis, which are separated by a basal membrane, and hypodermis (Wysocki, 1999).

The epidermis is the outermost layer of the skin derived from the embryonic ectoderm. The epidermis has four to five layers (stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale), that rely on the thickness of the skin (Wysocki, 1999). Stratum corneum is the outermost layer and is located directly on the top of the epidermis and provides real protection and a barrier to prevent water loss and penetration of xenobiotics. The epidermis consists mainly of keratinocytes but it also contains cells such as melanocytes and Langerhans cells, which are part of the immune system (Kolarsick et al., 2011). The epidermis is an avascular layer dependent on the capillary beds in the dermis for the supply of oxygen and nutrition (Wysocki, 1999).

The dermis, that is derived from mesoderm, is located beneath the epidermis and is the thickest of the three layers of the skin (Wysocki, 1999). It can be characterised as an integrated system of fibrous and amorphous connective tissue. Elastic and collagen fibres are two main components of fibrous connective tissue (Prost-squarcioni et al., 2008). Moreover, the dermis is composed of nerve endings, sweat glands, hair follicles, blood vessels and mast cells. Its properties such as pliability, elasticity and tensile strength are provided by elastic and collagen fibers (Kolarsick et al., 2011; Prost-squarcioni et al., 2008).

The hypodermis is a loose connective tissue layer that functions as a storehouse of energy (Kolarsick et al., 2011). It consists of adipocytes or fat cells and fibroblasts, but also macrophages, nerves and blood vessels are included in this layer (Wysocki, 1999).

2.2 Cutaneous nervous system

The nervous system consists of two parts, the central nervous system (CNS) and peripheral nervous system (PNS). CNS is composed of the brain and spinal cord. PNS includes the nerves through which information flow between the CNS and the rest of the body (Démarchez, 2015). The cutaneous nervous system is a part of the PNS with a dense network of afferent sensory and efferent autonomic nerves that occurs in all cutaneous layers (Cooper, 2002). CNS also regulates skin functions by the adrenal glands and immune cells (Roosterman et al., 2006).

2.2.1 The peripheral nervous system in the skin

The innervated skin is a vital barrier with direct contact to the other parts of the nervous system. Afferent nerve impulses from the periphery are transmitted by fibers of primary sensory neurons to trigeminal or dorsal root ganglia. Efferent axons of the sympathetic paravertebral ganglia neurons terminate in the skin where they innervate a variety of cutaneous structures such as sweat glands, hair follicles and blood vessels (McGlone and Reilly, 2010).

2.2.1.1 Sensory system

The main function of somatosensory axons is sense perception. Each type of sensory receptor converts information from the external environment into action potentials that are subsequently conveyed and processed in the CNS (Oaklander and Siegel, 2005). The sensory system consists of receptors for touch, temperature, pain and other chemical and physical stimuli. Morphologically, cutaneous sensory axons contain myelinated (Aβ, δ) as well as unmyelinated (C) nerve fibers (Table I) (Purves et al., 2001a).

Table 1. The major classes of somatic sensory receptors

Receptor type	Anatomical characteristics	Associated axons	Location	Function	Rate of adaptation	Threshold of activation
Free nerve endings	Minimally specialized nerve endings	C, Aδ	All skin, epidermis	Pain, temperature, crude touch	Slow	High
Meissner's corpuscles	Encapsulated; between dermal papillae	Aβ	Principally glabrous skin, dermis	Touch, pressure (dynamic)	Rapid	Low
Pacinian corpuscles	Encapsulated; onion like covering	Aβ	Subcutaneous tissue, interosseous membranes, viscera	Deep pressure, vibration (dynamic)	Rapid	Low
Merkel's disks	Encapsulated; associated with peptide-releasing cells	Aβ	All skin, hair follicles	Touch, pressure (static)	Slow	Low
Ruffini's corpuscles	Encapsulated; oriented along stretch lines	Aβ	All skin, dermis	Stretching of skin	Slow	Low

Table was adjusted according to Purves et al., 2001b.

The sensory receptors can be classified as encapsulated or free nerve endings. The free nerve endings are associated with non-myelinated C-fibers and of thinly myelinated Aδ-fibers form the majority of nociceptive peripheral pathways. The free nerve endings are not encapsulated, they are freely lying in the extracellular space or they are closely deposited on cells of innervated tissues (Purves et al., 2001a).

Encapsulated receptors function as mechanoreceptors and respond to stimuli such as touch, pressure, vibration and cutaneous tension. There are four major types of these mechanoreceptors: Meissner's, Pacinian, Ruffini´s corpuscles and Merkel´s disks (Tab.1). They can be also referred as low-threshold mechanoreceptors because even a weak stimulation is

sufficient to induce the action potential. All signals from low-threshold mechanoreceptors are conducted by myelinated Aβ-fibers. The Meissner's corpuscles are elongated receptors that lie just beneath the epidermis of the fingers, palms, and soles and they are efficient in transducing information about the low-frequency vibrations. The Pacinian corpuscles are large structures located in subcutaneous tissue with sensitivity to high-frequency stimuli, such as vibration. The Merkel's disks are located at the dermo-epidermal junction and their stimulation produces a sensation of light pressure. The Ruffini's corpuscles have an elongated spindle shape and they are localized deeply in the skin. They respond to skin stretch (Purves et al., 2001b) (Fig. 1).

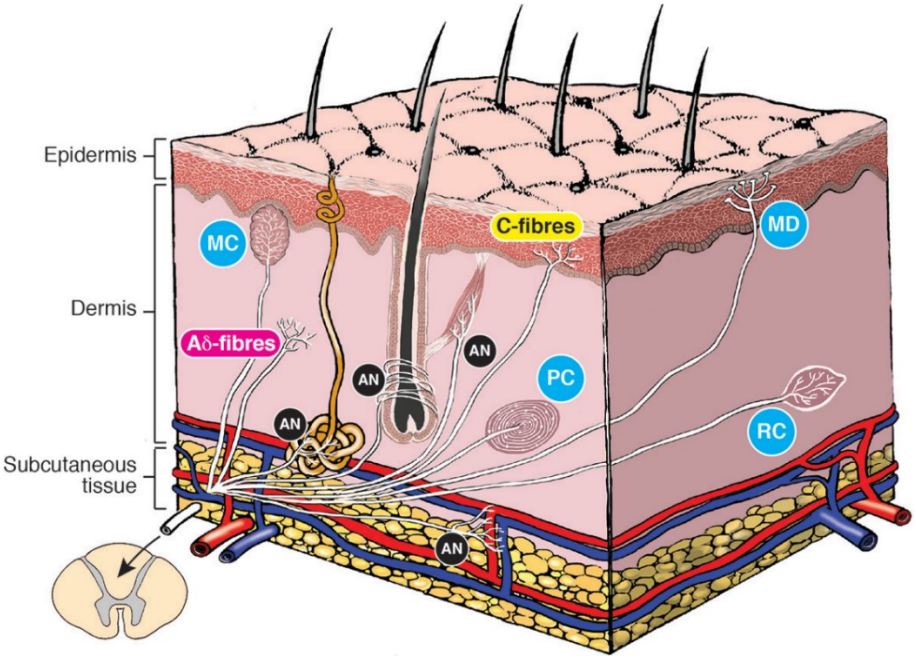


Fig.1. Distribution of cutaneous nervous system across the skin. *MC: Meissner's corpuscle, MD: Merkel disc, PC: Pacinian corpuscle, RC: Ruffini's corpuscle, AN: autonomic nerves. The figure was taken from Ashrafi et al., 2016.*

2.2.1.2 Autonomic innervation of the skin

In comparison with sensory nerves, the autonomic nervous system constitutes only the minority of the nerve fibers of the skin and they are located in the dermis where they innervate blood vessels, lymphatic vessels, eccrine glands, apocrine glands, sweat glands and hair follicles (Roosterman et al., 2006).

2.2.2 The nerve fibers

In the PNS, sensory fibers can be divided into four groups according to their degree of myelination (Fig. 2). Unmyelinated fibers, also called C-fibers, have axons encircled by a single layer of noncompact Schwann cell membrane whereas myelinated fibers, including Aα-, Aβ- and Aδ-fibers, have axons encased by multiple layers of Schwann cell membrane forming compact myelin. Myelinated fibers have different extent of myelination. Aα-fibers are characterized by a thick coat of myelin with diameter of 12-22 µm, Aβ-fibers have a thinner myelin coat with diameter of 6-12 µm, while Aδ-fibers are thinly myelinated (diameter: 1-5 µm). The skin is innervated by the Aβ-, Aδ- and C-fibers. Encapsulated c mechanoreceptors are innervated by large myelinated Aβ-fibers. Small fibers (thinly myelinated Aδ- and unmyelinated C-fibers) are localized in the epidermis as free nerve endings that are responsible for thermal and nociceptive stimuli. Nerve fibers can be also classified according to the conduction velocity. While Aδ-fibers have conduction velocity 4-30 m/s, the C- fibers have slow conduction velocity 0,4-2 m/s so they are responsible for conveying a different type of pain (Coutaux et al., 2005; Myers et al., 2013).

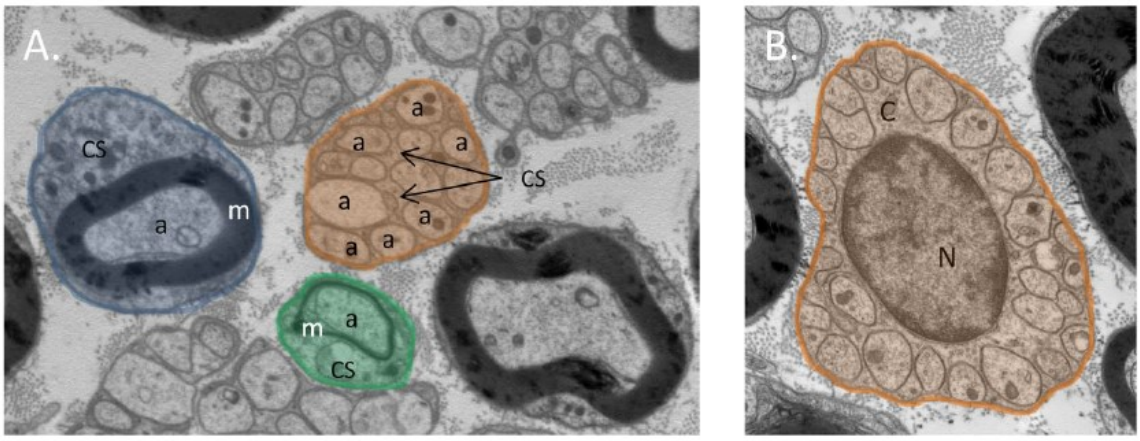


Fig. 2. Morphology of three types nerve fibers Aβ, Aδ and C on a cross section of mouse nerve. *(A) Myelinated Aβ-fibers (blue), thinly myelinated Aδ-fibers (green) and unmyelinated C-fibers (orange). CS: cytoplasm of the Schwann cell, a: cytoplasm of an axon, m: myelin sheath. (B) the unmyelinated axons are enveloped by invaginations of the cytoplasm of a non-myelinating Schwann cell. C: Schwann cell, N: neuron. The figure is an unpublished work of Danigo.*

2.3 Small fiber neuropathy

Neuropathy is a term that refers to general diseases or malfunctions of the nerves (Stöppler and Shiel, 2017). Disease or injury can affect the cell body of neurons, but also their axon or myelin (Vaillancourt and Langevin, 1999). In general, peripheral neuropathy can be classified according to the function of the involved nerve fibres or their diameter and conduction velocity. They are three types that can be distinguished on the grounds of functions of nerve fibres: motor nerve damage, sensory nerve damage and autonomic nerve damage. Another classification is based on the size: damage to large sensory fibers and small sensory fibers (NINDS, 2017). Peripheral neuropathies can be also defined as polyneuropathy or mononeuropathy. While polyneuropathies affect all nerves, especially on the feet where they are longest, mononeuropathies affect peripheral nerves focally (Vaillancourt and Langevin, 1999). Small fibre neuropathies (SFN) affect small myelinated A-δ nerve fibres or unmyelinated C-fibres. Aδ-fibres and C-fibres mediates pain and temperature sensation and have also autonomic functions, therefore their damage can be accompanied by symptoms of autonomic dysfunction (Hoitsma et al., 2004; NINDS, 2017). SFN is an increasing public health problem and it can be characterised as a structural abnormality of small C- and Aδ-fibres with degeneration of distal terminal endings (Themistocleous et al., 2014). However, the pathogenesis is not very understood, SFN can be a consequence of many diseases such as diabetes mellitus, thyroid dysfunction, HIV infection, vitamin B12 deficiency or drug neurotoxicity. Despite this, many neuropathies are of idiopathic or complex origin. Indeed, the most common cause is diabetes, where a complex interplay of metabolic factors, ischemia and impaired recovery predispose peripheral neurons, glial cells and vascular endothelial cells to a damage that ultimately leads to neuronal injury and peripheral neuropathy (Hovaguimian and Gibbons, 2012; Themistocleous et al., 2014).

2.4 Transient potential receptor

Transient receptor potential (TRP) genes were first described in the fruit fly *Drosophila melanogaster* (Nilius and Owsianik, 2011). TRP comprise of six subfamilies: TRP Ankyrin (TRPA), TRP Canonical (TRPC), TRP Melastin (TRPM), TRP Mucolipin (TRPML), TRP Polycystin (TRPP), and TRP Vanilloid (TRPV) (Premkumar, 2014). TRP ion channels participate on diverse physiological processes, such as sensation of different stimuli or ion homeostasis and are expressed in many different tissues and cell types (Nilius and Owsianik, 2011).

2.4.1 TRPV1

The transient receptor potential vanilloid 1 (TRPV1) ion channel is a polymodal receptor with tetrameric structure and six transmembrane segments (S1-S6) per unit, with a free region between the fifth and sixth segment that forms the channel conductive pore. The N- and C-termini of the proteins are located intracellularly and the ankyrin domains are located in the N-terminus (Fig.3) (Messeguer et al., 2006). The amino acid sequence also contains multiple phosphorylation sites for protein kinase C (PKC), protein kinase A (PKA) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) (Rosenbaum and Simon, 2007).

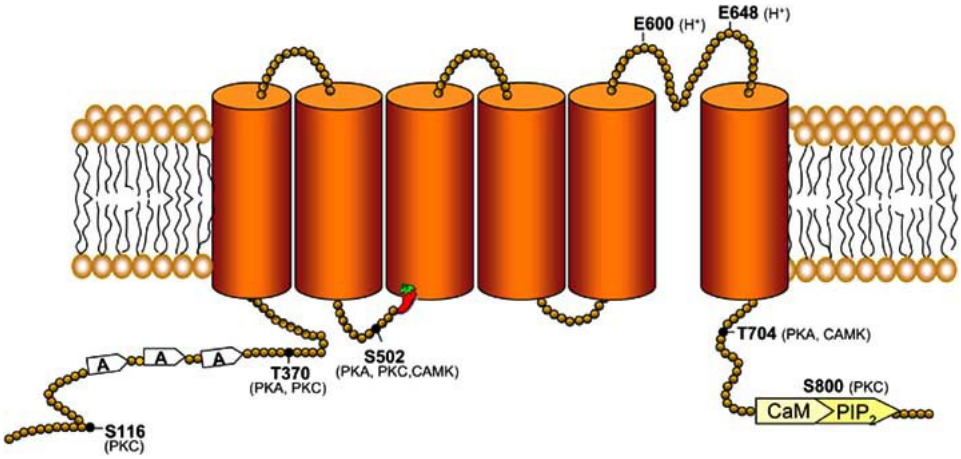


Fig. 3. Structure of TRPV receptor. *The capsaicin binding site is located intracellularly and is labelled as chili icon. The figure was taken Messeguer et al., 2006***.**

Activation of these receptor mediates influx of cations (mainly Ca^{2+}) across the plasma membrane (Diaz-Franulic et al., 2016). TRPV1 is expressed on cutaneous sensory neurons especially on C- and Aδ-fibers. Moreover, it was found to be expressed in non-neuronal skin cells such as keratinocytes, dermal mast cells, dendritic cells, dermal blood vessels, hair follicles and sweat glands where it also acts as pain and chemical sensor (Gouin et al., 2017).

TRPV1 can be activated by various stimuli that can have endogenous or exogenous origins (Table 2) (Gouin et al., 2017). The chemical activators can be divided into the group that directly activate the channel and those that allosterically modulate its activation (Messeguer et al., 2006).

Table 2. Endogenous and exogenous agonists involved in TRPV1 activation and sensitization

Exogenous activators	Endogenous activators	Indirect activators/sensitizer	Inhibitors/ desensitizers
High temperature (>43°C)	Anandamide	Bradykinin	Calcineurin
Protons (pH < 5,9)	Lipooxygenase products	NGF	PIP2
Ultraviolent	DAG	Serotonin	-
Vanilloids:	CAMKII	Histamine	
-Capsaicin	PIP2	Proteases	-
-Resiniferatoxin		ATP	-

DAG: diacylglycerol; NGF: nerve growth factor; PIP2: phosphatidylinositol-4,5-bisphosphate. The table was adjusted from Gouin et al., 2017.

2.4.1.1 Mechanism of action

The binding of vanilloid activators to TRPV1 leads to opening of the channel and an increase in the concentration of Ca^{2+} in the cell with its subsequent depolarization. The action potential is generated after the membrane depolarization reaches the threshold level and it can be perceived as pain or itch. The vanilloids (like capsaicin and resiniferatoxin) cause also release of neuropeptides such as substance P and CGRP (Kissin, 2008). These neuropeptides are released from the peripheral terminals of sensory nerves after their depolarization (Brain, 1997) and initiate neurogenic inflammation (Kissin, 2008).

Sensitization of the TRPV1 receptor may be a consequence of direct activation or of modulation of its activity. The main mechanism of sensitization the TRPV1 receptor is phosphorylation. Mediators of inflammation can act directly on the TRPV1 receptor. Above all, they represent ligands of metabotropic receptors whose activation triggers the specific signalling pathways that lead to activation of protein kinases and phosphorylation of the TRPV1 receptor. Therefore,

phosphorylation itself does not cause immediate opening of the ion channel but allosterically modulates its sensitivity to specific stimuli (Fig 4.). Signal pathways include calcium / calmodulin-dependent protein kinase II (CaMKII), protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA) as was described in chapter 2.4.1 (Sušánková and Vlachová, 2006).

PKA-dependent pathway that influences capsaicin or heat mediated actions on TRPV1 is triggered by inflammatory mediators such as prostaglandins. Phosphorylation sites as Ser-116 and Thr-370 of the amino acid sequence are implicated in desensitization. Contrarily, phosphorylation sites Thr-144, Thr-370 and Ser-502 are implicated in sensitization (Rosenbaum and Simon, 2007).

Phosphorylation of TRPV1 by PKC can be induced by extracellular ATP, proteases, IL-1β, and bradykinin at phosphorylation sites Ser-502 and Ser-800. Different isoforms of PKC (PKCα, PKCε and PKCμ) are involved in TRPV1 sensitization. Phosphorylation by PKC potentiates capsaicin- or proton-evoked responses but may also lead to a decrease of temperature threshold level, leading to the activation of the channel at body temperature (Messeguer et al., 2006; Rosenbaum and Simon, 2007).

CaMKII phosphorylates TRPV1 at sites Ser-502 and Thr-704 and contributes to an activation similarly to the response induced by capsaicin application. Moreover, calcineurin mediates dephosphorylation at the same sites and can hence produce desensitization of TRPV1 (Devesa et al., 2011). Desensitization of TRPV1 depends on the presence of intracellular Ca^{2+} and may rapidly occur after a prolonged application of an agonist or repeated agonist applications (Devesa et al., 2011; Rosenbaum and Simon, 2007).

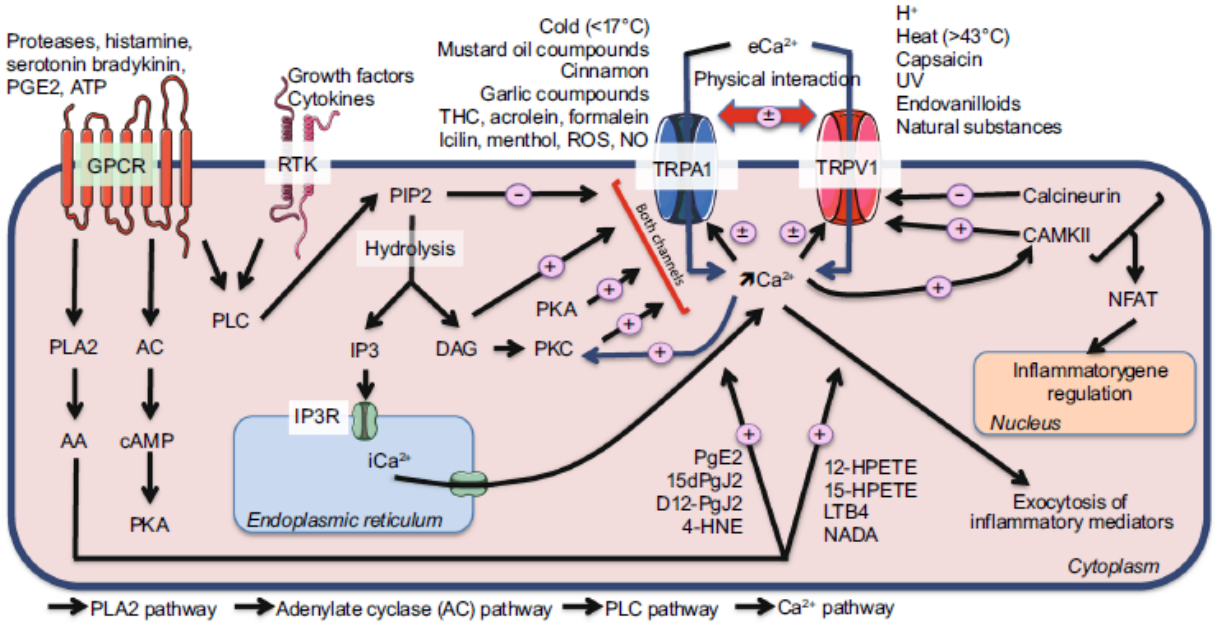


Fig. 4. Intracellular pathways via the activation and sensitization od TRPV1. *The G protein-coupled receptor (GPCR) and receptor tyrosine kinase (RTK) activation stimulate phospholipase C (PLC), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG activates PKC and IP3, which binds to the endoplasmic reticulum receptor and contributes to increases in the intracellular concentration of Ca^{2+}. In addition, PIP2 is constitutively associated with TRPV1, causing its inhibition. Upon activation of PLC, this inhibition is relieved by PIP2 hydrolysis and this leads to channel sensitization. GPCRs can also activate the phospholipase A_2 (PLA2) and adenylate cyclase pathways, which lead to protein kinase A (PKA) stimulation and the formation of arachidonic acid (AA) metabolites. PKA directly sensitizes the TRP channels, while AA metabolites and products directly activate the TRP channels. Thus, TRPV1 can be directly or indirectly regulated by direct interaction or via changes in the intracellular Ca^{2+} concentration, resulting in a sensitization/desensitization process. The elevation of intracellular Ca^{2+} initiates the exocytosis of inflammatory mediators and stimulates both Ca^{2+}/calmodulin-dependent kinase II (CAMKII) and calcineurin, which sensitize and desensitize TRPV1, respectively. The figure was taken from Gouin et al. 2017.*

2.4.1.2 Capsaicin and Resiniferatoxin

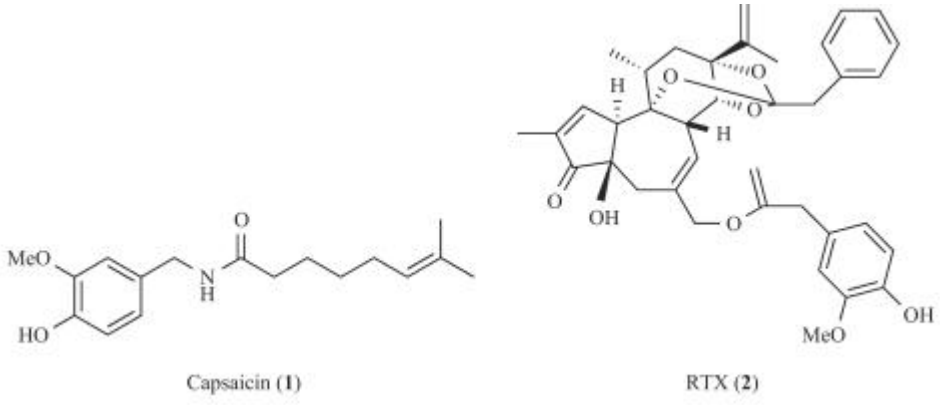


Figure 5. *Chemical structure of capsaicin and resiniferaroxin (RTX).*

Capsaicin and resiniferatoxin (RTX, Fig. 5) are vanilloid agonists. Capsaicin is an alkaloid found in the fruit of the genus *Capsicum*. RTX is an ultrapotent capsaicin analog derived from the plant *Euphorbia resinifera*. They have been collectively named as vanilloids because both of them have vanillyl group that is essential for their activity. The rest of the molecule is different. Capsaicin is used topically because of its analgesic response due to desensitizing of the sensory neurons (De Lourdes Reyes-Escogido et al., 2011; Kissin, 2008)

2.4.1.3 Neuropathy caused by RTX

Intraperitoneal administration of RTX leads to SFN in mouse. The study by Danigo et al. (2014b) demonstrates that it is characterised by functional sensory nerve impairment without overt nerve degeneration however with large depletion of substance P but only with mild CGRP depletion,. It was also shown that significant thermal and mechanical hypoalgesia was induced seven days after RTX administration. Depletion of neuropeptides, blockade of the axonal transport of macromolecules, depolarization block that prevents action potential generation and other effects mediated by TRPV1 can be responsible for vanilloid-induced inactivation of sensory neurons (Kissin, 2008).

2.5 Neurogenic inflammation

Cutaneous neurogenic inflammation is inflammation of the skin involving the release of neuropeptides from sensory nerve endings (Gouin et al., 2017). The nervous and the immune systems are extensively interconnected anatomically and through different mediators (Gaudillere et al., 1996; Planells-Cases et al., 2005). The ability of the PNS to communicate directly with the immune system ensures the formation of an integrated protective mechanism. The dense innervation network of sensory and autonomic fibers in peripheral tissue allows high speed of neural signal transduction with systemic modulation of immunity. The PNS plays an important role in the innate and adaptive immunity (Chiu et al., 2013).

The pro-inflammatory response on a target tissue can be explained by the axon-reflex model of neurogenic inflammation. This model proposes that tissue damaged by chemical, thermal or mechanical way, elicits by sensory nerves an immediate orthodromic signal which is conveyed from the tissue to the dorsal root ganglia and to the CNS in order to allow quick response to the injury. The sensory nerves are able to generate a second impulse in the reverse direction (back to the tissue) which can be called the "antidromic response". This antidromic response induces release of neuropeptides into the damaged tissue where they can interact with potential target cells located near the activated nerve fibers, Ansel et al., (1997). Afferent nerves also possess specific receptors for neuropeptides, prostaglandins, histamine, proteases, vanilloids and cytokines (Zegarska et al., 2006a).

Activation of receptors on sensory nerve endings by various exogenous stimuli like mechanical skin injuries, exposure of skin to heat and cold, ultraviolet and chemical irritants leads to the release of neuropeptides, substance P and CGRP, that are involved in neurogenic inflammation. Except for the exogenous factors, also the endogenous stimuli, such as protons (pH changes), hormones, cytokines, proteinases, kinins, and other mediators can initiate neurogenic inflammation (Richardson and Vasko, 2002). These neuropeptides are located in a subset of small dorsal root ganglion neurons and have autocrine action on the nociceptor terminals and paracrine action on peripheral target cells such as mast cells, immune cells and vascular smooth muscle cells. All these latter cells can be involved in tissue inflammation (Planells-Cases et al., 2005; Richardson and Vasko, 2002). However, nociceptors do not release only substance P and CGRP, but also many other neuropeptides such as neurokinins A and B, neuropeptide Y , adrenomedullin, the vasoactive intestinal peptide (VIP), as well as other mediators like glutamate, nitric oxide and cytokines (Chiu et al., 2013). However, substance P and CGRP are

considered as major initiators of neurogenic inflammation (Richardson and Vasko, 2002). Neuropeptides can be also released by skin cells such as keratinocytes, microvascular endothelial cells, Merkel cells, Langerhans cells, leukocytes and mast cells (Zegarska et al., 2006b).

The neuropeptide activity in the inflammation of skin can result in erythema, oedema, hyperthermia and pruritus. Local erythema is a result of the axon reflex. The antidromic sensory nerve stimulation induces release of factors implicated in the regulation of vessels (substance P, histamine, purines and CGRP) (Zegarska et al., 2006b). The release of mediators from sensory neurons in the periphery does not solely act on the level of vasculature, but it also leads to the activation of the innate cell (such as mast cells, dendritic cells) and adaptive immune cells (T lymphocytes) (Chiu et al., 2013). Substance P can mediate the release of histamine from mast cells of the skin (Zegarska et al., 2006b). Both substance P and histamine can have a dual mediator role. On one side, the action potential generated by activation of sensory nerve endings leads to the release of substance P. On the other hand, vasodilatation as a consequence of releasing substance P induces the release of histamine from the adjacent mast cells and then activates other sensory nerve endings, leading to the amplification of the neurogenic inflammation (Rosa and Fantozzi, 2013).

2.5.1 Neuropeptides

Neuropeptides are a group of small peptides composed of 4 to more than 40 amino acids (Steinhoff et al., 2003). They belong to the large family of extracellular signalling molecules, whose function ranges from neurotransmitters to hormones. The mostly studied and hence known neuropeptides in the skin are tachykinins; substance P, neurokinin-A and CGRP (Fig.6). Substance P and CGRP contribute to the transmission of nociceptive signal to the spinal cord. In the presence of a nociceptive stimulus, these neuropeptides are also released into the peripheral tissues through the free nerve endings of the TRPV1 expressing fibres and participate in neurogenic inflammation.

2.5.1.1 Substance P

Substance P has been shown to localize in blood vessels, hair follicules or mast cells (Reilly et al., 1997). Substance P consists of 11 amino acids and is a member of tachykinin peptide hormone family, which also contains other neuropeptides as neurokinin A, neuropeptide K and neuropeptide Y. The most known roles of substance P are nociception and neurogenic inflammation and both are mediated by the specific receptors (Mashaghi et al., 2016).

Neurokinin receptors (NKR) are of three canonical types, labelled as NK1R, NK2R and NK3R and are often co-expressed by the same cells. NK1R is a G-protein coupled receptor and have the highest affinity for substance P (Mashaghi et al., 2016).

Substance P participate in inflammatory effects via direct or indirect way. Directly, substance P affects smooth muscle contraction, modifies endothelial permeability and causes vasodilatation (Stanisz, 2001). Indirectly, substance P stimulates the amplification of the inflammatory response by inducing the degranulation of mast cells and releasing tumor necrosis factor-α (TNF-α) and histamine, inducing synthesis and release of interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β) by keratinocytes. It affects the production of TNF-α, IL-8, IL-2 and IL-6 by leukocytes as well (Ansel et al., 1993; Delgado et al., 2003; Wei et al., 2012).

2.5.1.2 Calcitonin gene-related peptide (CGRP)

CGRP is ubiquitously expressed in the nervous system with predominant presence in peptidergic fibres Aδ and C, where it is co-located with substance P (Maggi CA, 1995). CGRP is a peptide of 37 amino acids that belongs to the calcitonin family along with calcitonin, adrenomedullin and amylin. CGRP is stored in the vesicles within the sensory nerve terminal. Following neuronal depolarization, CGRP is released from the terminal via calcium-dependent exocytosis mediated by classical exocytotic pathways (Russell et al., 2014). The CGRP receptor is a G protein-coupled receptor that consists of the assembly of a 7-transmembrane domain known as calcitonin receptor-like receptor (CLR) and an associated single transmembrane domain protein called receptor activity-modifying protein 1 (RAMP1), that is required for full functionality and for transportation of CLRs from the endoplasmic reticulum to the plasma membrane (Benarroch, 2011). The CGRP receptor is also expressed in non-neuronal cells, such as endothelial cells, vascular smooth muscle cells, keratinocytes, Langerhans cells, macrophages and mast cells (Albertin et al., 2003; Hagner et al., 2002). The CGRP is the most powerful known vasodilator involved in the pathophysiology of inflammatory and neuropathic pain. In addition to its vascular effects, CGRP modulates immunity and stimulates cell proliferation (Peters et al., 2006). CGRP stimulates mast cells and keratinocytes to secrete TNFα and IL-1α, respectively (Niizeki et al., 1997).

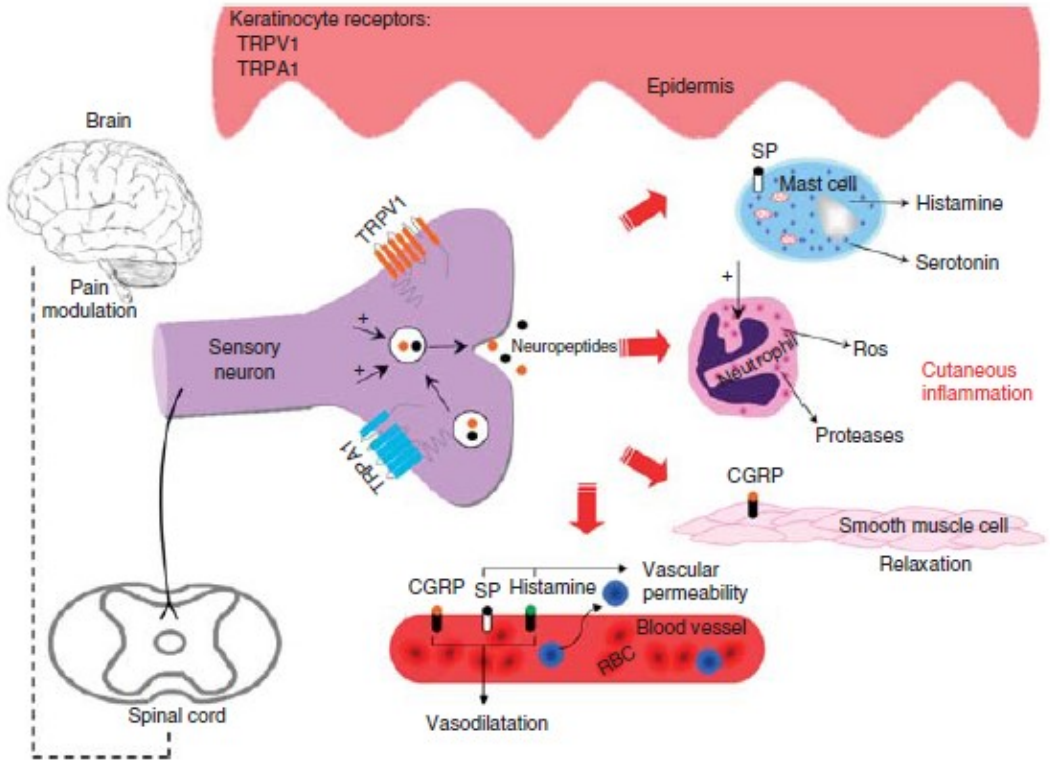


Fig. 6. Role of TRPV1, substance P and CGRP in neurogenic inflammation. *The activation of TRPV1 leads to opening of the channel and an increase in intracellular calcium concentration that mediates the release of substance P and CGRP. Substance P can evoke an increase vascular permeability and CGRP vasodilation, leading to oedema and erythema as manifestations of inflammation. CGRP and substance P also activate several immune cells, for example, mast cells that release histamine that can sensitize TRPV1, responsible for amplification of the inflammation process. The figure was taken from Aubdool and Brain, 2011*.

2.6 Cutaneous inflammatory markers

The wound healing process is classically described as a coordinated succession of cellular and biochemical events that can be classified in three time-dependent phases: the inflammatory phase, the proliferative phase (extracellular matrix deposition, angiogenesis, reepithelialisation) and the regeneration phase (remodelling of the scar in a functional tissue). The first stage of wound healing is immediately initiated after injury, with vascular constriction. Blood platelets begin to stick to the injured site and this activates release of chemical signals to promote clotting. This results in the activation of fibrin, which forms a mesh that is incorporated into and around the platelet plug. The clot and surrounding wound tissue release growth factors, hormones and cytokines contributing to the recruitment of leukocytes and the initiation of the inflammatory response (Gale, 2011; Guo and DiPietro, 2010).

Neutrophils quickly migrate to the wound followed 24 hours after by monocytes and lymphocytes (). These additional immune cells secrete growth factors and cytokines attracting more cells implicated in wound healing and stimulating their proliferation and survival. Immune cells also secrete antibacterial products (reactive oxygen species, proteinases). Cells with phagocytic activities such as macrophages help to prevent infection. Platelets also contribute to the recruitment of fibroblasts and endothelial cells via the release of chemokines (Steed, 1997; Gawaz and Vogel, 2013). Mast cells are also involved in the inflammatory phase via the secretion of histamine and pro-inflammatory mediators evoking vascular permeability and cells recruitment (Wulff and Wilgus, 2013).

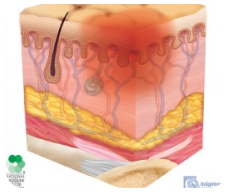
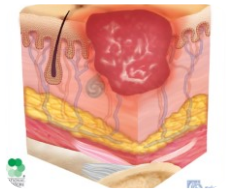
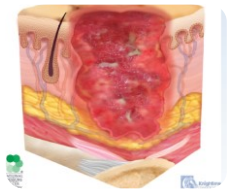
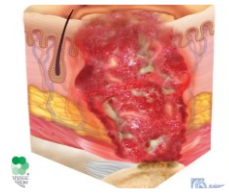
In addition to their role in pain transduction, sensory nerves are also important players in the wound healing process. It has been shown that sensory neurons can produce and release neuropeptides involved in the inflammatory (neurogenic inflammation) and proliferative phases, which allow cross-talks with different cell populations participating in tissue regeneration.

Cytokines are soluble glycoproteins of low molecular weight produced by different cell types in all organs. Cytokines form a heterogeneous group of receptor agonists that together with cellular and matrix bound molecules, contribute to a complex cellular signalling network. They are classified as interleukins (ILs), colony-stimulating factors (CSFs), interferons (IFNs), and tumor necrosis factors (TNFs) (Sprague and Khalil, 2010).

2.7 Pressure ulcers (PUs)

Pressure sores (decubitus or ulcers) can be defined according to NPUAP (The Nation Pressure Ulcer Advisory Panel) as localized damage to the skin and underlying soft tissue over a bony prominence. Injury can be present as intact skin or open ulcer and can cause pain. The damage occurs as a result of intense and/or prolonged pressure or pressure in combination with shear. The classification of the pressure ulcers stages is shown in Tab.3. The detailed mechanism of pressure sores remains largely unknown, but local ischemia and prolonged pressure can be considered as the primary etiological factors of PUs (Liao et al., 2013). Pressure ulcers are commonly associated with inflammatory reaction, and frequently with local bacterial colonization or systemic infection. Exudation from large areas of injured skin leads to fluid and protein loss. Their severity is classified according to the depth of extension (Anders et al., 2010).

Table 3. Classification of pressure ulcers by NPUAP

	Stage 1 : Non- blanchable erythema of intact skin Intact skin with a localized area of non-blanchable erythrema. Presence of blanchable erythema or changes in sensation can lead to visual changes. No purple or maroon discoloration is present.
	Stage 2 : Partial-thickness skin loss with ecposed dermis The wound bed is viable, pink or red, moist, and may present as an intact or ruptured serum-filled blister. Adipose and deeper tissue are not visible.
	Stage 3 : Full-thickness skin loss Adipose tissue is visible and granulation tissue is presented. Slough and/or eschar may be visible. The depth of tissue injury varies by anatomical location.
	Stage 4 : Full-thickness skin and tissue loss Tissue loss with exposed muscle, tendon, ligament or bone in the ulcer. Slough and/or eschar may be visible.

The table was adapted from Edsberg et al., 2016.

3 Aim of the study

This study aimed at demonstration of the modulation of genes involved in SFN by transcriptomic analyses. Two mice models of mice were selected: mice with PUs and without PUs.

The main goal of the study was:

- to explore which genes are involved in changes of cutaneous inflammatory state during ischemic condition or in the context of SFN by using transcriptomic analysis

Second goal was:

- to confirm the results of the transcriptomic analysis by qPCR

4 Materials and Methods

4.1 Animal experiment

The study was carried out according to the guidelines for ethical care of experimental animals of the European Community and was approved by the French Agriculture Ministry (authorization n°87-019). The protocol was approved by the Ethics Committee of Animal Experiments of Limousin (Comité Régional d'Ethique pour l'Expérimentation Animale, CREEAL. Permission numbers: 1-2013-1 and 2-2013-2). According to the experiments, animals were anesthetized by isoflurane inhalation and euthanatized by cervical dislocation.

4.1.1 Animal and RTX treatment

Experiments were performed on young male Swiss mice (20-25 g). The mice were randomly assigned to four weight-matched groups of 4 mice per group: control-US (uninjured skin), RTX-US, control-PU (pressure ulcer) and RTX-PU. Animals were housed in plastic cages and maintained on a 12 h light/dark cycle with food and water *ad libitum*. The animals were allowed to adapt to this environment for a period of 7 days before the experiments. SFN was induced by a single injection of RTX (50 µg/kg, i.p. Sigma-Aldrich, Lyon, France) and the control mice received a vehicle (10% DMSO, i.p., 1 ml / kg).

4.1.2 Protocol A: Pressure Ulcer (PU) model

PU was created on the dorsal skin. Six days after intraperitoneal injection of the vehicle or RTX, the dorsal hair was shaved and the remaining hair were removed by use of depilatory lotion. After 24 hours, the skin was gently pulled up and placed between two round ceramic magnetic plates (10 mm diameter and 1 mm thick, with an average weight of 0,5 g and 10 000 Gauss magnetic force). This process created a compressive pressure of approximately 2000 mmHg between the two magnets. Epidermis, dermis, and subcutaneous tissue layer including panniculus carnosum muscle were pinched with the magnetic plates for 12 hours that induces PUs (stage ≥ 2) in healthy mice. Twenty-four hours after removal of the magnets, the animals were euthanized by cervical dislocation and the ulcers were removed and preserved in an RNA stabilization reagent called RNAlater. The procedure is summarized in Fig. 7.

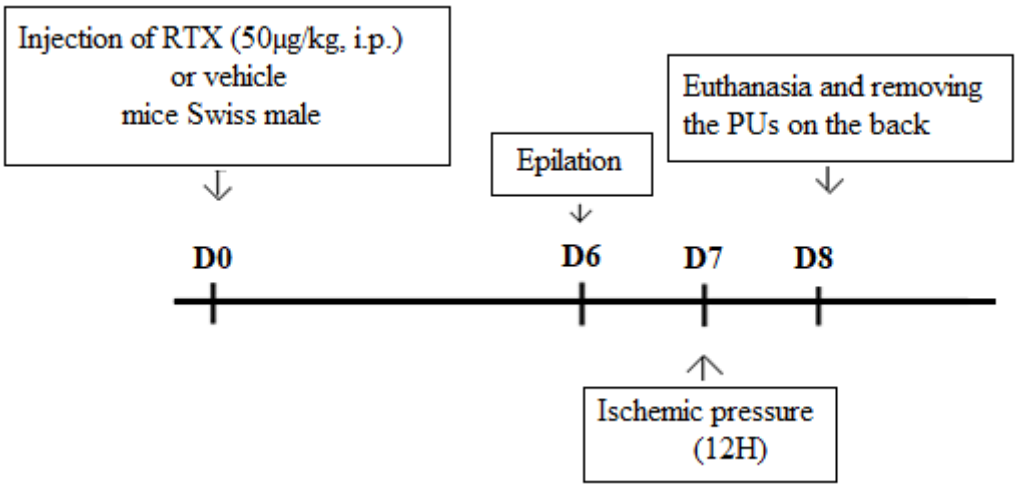


Fig. 7. *Chronology of the Protocol A-Pressure Ulcer model. D: day, 12H: 12 hours*

4.1.3 Protocol B: Uninjured model of mouse

Six days after injection, the dorsal hair was shaved and the remaining hair were removed by use of depilatory lotion. After 24 hours, mice were euthanized by cervical dislocation and the back skin was removed and preserved in RNAlater. The procedure is summarized in Fig. 8.

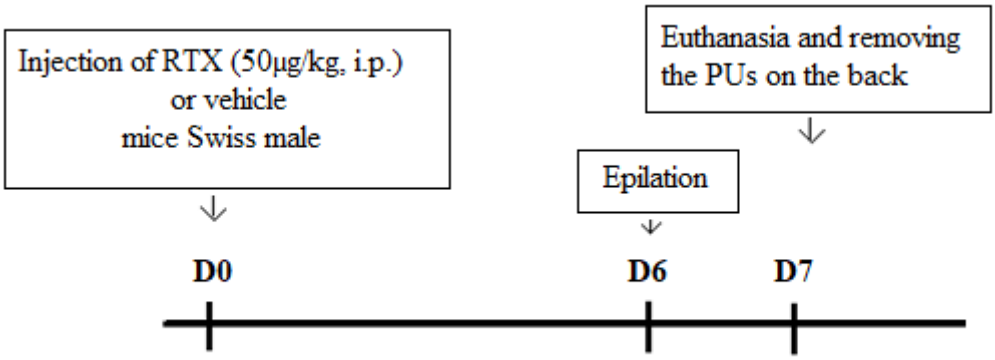


Fig. 8. *Chronology of the Protocol B-Uninjured model*

4.2 Molecular biology

Isolation of RNA was carried out on tissues collected from the skin on the back. This RNA was divided into two parts:

- One part was used for transcriptomic analysis. The quality and quantity of RNA was evaluated before this analysis.
- Second part was transformed into cDNA and was used to validate the results obtained on microarray RNA by PCRq.

All steps are summarized in Fig.9

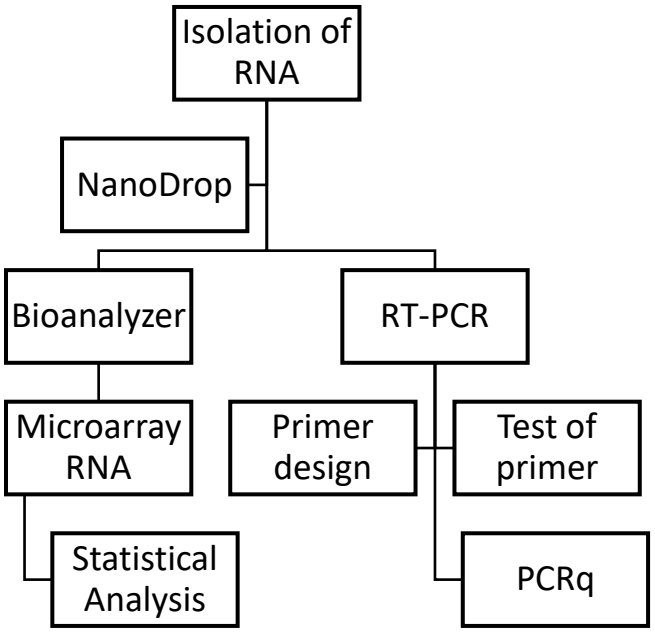


Fig. 9. *This diagram illustrates the various steps of the methods used to reach the results.*

4.2.1 Isolation of RNA

The skin and the subcutaneous tissue were dissected into small pieces, and placed in RNAlater (Qiagen, Hilden, Germany) before proceeding to RNA isolation. RNA isolation was performed using TRIzol Reagent (Qiagen, Hilden, Germany) followed by purification using the RNeasy mini kit (Qiagen, Hilden, Germany). This kit is based on the principle of phenol/chloroform extraction. The purity of RNA was checked with Nanodrop (Thermo Fisher Scientific). Each sample of RNA was stored in a refrigerator at -80°C.

4.2.2 RNA quantification and RNA integrity control

Total RNA concentration was quantified with the NanoDrop (Thermo Fisher Scientific) by measuring the absorption at 260 nm. The optical density (OD) was measured at different wave lengths: 230 nm (absorption of contaminants and background absorption), 260 nm (absorption maxima of nucleic acids) and 280 nm (absorption maxima of proteins). Additionally, the OD260/230 and the OD260/280 ratio showing RNA purity were examined. Quality and quantity control of RNA was performed with the 2100 Bioanalyzer using ‘‘Eukaryote total RNA Nano Assay” (Agilent Technologies) based on the principle of capillary electrophoresis. The RNA integrity number (RIN) is used to estimate the integrity of RNA samples. RIN is the ratio of 28S rRNA to 18S rRNA areas under the peaks. It is based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. The samples used had RIN close to 7.

4.2.3 Microarray RNA

Microarray was performed according to the protocol of manufacturer (Agilent, CA, U.S.A). Double-stranded cDNA was synthesized from total RNA. An *in vitro* transcription was performed to produce cyanin 3-labeled cRNA from cDNA. Finally, cRNA was hybridized to a Sureprint G3Mouse Gene expression 8x60k Microarray (Agilent, CA, U.S.A) for 17 hours at 65°C. This microarray chip provides the entire transcribed mouse genome on a single array for 8 samples, and analyses more than 36,000 genes by a sample at one time. After hybridization, chips were washed and dried and then scanned by the Agilent SureScan Microarray Scanner. To calculate the fold change, the expression value of each gene from the RTX group was divided by the expression value from the control group.

4.2.4 Reverse transcription

RNA samples were converted to cDNA using QuantiTect Reverse Transcription kit (Qiagen). Therefore, 500 ng total RNA were diluted to a final volume of 12 µl in RNAse free water. For achieving accurate gene expression, elimination of genomic DNA (gDNA) contamination in an RNA sample is essential: gDNA Wipeout Buffer was added (2µl) to the diluted sample. Removal gDNA protocol was performed at 42°C for 5 min; maintained at 4°C. The master mix for reverse transcription was prepared as follows: 4µl of quantiscript RT buffer 5X, 1 µl of RT Primer Mix (dNTP + primer), and 1 µl of Quantiscript Reverse Transcriptase. After adding 6 µl of the master mix to the diluted sample, the plate was inserted in the 8-Strip PCR Tubes

(Axygen) and the protocol was started (42°C for, 30 min) followed by 3 min at 95°C, 3 min and maintained at 4°C.

4.2.5 Primer design

Primer pairs were designed using published genome browser - Ensembl genome browser 91 (https://www.ensembl.org/index.html). Suitable primer pairs were found by primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with following parameters:

- PCR product size 80-120;
- primer melting temperatures (Tm): minimum 61°C, optimum 62°C;
- primer size 19-21 and primer GC content 50-60%.

The uniqueness of eventual primer (forward and reverse) was checked up by UCSC Genome Browser (https://genome.ucsc.edu/) and potential dimers were checked up by Oligo Cal (http://biotools.nubic.northwestern.edu/OligoCalc.html). Primer function was verified by quantitative PCR using the Rotor-Gene SYBER Green PCR kit (Qiagen) on a Rotor Gene 6000.

4.2.6 Quantitative PCR

Inflammatory biomarkers were analysed by quantitative PCR, because it may be more reliable for quantification than microarray analysis. Total skin RNA was isolated and reverse transcribed as previously described. Quantitative PCR was performed using the Rotor-Gene SYBER Green PCR kit (Qiagen) on a Rotor Gene 6000. The master mix was prepared as follows: 12.5 µl of RotorGene SYBR Green PCR Master Mix 2X; 6,5µl of nuclease free water and 3 µl of cDNA diluted (around 100 ng). The master mix (22 µl) was added to 3 µl primers 5 µM containing as forward as reverse primers. After then, samples were incubated at 95°C for 10 minutes to activate polymerase. The amplification conditions were 40 cycles at 95°C for 15 seconds (denaturation), 62°C for 15 seconds (hybridization) and 62°C for 30 seconds (elongation). One cycle for melting curve at 72°-95 °C for 5 seconds. Each of sample was analysed two times. Data were analysed by the Rotor Gene 6000 Series software 1.7. The threshold line for quantification was determined by the software automatically.

4.3 Statistical analysis

Statistical analyses were performed by using software GeneSpring (Agilent, CA, U.S.A). The student T-test was used for comparison of results between the controls and RTX group. The minimal level of statistical significance was set to $p<0.05$

5 Results

5.1 Protocol A – PUs model

5.1.1 Macroscopic analysis of pressure ulcer

The macroscopic analysis was performed to compare the different development of pressure ulcers between control and RTX mice. The skin ulcer area percentage was calculated in the total compressed area. Progression of pressure ulcers was visible in RTX as well as in the control mice. However, a larger PU area was observed at 24 h after pressure release in RTX mice compared with the controls (Fig. 10).

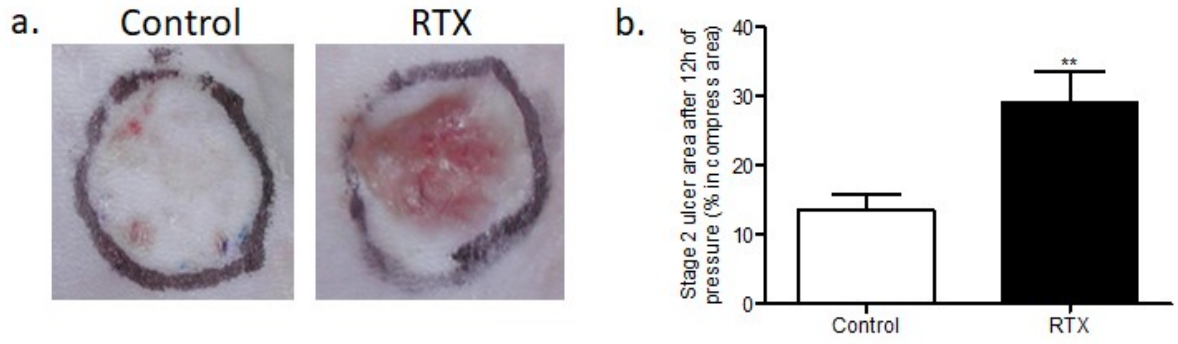


Fig. 10. Effect of resiniferatoxin (RTX) on cutaneous macroscopic findings. *The data were analysed after 24 hours from pressure release. The pressure was maintained for 12 hours. (a) Macroscopic appearance of PUs in the control and RTX mice. (b) corresponding macroscopic stage 2 ulcer areas. **p < 0.01 control vs. RTX. n = 6 in each group.*

5.1.2 Microarray RNA

The data of this chip were obtained by bioinformatician and the results obtained were analysed using the software GeneSpring. This analysis gave us a list of genes whose level of expression differed significantly between the two groups control and RTX.

5.1.2.1 Results with stat GeneSpring

a. Statistical analysis

The Student t-test was used to determinate what genes were differentially expressed between control and RTX groups. This analysis allows to extract 8731 probes for which there was a significant difference of expression between the control and RTX groups at the selected 1.5 fold change (Fig. 12). All these significant changes were later expressed as fold change (FC).

For example, in our study, if a gene has a FC = + 3, it means that this gene is three times more expressed in RTX mice than in the control mice. Similar results are shown for down-regulation.

a.

Test Description

Selected Test:	T Test unpaired
Entity List:	All Entities
Interpretation:	Treatment
p-value computation:	Asymptotic
Multiple Testing Correction:	No Correction

Result Summary

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.005	P < 0....
FC all	56745	10705	6137	3891	2391	682
FC > 1.1	48399	10699	6137	3891	2391	682
FC > 1.5	19031	8731	5242	3365	2105	616
FC > 2.0	7690	4633	2938	1919	1236	364
FC > 3.0	1993	1491	1030	694	459	146
Expecte...		2837	1134	567	283	56

b.

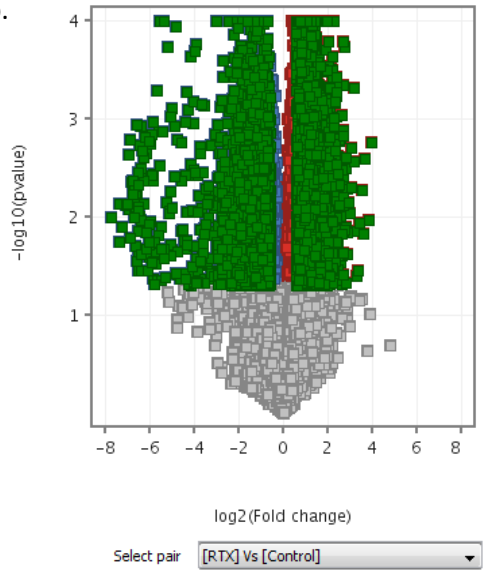


Fig. 12. Results of the t-test of Student on the first microarray RNA (Pressure ulcer model). *(a) The red rectangle highlights 8731 genes which were differentially expressed between RTX and control mice with a FC (fold change) > 1.5 and a significant p value < 0.05. (b) The Volcano plot is a graphical representation of the distribution of genes according to their difference between RTX and control groups.*

The volcano plot shows the fold change on the x-axis and the statistical significance on the y-axis. Thus, the grey points have a p value > 0.05 (they are considered non-significant and were not further analysed). Other points with p value < 0.05 were further analysed. Points at the right side of the zero x axis represent genes which are overexpressed in RTX mice compared to the control mice, and points at the left side of the zero x axis represent genes which are underexpressed.

b. Genes of interests

The 8731 genes were confronted to the literature, and several genes have been selected that seemed to be implicated in the pathophysiology of neuropathies or skin diseases. The following tables 4-6 show several significantly up- or down-expressed genes.

Interleukins

Table 4. List of Interleukin that are differentially regulated in pressure ulcers between RTX mice and control mice

p	Regulation	FC	GeneSymbol
Upregulated Interleukin			
0,018	Up	3,59	Il1f5
0,011	Up	2,95	Il1f6
0,003	Up	2,12	Il11
0,038	Up	1,74	Il17d
0,002	Up	4,22	Il20
0,034	Up	2,54	Il34
Upregulated Interleukin receptors			
0,017	Up	1,50	Il11ra1
0,005	Up	1,75	Il17rc
0,005	Up	1,63	Il17rd
0,008	Up	2,18	Il1rl1
0,003	Up	2,80	Il20ra
0,021	Up	2,05	Il20rb
0,005	Up	2,11	Il31ra
0,038	Up	3,42	Il2ra
0,038	Up	1,96	Il3ra
0,005	Up	6,17	Il5ra
Downregulated Interleukin			
0,004	Down	14,18	Il16

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

First analysis of interleukins showed that interleukins and their receptors, whom expression significantly differs between RTX and control, are almost all upregulated in RTX-PUs, suggesting that RTX-induced neuropathy could affect cutaneous inflammatory response to ischemic injury (Table 4).

Upregulation of **Ilf5** and **Ilf6** has been highlighted in the human psoriatic skin (Blumberg et al., 2007). Psoriasis is an inflammatory skin disorder, confirming relationship between small fiber neuropathy and cutaneous inflammatory dysregulation.

Il11 is the only anti-inflammatory cytokine which was differentially regulated in RTX mice compared with control mice. Il11 and its receptor Il11ra1 are overexpressed in RTX mice.

Il17 is a proinflammatory cytokine that is produced by Th cells and it plays important role in inflammatory disease (autoimmune such as rheumatoid arthritis or psoriasis) (Kuwabara et al., 2017). It has been shown that also IL17 can stimulate keratinocytes in the skin with their

activation and expression of chemokines CXCL1 and CXCL8, as well as inducing proliferation of other cells (Guilloteau et al., 2010; Kuwabara et al., 2017).

Here, PU lesion of RTX mice exhibited significant high levels of **Il20** and its receptors **Il20ra** and **Il20rb** compared to control mice. Il-20, an effector of skin inflammation, is upregulated in the wound of diabetic mice (Finley et al., 2016) and in the skin lesions of psoriasis and spongiotic dermatitis (another inflammatory skin disorder) (Wei et al., 2005). Il34 is a cytokine that promotes the proliferation, survival and differentiation of monocytes and macrophages. It promotes the release of proinflammatory chemokines, and thereby plays an important role in innate immunity and in inflammatory processes. In the skin, **Il34** is exclusively produced by keratinocytes. Il34 and its homologue CSF1 (also upregulated in RTX: $p = 0.02$, FC = 2.28) are required for the development and the maintenance of cutaneous Langerhans cells, which are dendritic cells crucially involved in the immunity in the skin.

Il16 is the only interleukin, which is downregulated in RTX mice compared with the control mice. Il16 is a chemotactic cytokine involved in the recruitment of CD4+ cells (macrophages, monocytes). One hypothesis suggested that IL-16 potentiates inflammatory immune responses. Here, Il16 mRNAs are 14-fold less expressed in RTX mice than in control mice, suggesting that immune response of RTX skin to injury is inadequate.

Chemokines

Table 5. List of chemokines that are differentially regulated in pressure ulcers between RTX mice and control mice

p	Regulation	FC	GeneSymbol
Upregulated chemokines			
0,038	Up	4,04	Ccl1
0,003	Up	12,82	Ccl17
0,029	Up	3,16	Ccl19
0,010	Up	3,07	Ccl24
0,018	Up	2,43	Ccl27a
0,038	Up	2,47	Ccl5
0,008	Up	5,78	Cxcl10
0,005	Up	8,34	Cxcl9
0,013	Up	3,59	Ccl8
0,006	Up	4,49	Xcl1
Upregulated chemokine receptors			
0,012	Up	2,04	Ccr10
0,034	Up	3,11	Ccr3
0,046	Up	1,93	Ccr4
0,029	Up	1,94	Ccr6
0,036	Up	1,67	Ccr9
0,032	Up	1,88	Cxcr3
0,047	Up	1,76	Xcr1
Downregulated chemokines			
0,041	Down	1,89	Ccl25
0,016	Down	2,10	Cxcl12
0,018	Down	1,98	Cxcl14
Downregulated chemokine receptors			
0,001	Down	31,86	Ccr5

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

Chemokines are mainly overexpressed in the pressure ulcer of RTX mice compared with PU of control mice (Table 5).

Ccr5 is chemokine receptor involved in wound healing. It is needed for the recruitment of endothelial progenitor cells (Ishida et al., 2012). This process is necessary for the neovascularization stage, the crucial phase of the normal wound healing. In our case, downregulation of Ccr5 (FC = 31.86) in RTX mice could be associated with a delay of the pressure ulcer closure.

CD (Cluster of differentiation)

Table 6. List of CDs that are differentially regulated in pressure-ulcers between RTX mice and control mice

p	Regulation	FC	GeneSymbol
Upregulated CDs			
0,031	Up	2,06	CD19
0,005	Up	2,19	CD2
0,021	Up	2,73	CD207
0,017	Up	2,38	CD209a
0,004	Up	4,02	CD209d
0,006	Up	4,33	CD209e
0,012	Up	1,87	CD22
0,030	Up	2,50	CD247
0,010	Up	2,44	CD274
0,031	Up	1,61	CD28
0,030	Up	2,16	CD300a
0,007	Up	1,76	CD320
0,023	Up	2,50	CD34
0,004	Up	1,79	CD37
0,008	Up	3,26	CD3d
0,037	Up	1,85	CD40
0,009	Up	2,08	CD47
0,004	Up	1,83	CD5
0,007	Up	3,01	CD52
0,008	Up	2,36	CD6
0,007	Up	1,58	CD63
0,006	Up	2,10	CD68
0,037	Up	3,29	CD70
0,025	Up	2,05	CD74
0,024	Up	2,34	CD79b
0,018	Up	2,17	CD83
0,020	Up	2,26	CD86
0,024	Up	1,51	CD93
Downregulated CDs			
0,018	Down	2,08	CD180
0,022	Down	1,79	CD200
0,050	Down	4,62	CD4
<0,001	Down	2,43	CD44
0,011	Down	3,75	CD82

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

CDs are involved in interactions between different immune cells. These genes are mainly overexpressed in the pressure ulcer of RTX mice compared with PU of the control mice. This may testify an exaggerated inflammatory response of the skin to pressure, probably linked with the SFN (Table 6).

Other genes

Ackr2 (up, FC = 3.29, p = 0.003) is a chemokine receptor expressed by dermal lymphatic endothelial cells and keratinocytes. Ackr2 expression is elevated in many inflammatory diseases including psoriasis. Ackr2 expression helps to compartmentalize tissue inflammatory responses to insult and infection by controlling the position of inflammatory leukocytes (Lee et al., 2013; Shams et al., 2017). In PU of RTX mice, Ackr2 is overexpressed, suggesting that RTX-induced neuropathy is responsible for an inappropriate inflammatory response to ischemic injury.

Asic3 (down, FC = 19.10, p = 0.048) is a gene for acid-sensing ion channel 3, which is a cation channel stimulated by protons. Asic3 has also been demonstrated to act as a mechanosensory for local pressure. Asic3 could be a critical effector in skin against pressure ulcer (Fromy et al., 2012). Thus, in our study, RTX-induced a marked decrease of skin Asic3 expression in response to pressure, leading to larger pressure ulcer development.

5.1.3 Quantitative PCR

The validation of RNA microarray results necessitates the use of another independent mRNA quantitation technique. Thus, we compared expression scores obtained with the microarray for the genes Il1f5, Il16 and Il20 and the expression levels for the same genes measured by qPCR. Only these three interleukin genes were analysed because of difficulties to design probes for Il4 and Il11. Two other genes were included in this qPCR analyses: CD4 (a glycoprotein found on the surface of immune cells) and Il1β (one of the major proinflammatory cytokines responsible for the early inflammatory response) (Table 7).

Table 7. List of the probe sequences used for the study of the gene expression level by qPCR

Name	Sequence	Number of exons	Tm (Fusion temperature)
IL1f5-MM-201F	CCTGGGCGTTCAAGGAGGAA	5	62,12
IL1f5-MM-201R	GGTGAGGTGCAGAGGAACCA	6	61,77
IL16-MM-177F	TCCTCCAAACCCAAGCCTCC	12	62
IL16-MM-177R	GGCTCTCCCTGCAGTGTCTT	13	62
IL20-MM-187F	CTGGGCTCAAGACCCTCCAT	1	61,28
IL20-MM-187R	TGACGAAGGAAGCAGCACCT	3	61,76
IL1b-MM-223F	AACTGCACTACAGGCTCCGA	5	61
IL1b-MM-223R	GTGGGTGTGCCGTCTTTCATT	6	61
CD4-MM-134F	CAGAACTGCCCTGCGAGAGT	3	62,16
CD4-MM-134F	ACGATCAAACTGCGAAGGCG	4	61,63
Housekeeping genes			
HPRT-MM-185F	TGATCAGTCAACGGGGGACAT	4	61,18
HPRT-MM-185R	AGGTCCTTTTCACCAGCAAGC	6 and 7	61,09
TBP-MM-128F	CCACGGACAACTGCGTTGATTT	5	62,2
TBP-MM-128R	TTAGCTGGGAAGCCCAACTTCT	6 and 7	61,63
Gusb-MM-199F	CAACGAGTCACTTCGGCACC	8	61,56
Gusb-MM-199R	GGCGTTGCTCACAAAGGTCA	9	61,44

Three samples per group were for this qPCR study. To quantify gene expression, the Ct (the number of cycles at which the fluorescence exceeds the threshold) for cDNA from the gene of interest was subtracted from the Ct of cDNA from a housekeeping gene in the same sample to normalize for variation in the amount and quality of cDNA between different samples. The housekeeping genes are required for the maintenance of basic cellular function and, therefore, are expected to maintain constant expression levels in all cells and under normal and pathophysiological conditions. Three housekeeping genes HPRT (hypoxanthine phosphoribosyltransferase), TBP (TATA-box binding protein) and Gusb (glucuronidase β) were selected (Tables 7 and 8).

Table 8. Results of the qPCR study and normalization

		Normalization to Gusb					Normalization to HPRT				
		Ilf5	**Il1b**	**Il16**	**Il20**	**CD4**	**Ilf5**	**Il1b**	**Il16**	**Il20**	**CD4**
PU control	1	0.819	4.117	0.101	0.002	0.033	0.632	3.179	0.078	0.001	0.025
	2	0.535	14.010	0.170	0.005	0.037	0.187	4.896	0.059	0.002	0.013
	3	0.395	4.724	0.121	0.006	0.031	0.244	2.915	0.075	0.004	0.019
	Mean	**0.583**	**7.617**	**0.131**	**0.004**	**0.034**	**0.354**	**3.663**	**0.071**	**0.002**	**0.019**
PU RTX	1	0.715	12.611	0.145	0.003	0.027	0.452	7.963	0.091	0.002	0.017
	2	0.326	14.808	0.166	0.003	0.035	0.171	7.772	0.087	0.002	0.018
	3	0.487	24.562	0.135	0.006	0.033	0.240	12.11	0.067	0.002	0.016
	Mean	**0.509**	**17.327**	**0.149**	**0.004**	**0.032**	**0.288**	**9.282**	**0.082**	**0.002**	**0.017**
RTX/control		**0.8736**	**2.2748**	**1.1378**	**0.9231**	**0.9406**	**0.812**	**2.534**	**1.157**	**0.908**	**0.900**

Significant result

The TBP reference gene was not used in the analysis of the results because it was too variable.

Based on these results, only the Il1β gene was significantly overexpressed in PU of RTX mice compared with the control mice (Table 8). However, results from the RNA microarray indicated that this gene was not significantly overexpressed compared to the control mice (data not shown).

In addition, Il1f5, Il16 and Il20 genes that showed a difference in expression on the microarray showed no evidence of over or under expression with qPCR results.

5.2 Protocol B – uninjured model

5.2.1 Microarray RNA

The data of microarray were obtained by bioinformatician using the software GeneSpring. The statistical test used to evaluate whether the differences between RTX and control were significant was the Student´s t-test. It was found that 1947 genes have different expression with at least 1.5 fold difference at the level of significance at $p<0.05$ (Fig. 16).

5.2.1.1 Results with stat GeneSpring

a) Statistical analysis

a.

Test Description

Selected Test:	T Test unpaired
Entity List:	All Entities
Interpretation:	Treatment
p-value computation:	Asymptotic
Multiple Testing Correction:	No Correction

Result Summary

	P all	P < 0.05	P < 0.02	P < 0.01	P < 0.005	P < 0....
FC all	56745	7156	2142	967	453	103
FC > 1.1	39322	7010	2087	941	445	101
FC > 1.5	9406	1947	782	349	177	49
FC > 2.0	2904	599	269	171	94	31
FC > 3.0	519	131	65	48	31	16
Expecte...		2837	1134	567	283	56

b.

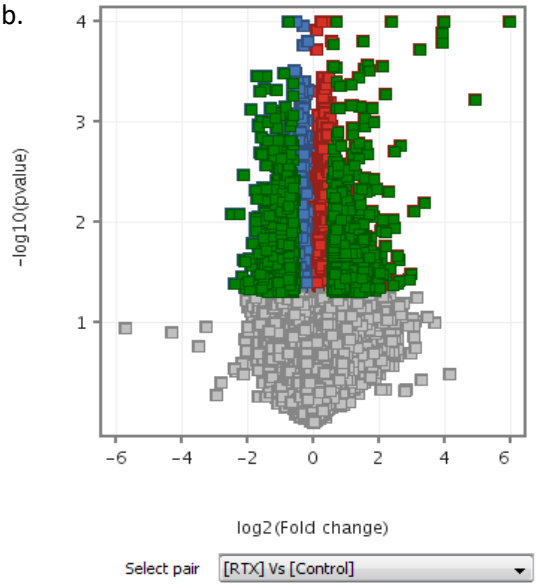


Fig. 16 Results of the t-test of Student on the second microarray RNA (Uninjured skin model). *(a) The red rectangle highlights 1947 genes which were differentially expressed between RTX and control mice with a FC (fold change) > 1.5 and a significant p value < 0.05. (b) The Volcano plot is a graphical representation of the distribution of genes according to their difference between RTX and control groups.*

The volcano plot plots the fold change on the x-axis and the statistical significance on the y-axis. Thus, the grey points that have a p value > 0.05 were not further analysed. Points at the right side of the zero x axis represent genes which are overexpressed in RTX mice compared to control mice, and points at the left of the zero x axis represent genes which are underexpressed (Fig. 16).

b) Genes of interests

The 1947 genes were confronted to the literature, and several genes seemed to be implicated in the pathophysiology of neuropathies or skin diseases. The following tables 9-12 show several differentially expressed genes.

Interleukins

Table 9. List of Interleukin that are differentially regulated in back skin between RTX mice and the control mice

p	Regulation	FC	GeneSymbol
Upregulated Interleukins			
0,017	Up	1,92	Il24
0,016	Up	1,63	Il33
0,048	Up	2,97	Il6
Downregulated Interleukins			
0,004	Down	1,51	Il15
0,017	Down	1,63	Il34

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

While the back skin was uninjured in this study, some interleukins were differentially regulated between RTX and control mice (Table 9).

Il24 is a proinflammatory cytokine, of the Il10 cytokine family, involved in regulation of wound healing responses. It was demonstrated that Il24 is crucially involved in the initiation of psoriasis-like skin inflammation (Kumari et al., 2013).

Like many other cytokines, Il6 and Il33 have both pro-inflammatory and anti-inflammatory properties. Il33 is upregulated in atopic dermatitis, an inflammatory skin disorder. Il33 could attenuate pathologic inflammation and promote wound healing (DaSilva-Arnold et al., 2018). Il6 has long been regarded as a proinflammatory cytokine. Although Il6 is a potent inducer of the acute-phase protein response, it has anti-inflammatory properties as well, by downregulating the synthesis of proinflammatory cytokines such as IL-1 and TNF.

In our case, we could suppose that **Il33** and **Il6** are overexpressed in response to an abnormal inflammatory state.

Il15 is secreted by hair follicle cells, and it is important in the generation and maintenance of memory T cells in the epidermis (Adachi et al., 2015). Thus, lack of Il15 could lead to an impaired immune response in case of RTX mice.

Chemokines

Table 10. List of chemokines that are differentially regulated in back skin between RTX mice and the control mice

p	Regulation	FC	GeneSymbol
Downregulated chemokines			
0,039	Down	2,43	Ccl4
0,010	Down	2,90	Ccl5
0,020	Down	2,43	Ccr10
Downregulated chemokine receptors			
0,041	Down	1,96	Ccr2
0,014	Down	2,75	Ccr5

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

Ccl4 and **Ccl5** are two pro-inflammatory chemokines involved in macrophage recruitment and migration. Both chemokines and receptors are underexpressed in RTX mice indicating that neuropathy already influences the inflammatory state of the skin, although there is no lesion (Table 10).

CD (Cluster of differentiation)

The CD are antigens expressed at the surface of immune cells that determinate cell type and function.

Table 11. List of CDs that are differentially regulated in back skin between RTX mice and the control mice

p	Regulation	FC	GeneSymbol
Downregulated CD (Cluster of differentiation)			
0,022	Down	1,51	CD3e
0,017	Down	1,85	CD52
0,041	Down	1,66	CD6
0,040	Down	1,74	CD72
0,016	Down	1,88	CD72
0,040	Down	1,74	CD74
0,047	Down	1,82	CD8b1

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

CDs are involved in interactions between different immune cells. These genes are globally underexpressed in the normal skin of RTX mice. This may testify a dysregulation of immunity, probably associated with SFN (Table 11).

Prostaglandins

Table 12. List of prostaglandin receptors that are differentially regulated in back skin between RTX mice and the control mice

p	Regulation	FC	GeneSymbol
Downregulated prostaglandin receptors			
0,028	Down	1,66	Ptgdr
0,048	Down	1,55	Ptger3

p: level of significance between control and RTX mice; FC: fold change - is expressed in absolute values

Ptgdr and **Ptger3** are two receptors for prostaglandins expressed by keratinocytes and strongly involved in regulation of inflammation. For example, EP3 deficient mice (Ptger3 gene) have an exaggerated inflammatory response. Here, prostaglandin receptors are underexpressed in RTX mice suggesting further deregulation during the inflammatory process (Table 12).

Other genes

Cacnab1, overexpressed (FC = 2.6, $p < 0.001$): gene that codes a subunit of a calcium channel involved in pain. Mice deficient in this gene show sensitivity disorders with strong mechanical hypoalgesia. Here, RTX mice overexpress this gene and present mechanical allodynia and thermal hypoalgesia.

CamK2, overexpressed (FC = 1.56, $p = 0.018$): gene that codes for an enzyme involved in sensitization of the TRPV1 receptor.

Stx2, overexpressed (FC = 2, $p = 0.016$): gene involved in keratinocyte differentiation and epidermal barrier regulation.

5.2.2 Quantitative PCR

This method was not completed during my fellowship.

6 Discussion

Excitation of sensory neurons by vanilloids is followed by a refractory state, in which neurons do not respond or are resistant to various stimuli. These processes are generally referred as desensitization or inactivation (Kissin, 2008). As mentioned in the study by Devesa et al., (2011), TRPV1 may have a proinflammatory but also anti-inflammatory action depending on pathological conditions. Therefore, it is proposed that the contribution of TRPV1 should be analysed for each inflammatory condition separately. Our first transcriptomic analysis suggests that inflammation process is exaggerated in pressure ulcers of RTX mice, due to predominant upregulation of inflammatory molecules. We also showed that the pressure ulcer area is larger and that necrosis was more expanded in RTX mice than in control mice 24 hours after pressure release as was demonstrated earlier in the study by Danigo et al. (2014a). We suppose that depletion of the two major mediators of neurogenic inflammation, substance P and CGRP, induced by RTX, could lead to a dysregulation of the normal inflammatory response in the context of ischemia/reperfusion. Thus, the depletion of substance P and CGRP in the skin of RTX mice induced an exaggerated response to ischemic pressure, which was deleterious for the development of pressure ulcers and skin wound healing. This hypothesis was also supported by authors who showed that TRPV1 deficiency could promote the infiltration of macrophages and increase the expression of TNFα, IL-1β, and IL-6 in a model of contact allergic dermatitis (Feng et al., 2017). This is consistent with the idea that TRPV1 deficiency or ablation of the sensory fibers that express TRPV1 could promote skin inflammation in specific conditions.

Based on these first results, we hypothesize that in the uninjured skin of RTX mice, the steady state of inflammatory molecules could be dysregulated because of desensitization of sensory neurons and substance P/CGRP depletion. As unexpected, our transcriptomic analysis showed that inflammatory molecules in RTX mouse skin are globally underexpressed compared with the control group. We also observed downregulation of receptors for prostaglandins, what may be associated with lower production of prostaglandins E and D. One study showed that RTX decreased the serum levels of IL-12, INF-γ, IL-1β, TNF-α, NO, and PGE_2 in *Trichinella spiralis* infection (Muñoz-Carrillo et al., 2017). Similarly, another study showed that RTX inhibited the expression of iNOS and COX-2 in macrophages stimulated with LPS (lipopolysaccharide) and IFN-γ (interferon-γ), resulting in a decrease in PGE_2 and NO (Chen et al., 2003).

Thus, desensitization of TRPV1 in sensory nerve endings could lead to two paradoxical inflammatory response, depending on physiological or pathological condition. Therefore, the

TRPV1-expressing sensory fibers can differentially regulate skin inflammation in an etiology-specific manner.

One of the goals of this study was to confirm the results of the transcriptomic analysis and this, unfortunately, was not succeeded. The large variability between individuals in the manifestation of pressure ulcer, which is also evident from the results of qPCR is one of the limits of this study. The second qPCR to verify the results of uninjured skin has not yet been performed. Therefore, it is difficult to assess whether there was a problem in methodologies or in variability among individuals. RNA chips are a good way to get a global idea of the genes which are over- or underexpressed in a tissue, in physiological or pathological conditions, however one of the largest disadvantages of this technique is that it is not very precise and qPCR must systematically verify the results obtained by this technique. Furthermore, working with RNA is very demanding, as RNA is very fragile and is rapidly subjected to degradation. High quality of RNA is very important for other steps especially for transcriptomic analysis. It is also necessary to properly design the primers, which seems to be particularly difficult for interleukins precisely because of their small genome and a high percentage of identity between the different interleukins. Second goal was to identify molecules differentially expressed in uninjured model of mouse. Our transcriptomic analysis shows that there are some interleukins such as IL24, IL33, IL6, IL15 and IL34 that are differently expressed and which are influenced by activation of TRPV1 by RTX. Even, IL34 is differentially expressed in both of our models, model of PU and uninjured model of mice. However, we have not found any evidence to confirm or disprove our results in conjunction with the RTX effect on TRPV1.

7 Conclusion

The aim of this study was to explore which genes are involved in changes of cutaneous inflammatory state during ischemic condition or in the context of SNF. The transcriptomic analysis of pressure-induced ulcer showed that IL1f5, IL1f6, IL11, IL17, IL20 and IL34 were upregulated, whereas a marked downregulation of IL16 was noticed when compared RTX to the control group. Besides that, several CD molecules and chemokines were differentially expressed, in particular, chemokine receptor Ccr5 that was 31 times less expressed in RTX compared with the control mice. However, these data were not confirmed by quantitative PCR testing. The second RNA microarray of uninjured skin showed upregulation of IL24, IL33, IL6 as well as downregulation of IL15 and IL34. IL34 was the only gene that was differently expressed in both mouse models. The confirmation of this results by qPCR is not available yet, it is in the process of preparation.

Based on these results we suppose that (1) RTX-induced neuropathy lead to an abnormal and exaggerated inflammatory response in mouse skin in response to an ischemic pressure, (2) Inflammatory steady state of the skin back of RTX mice is dysregulated and associated with an underexpression of numerous genes involved in inflammation.

.

8 Abbreviations

CaMK II	Calmodulin dependent protein kinase II
CD	Cluster of differentiation
CGRP	Calcitonin gene related peptide
CLR	Calcitonin-receptor like receptor
CNS	Central nervous system
Ct	Number of cycle
DAG	Diacylglycerol
GUSB	Glucuronidase β
HPRT	Hypoxanthine phosphoribosyltransferase
IP3	Inositol-1,4,5-triphosphate
NKR	Neurokinin receptor
PCA	Principal analysis component
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PNS	Peripheral nervous system
PUs	Pressure ulcers
RAMP	Receptor activity modifying properties
RIN	RNA integrity number
RTX	Resiniferatoxin
SFN	Small fiber neuropathy
TBP	TATA-box binding protein
TNF	Tumor necrosis factor
TRPV1	Transient receptor potential vanilloid 1

9 References

Adachi, T., Kobayashi, T., Sugihara, E., Yamada, T., Ikuta, K., Pittaluga, S., Saya, H., Amagai, M., Nagao, K., 2015. Hair follicle-derived IL-7 and IL-15 mediate skin-resident memory T cell homeostasis and lymphoma. Nat. Med. 21, 1272–9. https://doi.org/10.1038/nm.3962

Albertin, G., Carraro, G., Parnigotto, P.P., Conconi, M.T., Ziolkowska, A., Malendowicz, L.K., Nussdorfer, G.G., 2003. Human skin keratinocytes and fibroblasts express adrenomedullin and its receptors, and adrenomedullin enhances their growth in vitro by stimulating proliferation and inhibiting apoptosis. Int. J. Mol. Med. 11, 635–9.

Anders, J., Heinemann, A., Leffmann, C., Leutenegger, M., Pröfener, F., von Renteln-Kruse, W., 2010. Decubitus ulcers: pathophysiology and primary prevention. Dtsch. Arztebl. Int. 107, 371–381; quiz 382. https://doi.org/10.3238/arztebl.2010.0371

Ansel, J.C., Armstrong, C.A., Song, I., Quinlan, K.L., Olerud, J.E., Wright Caughman, S., Bunnett, N.W., 1997. Interactions of the skin and nervous system. J. Investig. Dermatology Symp. Proc. 2, 23–26. https://doi.org/10.1038/jidsymp.1997.6

Ansel, J.C., Brown, J.R., Payan, D.G., Brown, M.A., 1993. Substance P selectively activates TNF-alpha gene expression in murine mast cells. J. Immunol. 150, 4478–4485.

Ashrafi, M., Baguneid, M., Bayat, A., 2016. The role of neuromediators and innervation in cutaneous wound healing. Acta Derm. Venereol. 96, 587–597. https://doi.org/10.2340/00015555-2321

Aubdool, A. a, Brain, S.D., 2011. Neurovascular aspects of skin neurogenic inflammation. J. Investig. Dermatol. Symp. Proc. 15, 33–9. https://doi.org/10.1038/jidsymp.2011.8

Benarroch, E.E., 2011. CGRP: Sensory neuropeptide with multiple neurologic implications. Neurology 77, 281–287. https://doi.org/10.1212/WNL.0b013e31822550e2

Blumberg, H., Dinh, H., Trueblood, E.S., Pretorius, J., Kugler, D., Weng, N., Kanaly, S.T., Towne, J.E., Willis, C.R., Kuechle, M.K., Sims, J.E., Peschon, J.J., 2007. Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. J. Exp. Med. 204, 2603–2614. https://doi.org/10.1084/jem.20070157

Brain, S.D., 1997. Sensory neuropeptides: Their role in inflammation and wound healing. Immunopharmacology 37, 133–152. https://doi.org/10.1016/S0162-3109(97)00055-6

Chen, C.-W., Lee, S.T., Wu, W.T., Fu, W.-M., Ho, F.-M., Lin, W.W., 2003. Signal transduction for inhibition of inducible nitric oxide synthase and cyclooxygenase-2 induction by capsaicin and related analogs in macrophages. Br. J. Pharmacol. 140, 1077–87. https://doi.org/10.1038/sj.bjp.0705533

Chiu, I.M., von Hehn, C.A., Woolf, C.J., 2013. Neurogenic Inflammation – The Peripheral Nervous System's Role in Host Defense and Immunopathology. Nat. Neurosci. 15, 1063–1067. https://doi.org/10.1038/nn.3144.Neurogenic

Cooper, S., 2002. The Biology of the Skin, in: Jrsm. Parthenon Pub. Group, pp. 109–109. https://doi.org/10.1258/jrsm.95.2.109

Coutaux, A., Adam, F., Willer, J.C., Le Bars, D., 2005. Hyperalgesia and allodynia: Peripheral mechanisms. Jt. Bone Spine 72, 359–371. https://doi.org/10.1016/j.jbspin.2004.01.010

Danigo, A., Magy, L., Richard, L., Desmoulière, A., Bourthoumieu, S., Funalot, B., Demiot, C., 2014a. Neuroprotective effect of erythropoietin against pressure ulcer in a mouse model of small fiber neuropathy. PLoS One 9, 1–19. https://doi.org/10.1371/journal.pone.0113454

Danigo, A., Magy, L., Richard, L., Sturtz, F., Funalot, B., Demiot, C., 2014b. A reversible functional sensory neuropathy model. Neurosci. Lett. 571, 39–44. https://doi.org/10.1016/j.neulet.2014.04.026

DaSilva-Arnold, S.C., Thyagarajan, A., Seymour, L.J., Yi, Q., Bradish, J.R., Al-Hassani, M., Zhou, H., Perdue, N.J., Nemeth, V., Krbanjevic, A., Serezani, A.P.M., Olson, M.R., Spandau, D.F., Travers, J.B., Kaplan, M.H., Turner, M.J., 2018. Phenotyping acute and chronic atopic dermatitis-like lesions in Stat6VT mice identifies a role for IL-33 in disease pathogenesis. Arch. Dermatol. Res. 310, 197–207. https://doi.org/10.1007/s00403-018-1807-y

De Lourdes Reyes-Escogido, M., Gonzalez-Mondragon, E.G., Vazquez-Tzompantzi, E., 2011. Chemical and pharmacological aspects of capsaicin. Molecules 16, 1253–1270. https://doi.org/10.3390/molecules16021253

Delgado, A. V., McManus, A.T., Chambers, J.P., 2003. Production of Tumor Necrosis Factor-alpha, Interleukin 1-beta, Interleukin 2, and Interleukin 6 by rat leukocyte subpopulations after exposure to Substance P. Neuropeptides 37, 355–361. https://doi.org/10.1016/j.npep.2003.09.005

Démarchez, M., 2015. Biologie de la peau [WWW Document]. URL https://biologiedelapeau.fr/spip.php?article30

Devesa, I., Planells-Cases, R., Fernández-Ballester, G., González-Ros, J.M., Ferrer-Montiel, A., Fernández-Carvajal, A., 2011. Role of the transient receptor potential vanilloid 1 in inflammation and sepsis. J. Inflamm. Res. 4, 67–81. https://doi.org/10.2147/JIR.S12978

Diaz-Franulic, I., Caceres-Molina, J., Sepulveda, R. V., Gonzalez-Nilo, F., Latorre, R., 2016. Structure Driven Pharmacology of Transient Receptor Potential Channel Vanilloid 1 (TRPV1). Mol. Pharmacol. 1, 300–308. https://doi.org/10.1124/mol.116.104430

Edsberg, L.E., Black, J.M., Goldberg, M., McNichol, L., Moore, L., Sieggreen, M., 2016. Revised National Pressure Ulcer Advisory Panel Pressure Injury Staging System. J. Wound, Ostomy Cont. Nurs. 43, 585–597. https://doi.org/10.1097/WON.0000000000000281

Feng, J., Yang, P., Mack, M.R., Dryn, D., Luo, J., Gong, X., Liu, S., Oetjen, L.K., Zholos, A. V., Mei, Z., Yin, S., Kim, B.S., Hu, H., 2017. Sensory TRP channels contribute differentially to skin inflammation and persistent itch. Nat. Commun. 8. https://doi.org/10.1038/s41467-017-01056-8

Finley, P.J., DeClue, C.E., Sell, S.A., DeBartolo, J.M., Shornick, L.P., 2016. Diabetic Wounds Exhibit Decreased Ym1 and Arginase Expression with Increased Expression of IL-17 and IL-20. Adv. Wound Care 5, 486–494. https://doi.org/10.1089/wound.2015.0676

Fromy, B., Lingueglia, E., Sigaudo-Roussel, D., Saumet, J.L., Lazdunski, M., 2012. Asic3 is a neuronal mechanosensor for pressure-induced vasodilation that protects against pressure ulcers. Nat. Med. 18, 1205–1207. https://doi.org/10.1038/nm.2844

Gale, A., 2011. Current understanding of hemostasis. Toxicol Pathol . 39, 273–280. https://doi.org/10.1177/0192623310389474.Current

Gaudillere, A., Misery, L., Souchier, C., Claudy, A., Schmitt, D., 1996. Intimate associations between PGP9.5-positive nerve fibres and Langerhans cells. Br. J. Dermatol. 135, 343–4.

Gawaz, M., Vogel, S., 2013. Platelets in tissue repair: control of apoptosis and interactions with regenerative cells. Blood 122, 2550–2554.

Gouin, O., L'Herondelle, K., Lebonvallet, N., Le Gall-Ianotto, C., Sakka, M., Buhé, V., Plée-Gautier, E., Carré, J.L., Lefeuvre, L., Misery, L., Le Garrec, R., 2017. TRPV1 and TRPA1

in cutaneous neurogenic and chronic inflammation: pro-inflammatory response induced by their activation and their sensitization. Protein Cell 8, 644–661. https://doi.org/10.1007/s13238-017-0395-5

Guilloteau, K., Paris, I., Pedretti, N., Boniface, K., Juchaux, F., Huguier, V., Guillet, G., Bernard, F.X., Lecron, J.C., Morel, F., 2010. Skin Inflammation Induced by the Synergistic Action of IL-17A, IL-22, Oncostatin M, IL-1 , and TNF- Recapitulates Some Features of Psoriasis. J. Immunol. 184, 5263–5270. https://doi.org/10.4049/jimmunol.0902464

Guo, S., DiPietro, L.A., 2010. Critical review in oral biology & medicine: Factors affecting wound healing. J. Dent. Res. 89, 219–229. https://doi.org/10.1177/0022034509359125

Hagner, S., Haberberger, R. V, Overkamp, D., Hoffmann, R., Voigt, K.H., McGregor, G.P., 2002. Expression and distribution of calcitonin receptor-like receptor in human hairy skin. Peptides 23, 109–16.

Hoitsma, E., Reulen, J.P.H., De Baets, M., Drent, M., Spaans, F., Faber, C.G., 2004. Small fiber neuropathy: A common and important clinical disorder. J. Neurol. Sci. 227, 119–130. https://doi.org/10.1016/j.jns.2004.08.012

Hovaguimian, A., Gibbons, C.H., 2012. Diagnosis and treatment of pain in small fiber neuropathy. Curr Pain headache Rep 15, 193–200. https://doi.org/10.1007/s11916-011-0181-7.Diagnosis

Ishida, Y., Kimura, A., Kuninaka, Y., Inui, M., Matsushima, K., Mukaida, N., Kondo, T., 2012. Pivotal role of the CCL5/CCR5 interaction for recruitment of endothelial progenitor cells in mouse wound healing. J. Clin. Invest. 122, 711–721. https://doi.org/10.1172/JCI43027

Kissin, I., 2008. Vanilloid-induced conduction analgesia: selective, dose-dependent, long-lasting, with a low level of potential neurotoxicity. Anesth. Analg. 107, 271–81. https://doi.org/10.1213/ane.0b013e318162cfa3

Kolarsick, P.A.J., Kolarsick, M.A., Goodwin, C., 2011. Anatomy and Physiology of the Skin. J. Dermatol. Nurses. Assoc. 3, 203–213. https://doi.org/10.1097/JDN.0b013e3182274a98

Kumari, S., Bonnet, M.C., Ulvmar, M.H., Wolk, K., Karagianni, N., Witte, E., Uthoff-Hachenberg, C., Renauld, J.-C., Kollias, G., Toftgard, R., Sabat, R., Pasparakis, M., Haase, I., 2013. Tumor Necrosis Factor Receptor Signaling in Keratinocytes Triggers Interleukin-

24-Dependent Psoriasis-like Skin Inflammation in Mice and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases. Immunity 39, 899–911. https://doi.org/10.1016/j.immuni.2013.10.009

Kuwabara, T., Ishikawa, F., Kondo, M., Kakiuchi, T., 2017. The Role of IL-17 and Related Cytokines in Inflammatory Autoimmune Diseases. Mediators Inflamm. 2017. https://doi.org/10.1155/2017/3908061

Lee, K.M., Nibbs, R.J.B., Graham, G.J., 2013. D6: the "crowd controller" at the immune gateway. Trends Immunol. 34, 7–12. https://doi.org/10.1016/j.it.2012.08.001

Liao, F., Burns, S., Jan, Y.-K., 2013. Skin blood flow dynamics and its role in pressure ulcers. J. Tissue Viability 22, 25–36. https://doi.org/10.1016/j.jtv.2013.03.001

Maggi CA, 1995. Tachykinins and calcitonin gene-related peptide (CGRP) as co- transmitters released from peripheral endings of sensory nerves. Prog. Neurobiol 45, 1–98.

Mashaghi, A., Marmalidou, A., Tehrani, M., Grace, P.M., Pothoulakis, C., Dana, R., 2016. Neuropeptide substance P and the immune response. Cell. Mol. Life Sci. 73, 4249–4264. https://doi.org/10.1007/s00018-016-2293-z

McGlone, F., Reilly, D., 2010. The cutaneous sensory system. Neurosci. Biobehav. Rev. 34, 148–159. https://doi.org/10.1016/j.neubiorev.2009.08.004

Messeguer, A., Planells-Cases, R., Ferrer-Montiel, A., 2006. Physiology and pharmacology of the vanilloid receptor. Curr. Neuropharmacol. 4, 1–15. https://doi.org/10.2174/157015906775202995

Muñoz-Carrillo, J.L., Muñoz-López, J.L., Muñoz-Escobedo, J.J., Maldonado-Tapia, C., Gutiérrez-Coronado, O., Contreras-Cordero, J.F., Moreno-García, M.A., 2017. Therapeutic Effects of Resiniferatoxin Related with Immunological Responses for Intestinal Inflammation in Trichinellosis. Korean J. Parasitol. 55, 587–599. https://doi.org/10.3347/kjp.2017.55.6.587

Myers, M.I., Peltier, A.C., Li, J., 2013. Evaluating dermal myelinated nerve fibers in skin biopsy. Muscle and Nerve 47, 1–11. https://doi.org/10.1002/mus.23510

Niizeki, H., Alard, P., Streilein, J.W., 1997. Calcitonin gene-related peptide is necessary for ultraviolet B-impaired induction of contact hypersensitivity. J. Immunol. 159, 5183–5186.

Nilius, B., Owsianik, G., 2011. The transient receptor potential family of ion channels. Genome Biol. 12, 218. https://doi.org/10.1186/gb-2011-12-3-218

NINDS, 2017. Peripheral Neuropathy Fact Sheet [WWW Document]. Natl. Inst. Neurol. Dosorders Stroke. URL https://www.ninds.nih.gov/Disorders/Patient-Caregiver-Education/Fact-Sheets/Peripheral-Neuropathy-Fact-Sheet (accessed 2.7.18).

Oaklander, A.L., Siegel, S.M., 2005. Cutaneous innervation: Form and function. J. Am. Acad. Dermatol. 53, 1027–1037. https://doi.org/10.1016/j.jaad.2005.08.049

Peters, E.M.J., Ericson, M.E., Hosoi, J., Seiffert, K., Hordinsky, M.K., Ansel, J.C., Paus, R., Scholzen, T.E., 2006. Neuropeptide control mechanisms in cutaneous biology: Physiological and clinical significance. J. Invest. Dermatol. https://doi.org/10.1038/sj.jid.5700429

Planells-Cases, R., Garcìa-Sanz, N., Morenilla-Palao, C., Ferrer-Montiel, A., 2005. Functional aspects and mechanisms of TRPV1 involvement in neurogenic inflammation that leads to thermal hyperalgesia. Pflugers Arch. Eur. J. Physiol. 451, 151–159. https://doi.org/10.1007/s00424-005-1423-5

Premkumar, L.S., 2014. Transient receptor potential channels as targets for phytochemicals. ACS Chem.Neurosci. 5, 1117–1130.

Prost-squarcioni, C., Fraitag, S., Heller, M., Boehm, N., 2008. Histologie fonctionnelle du derme. Ann. Dermatol. Venereol. 135, 5–20. https://doi.org/10.1016/S0151-9638(08)70206-0

Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.-S., McNamara, J.O., Williams, S.M., 2001a. Cutaneous and Subcutaneous Somatic Sensory Receptors.

Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.-S., McNamara, J.O., Williams, S.M., 2001b. Mechanoreceptors Specialized to Receive Tactile Information.

Reilly, D.., Ferdinando, D., Johnson, C., Shaw, C., Buchanan, K.D., Green, M.R., 1997. The epidermal nerve fibre network: characterization of nerve fibres in human skin by confocal microscopy and assessment of racial variations. Br. J. Dermatol. 137, 163–170.

Richardson, J.D., Vasko, M.R., 2002. Cellular mechanisms of neurogenic inflammation. J. Pharmacol. Exp. Ther. 302, 839–845. https://doi.org/10.1124/jpet.102.032797.characterized

Roosterman, D., Goerge, T., Schneider, S.W., Bunnett, N.W., Steinhoff, M., 2006. Neuronal Control of Skin Function: The Skin as a Neuroimmunoendocrine Organ. Physiol. Rev. 86, 1309–1379. https://doi.org/10.1152/physrev.00026.2005

Rosa, A.C., Fantozzi, R., 2013. The role of histamine in neurogenic inflammation. Br. J. Pharmacol. 170, 38–45. https://doi.org/10.1111/bph.12266

Rosenbaum, T., Simon, S.A., 2007. TRPV1 Receptors and Signal Transduction, TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades. CRC Press/Taylor & Francis.

Russell, F.A., King, R., Smillie, S.-J., Kodji, X., Brain, S.D., 2014. Calcitonin gene-related peptide: physiology and pathophysiology. Physiol. Rev. 94, 1099–142. https://doi.org/10.1152/physrev.00034.2013

Shams, K., Kurowska-Stolarska, M., Schütte, F., Burden, A.D., McKimmie, C.S., Graham, G.J., 2017. MicroRNA-146 and cell trauma downregulate expression of the psoriasis-associated atypical chemokine receptor ACKR2. J. Biol. Chem. 293, jbc.M117.809780. https://doi.org/10.1074/jbc.M117.809780

Sprague, A.H., Khalil, R.A., 2010. Inflammatory Cytokines in Vascular Dysfunction and Vascular Disease. Biochem Pharmacol. 78, 539–552. https://doi.org/10.1016/j.bcp.2009.04.029.Inflammatory

Stanisz, A.M., 2001. Neurogenic inflammation: Role of substance P. NeuroImmune Biol. 1, 373–378. https://doi.org/10.1016/S1567-7443(01)80033-8

Steed, D.L., 1997. The role of growth factors in wound healing. Surg. Clin. North Am. 77, 575–586.

Steinhoff, M., Ständer, S., Seeliger, S., Ansel, J.C., Schmelz, M., Luger, T., 2003. Modern aspects of cutaneous neurogenic inflammation. Arch. Dermatol. 139, 1479–1488. https://doi.org/10.1001/archderm.139.11.1479

Stöppler, M.C., Shiel, W.C., 2017. Neuropathy Types (Diabetic), Causes, Treatment, & Medication [WWW Document]. emedicinehealth. URL https://www.emedicinehealth.com/neuropathy/article_em.htm#what_is_neuropathy (accessed 2.7.18).

Sušánková, K., Vlachová, V., 2006. Molekulární mechanizmy modulace vaniloidního

receptoru TRPV1. Bolest 9, 236–240.

Themistocleous, A.C., Ramirez, J.D., Serra, J., Bennett, D.L.H., 2014. The clinical approach to small fiber neuropathy and painful channelopathy. Pract. Neurol. 14, 368–379. https://doi.org/10.1136/practneurol-2013-000758

Vaillancourt, P.D., Langevin, H.M., 1999. Painful peripheral neuropathies. Med. Clin. North Am. 83, 627–642. https://doi.org/10.1016/S0025-7125(05)70127-9

Wei, C.-C., Chen, W.-Y., Wang, Y.-C., Chen, P.-J., Lee, J.Y., Wong, T.-W., Chen, W.C., Wu, J., Chen, G., Chang, M.-S., Lin, Y., 2005. Detection of IL-20 and its receptors on psoriatic skin. Clin. Immunol. 117, 65–72. https://doi.org/10.1016/j.clim.2005.06.012

Wei, T., Guo, T.-Z., Li, W.-W., Hou, S., Kingery, W.S., Clark, J.D., 2012. Keratinocyte expression of inflammatory mediators plays a crucial role in substance P-induced acute and chronic pain. J Neuroinflammation 9, 181.

Weidner, C., Klede, M., Rukwied, R., Lischetzki, G., Neisius, U., Skov, P.S., Petersen, L.J., Schmelz, M., 2000. Acute effects of substance P and calcitonin gene-related peptide in human skin--a microdialysis study. J. Invest. Dermatol. 115, 1015–20. https://doi.org/10.1046/j.1523-1747.2000.00142.x

Wulff, B.C., Wilgus, T.A., 2013. Mast cell activity in the healing wound: more than meets the eye? Exp. Dermatol. 22, 507–510. https://doi.org/10.1111/exd.12169

Wysocki, A.B., 1999. Skin anatomy, physiology, and pathophysiology. Nurs. Clin. North Am.

Zegarska, B., Lelińska, A., Tyrakowski, T., 2006a. Clinical and experimental aspects of cutaneous neurogenic inflammation. Pharmacol. Rep. 58, 13–21.

Zegarska, B., Lelińska, A., Tyrakowski, T., 2006b. Clinical and experimental aspects of cutaneous neurogenic inflammation. Pharmacol. Rep. 58, 13–21.

10 Annexes

10.1.1 Bioanalyzer

It is the standard method utilized for qualitative and quantitative analysis of RNA samples prior to analysis on microarray system. Data is translated into gel-like images (bands) and electropherograms (peaks) (Fig. Annex I and II). With the help of a ladder that contains components of known sizes, a standard curve of migration time versus fragments size is plotted. From the migration times measured for each fragment in the sample, the size is calculated. For RNA only one marker is run with each of the samples bracketing the overall sizing range. The marker is internal standards used to align the ladder data with data from the sample wells. This is necessary to compensate for drift effects that may occur during the course of a chip run.

For the microarray assay, high quality of RNA is especially important. Thus, twelve samples (six of control and six of RTX group) were analysed by a bioanalyzer.

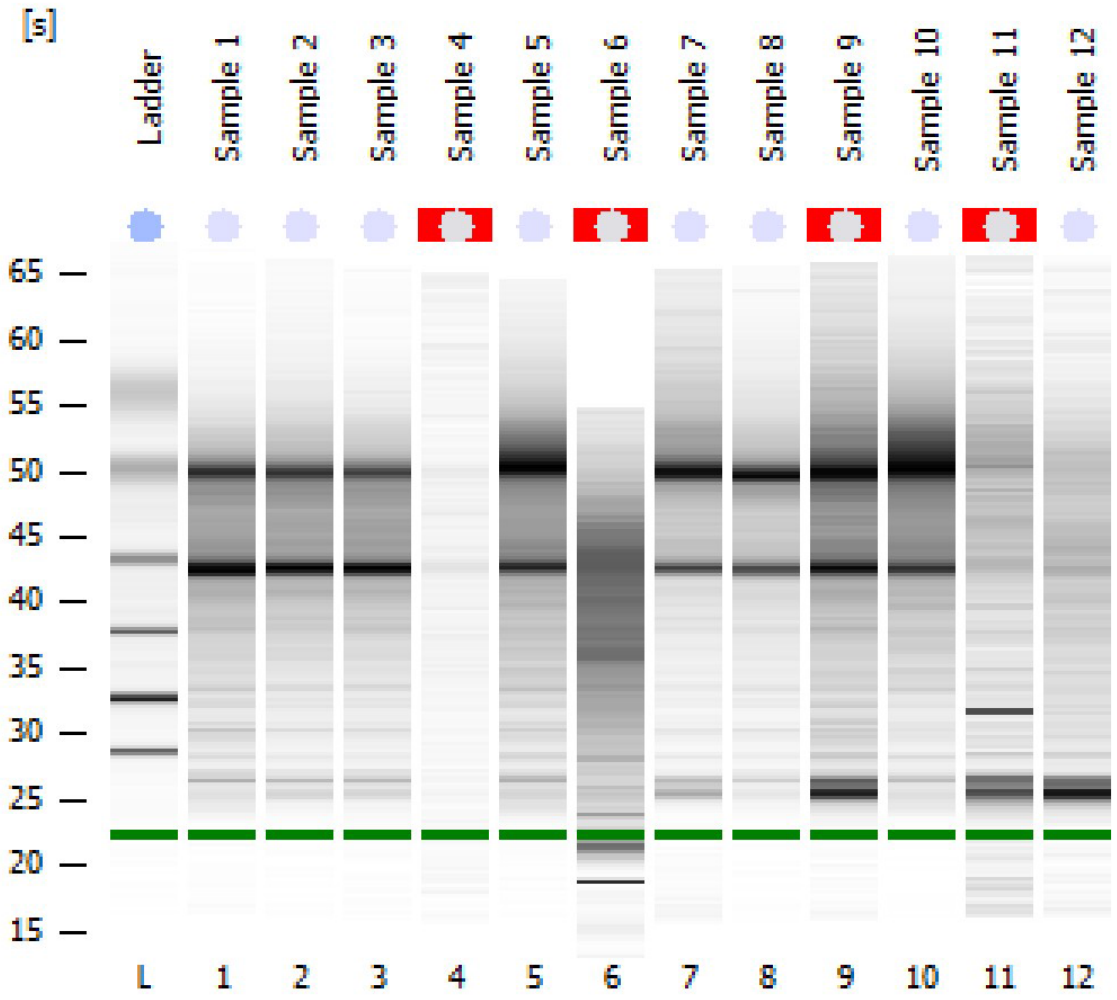


Fig. Annex I. Gel-like images. *Comparison of the samples from the ladder. It is obvious that four of them were degraded (samples 4, 6, 9 and 11).*

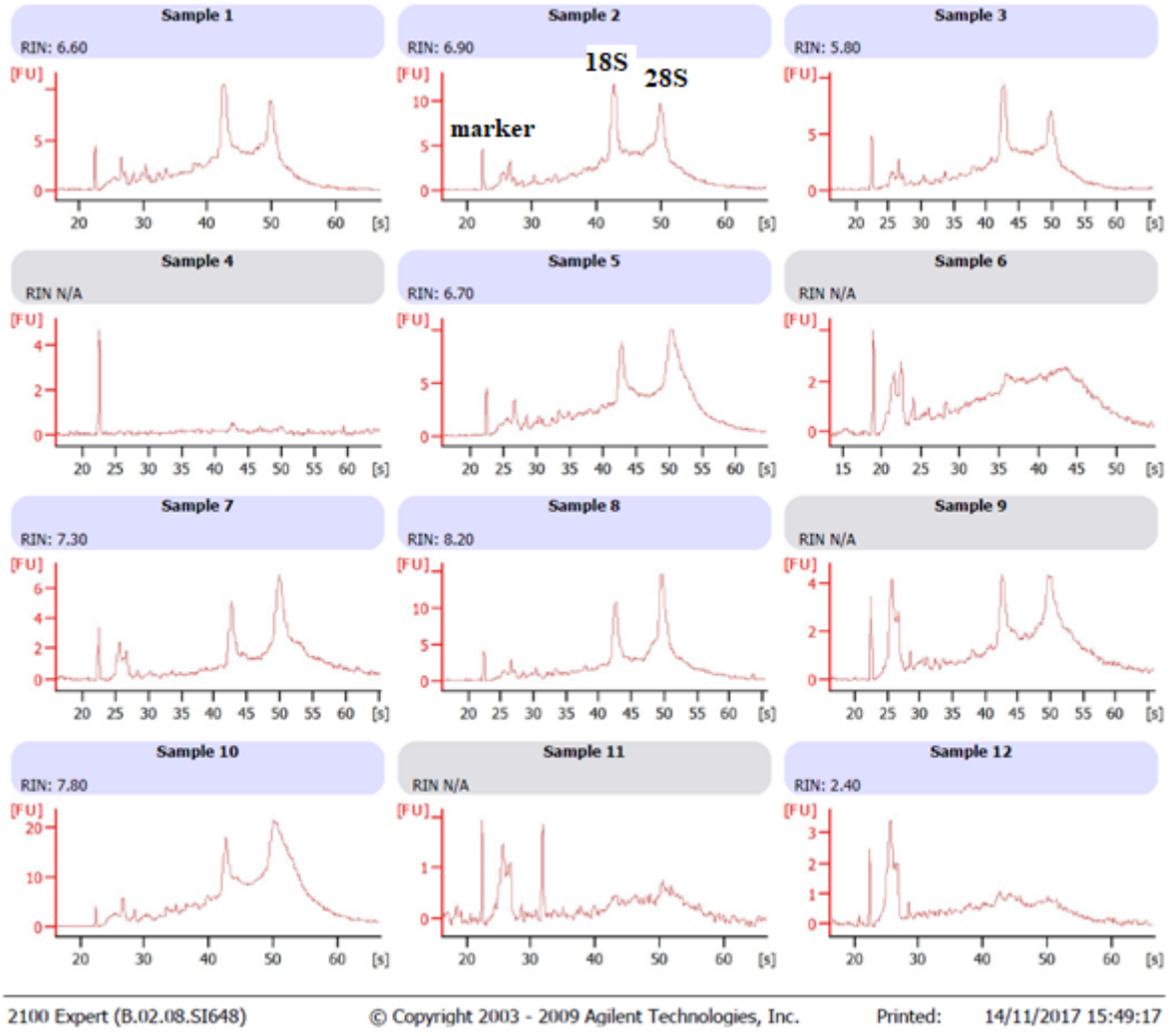


Fig. Annex II. Electropherogram. *The 2100 expert software plots fluorescence intensity versus size/migration time and produces an electropherogram for each sample. RIN is the ratio of the 28S to 18S ribosomal RNA peaks. 28S RNA degrades faster than 18S, thus if 28S RNA is degraded, RIN decreases. It is assumed that ribosomal RNA are less fragile than messenger RNA, and therefore the RIN is representative of total RNA.*