INHIBITION OF TRPV1 CATION CHANNEL FUNCTION BY RNA INTERFERENCE AND LIPID RAFT DISRUPTION

PhD thesis

Dániel Márton Tóth

Neuropharmacology Program
Program director: Erika Pintér MD, PhD, DSc
Supervisor: Zoltán Sándor MD, PhD

DEPARTMENT OF PHARMACOLOGY AND PHARMACOTHERAPY
FACULTY OF MEDICINE
UNIVERSITY OF PÉCS

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A BRIEF OVERVIEW OF THE TARGET TRPV1 RECEPTOR

Capsaicin-sensitive neurons and their triple function

Nociceptors are a group of primary sensory neurons. Their role is the transduction of potentially harmful stimuli in the periphery. The largest subset of nociceptors is polymodal nociceptors which can respond to all three causes of pain (e.g. painful mechanical stimuli, noxious heat and acid or other chemical damaging agents) [1]. It has been found that these polymodal nociceptors are capsaicin sensitive as well [2;3].

The capsaicin-sensitive polymodal nociceptors have triple function. The first is the classical **afferent function**, namely perception of painful stimulus and transmission of this nociceptive information into the central nervous system (CNS). Besides nociception, capsaicin treatment evokes exocytosis of several neuropeptides from the peripheral terminals in the innervated area causing arteriolar vasodilatation, plasma protein extravasation and accumulation of inflammatory cells [4;5]. This phenomenon is called neurogenic inflammation [6] and it is the **local efferent function** of capsaicin-sensitive neurons. But somatostatin is released also from these peripheral nerve terminals which enters the circulation and exerts systemic anti-inflammatory and antinociceptive actions, labeled as **systemic efferent function** of capsaicin-sensitive neurons [7a,b].

The capsaicin receptor: Transient Receptor Potential Vanilloid 1 (TRPV1)

Structure. The cDNA of TRPV1 was cloned as a ligand-gated cation channel and was termed capsaicin receptor or vanilloid receptor of type 1 [8]. Then, in consequence of its structure analogy with transient receptor potential (TRP) channels, it was classified and renamed as transient receptor potential vanilloid 1 (TRPV1) [9]. TRPV1 is a polytopic protein containing six transmembrane (TM) domains with an intracellular C-terminal and N-terminal region. Between the fifth and sixth TM domains there is a short, pore-forming hydrophobic stretch [8]. The receptor has a tetrameric structure in the plasma membrane where four TM5-pore loop-TM6 elements face the centre of the channel to form the gate and its selectivity filter [10].

Activators. The TRPV1 receptor is an integrative nocisensor protein because it is activated by vanilloids, noxious heat and protons [11]. Vanilloids as capsaicin and its analogues such as resiniferatoxin (RTX) are lipophilic, therefore they can pass through the cell membrane and bind to the intracellular surface of the receptor [12]. Not just vanilloids, but other endogenous substances such as *N*-oleoyldopamine (OLDA), anandamide (AEA), and lipoxygenase products [13-15] can also activate the receptor. All these agonists are highly lipophylic compounds and bind to the intracellular side of the TMs like vanilloids [16]. Furthermore, TRPV1 was identified as the first thermally gated cation channel in nociceptors which is activated by $>$43°C [11]. Finally, extracellular protons can open the channel at room temperature or potentiate its response to noxious heat and other stimuli [11;17]. Since TRPV1 has a low selectivity it can get through mono- and bivalent cations such as Na^+ and Ca^{2+} [8;18]. The former is mainly responsible for generating action potential and, consequently, for the development of nociception and the latter is principally responsible for formation of neurogenic inflammation. But after treatment with long-lasting or high concentration of capsaicin the increased influx of Ca^{2+} through the prolonged gated cation channel produces desensitization by enhancement of positive

membrane potential, which leads to a gradual inactivation of voltage-dependent Na^+ channels and antinociception [19].

Occurrence. The primary occurrence of TRPV1 receptor is the polymodal nociceptors, but its expression was demonstrated also in various non-neuronal cells such as keratinocytes, uroepithelial-, smooth muscle- and endothelial cells, as well as in cells of the immune system [20]. Its existence in CNS has also been reported in several studies using a variety of methods but experiments with knock-in mice confirmed its presence just within and adjacent to the caudal hypothalamus [21].

TRPV1 in pain relief. In the case of antagonists the gating function of TRPV1 channel is inhibited and they showed good efficacy in reversing both inflammatory and chronic neuropathic pain [27]. It was found that TRPV1 antagonists caused vasoconstriction and increased thermogenesis resulting in hyperthermia. Importantly, they did not elicit hyperthermia in *Trpv1* KO mice, suggesting that the hyperthermic effect of antagonists was TRPV1 mediated [26;28]. Since TRPV1 can be desensitized with its agonists, a conceptually different analgesic process can be achieved compared to using its antagonists. But TRPV1 agonists induce transient hypothermia which is due to a coordinated heat loss response accompanied by complete avoidance of the warm environment, as well as vasodilatation and fall in metabolic rate [22a,b-24]. This acute hypothermic effect of capsaicin and RTX does not exist in *Trpv1* knock out (KO) mice [25;26].

An other TRP channel: ankyrin repeat domain 1 (TRPA1) receptor

TRPA1 receptor is the only member of the TRPA branch of the TRP ion channel family in mammals [10;29].

TRPA1 is predominantly coexpressed with TRPV1 in polymodal nociceptors, but it is also expressed in lung fibroblasts and auditory hair cells [29]. It is insensitive to capsaicin, but can be specifically activated by other ligands such as isothiocyanate derivatives like allyl isothiocyanate (from mustard oil) or formaldehyde [30]. Furthermore, TRPA1 is considered as a noxious cold sensor which is activated and sensitized by painful cold temperatures, below 17°C [29;31]. Application of its activators causes a mild Ca^{2+}-influx into the sensory nerve terminals producing acute pain and neurogenic inflammation through peripheral release of neuropeptides [32].

I. GENERATION AND CHARACTERIZATION OF TRPV1 KNOCKDOWN TRANSGENIC MICE

I.1 INTRODUCTION

RNA interference (RNAi). RNAi is an evolutionarily conserved process of sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs (dsRNA). Several types of dsRNAs have been identified as inducers of RNAi, most importantly short interfering RNAs (siRNAs) and microRNAs (miRNAs).

siRNAs have mostly been considered extragenomic in origin and they defend the genome integrity in response against foreign or invasive nucleic acids such as viruses, transposons and transgenes [33]. siRNAs are 21-23 nt long double-stranded RNAs with 2 nt overhangs at both 3' ends, and are excised from long, fully complementary dsRNAs in the cytoplasm by Dicer. Then the double-stranded product of Dicer incorporates into RNA-induced silencing complex (RISC) where the

siRNA is unwounded [34-36] and the sense (passenger) strand, which has a higher relative stability on its 5' end, is discarded [35]. The antisense (guide) strand incorporates into the RISC and this RISC-antisense strand ribonucleoprotein complex is able to identify the complementary mRNA target by Watson-Crick base pairing then the mRNA is degraded by nuclease activity of RISC [34;36]. After cleavage the target dissociates from the RISC-complex which is able to cleave additional targets [36].
miRNAs are expressed endogenously from the host genome and they regulate a large number of endogenous genes. They are produced from endogenious RNAs containing several loops (pri-miRNA) [33] which is cleaved by Drosha producing pre-miRNA [37;38]. After all, this is exported into the cytoplasm where is cleaved by Dicer to form a mature miRNA duplex [39]. Finally, the mature miRNA assembled into the RISC where the antisense strand associates stably with the complex [38] and binds to the complementary, target mRNA with perfect or almost imperfect base pairing [36]. In the case of perfect match, target mRNA is cleaved, but in the case of imperfect match, only the translation of the target mRNA is arrested on ribosomes [40].
There are several possibilities to achieve a knock down phenotype. siRNA can be synthesized (chemically or enzymatically) then introduced into cells or animals. In this case, the exogenously delivered siRNAs can only result in transient silencing of targeted genes, due to the lack of intracellular reproduction of siRNAs [34]. Interfering RNAs can also be endogenously produced by miRNA or short hairpin RNA (shRNA). shRNAs are produced from a DNA construct consisting of the sense sequence of 21-29 nt of the target, a short loop region, the reverse complement of the target sequence and a short terminator (5-6 T residues) sequence [34]. When transcribed *in vivo* driven by Pol III promoters (such as U6 and H1), this short transcript folds back on itself to form a hairpin structure. Finally, this hairpin structure is converted into double-stranded siRNAs by Dicer and loaded to RISC complex as described previously [41].

Transgenesis with lentiviral vectors. Lentiviruses, such as Human Immunodeficiency Virus (HIV), are members of the family of retroviruses. Lentiviruses infect wide variety of dividing as well as resting and differentiated cells. After entry in the host cell, the viral RNA genome is reverse transcribed into cDNA, which is transported and stably integrated into the host genome.
In transgenesis replication-defective viruses are used, where all dispensable genes are eliminated from the viral genome. The *cis*-acting sequences can be found in the transfer plasmid which contains cloning sites for the insertion of the transgene between the virus long-terminal repeats (LTRs). LTRs are indispensable for the integration of the enclosed transgene into the host genome, but they also have roles as a promoter and an enhancer, thus unmodified LTRs can influence the transcription of the transgene. Therefore self-inactivating (SIN) lentiviral vectors were designed, where the viral promoter activity is abolished. Furthermore, the SIN design also prevents potential interferences between the viral LTRs and the internal promoter of the transgene [42]. Additionally, the *trans*-acting factors, that are absolutely required for viral particle production, infection and integration into the host genome, can be found in helper plasmids. These contain the genes of integrase, reverse transcriptase, structural proteins, and envelope glycoproteins [43].
Lifecycle of these improved replication-defective viruses is limited to a single round. Virions containing the transgene are generated from the transfer and helper plasmids

in transducer cells then the target cell is infected by the formed virus particles where the viral genome integrates into the host genome stably without generating progeny virions. In the case of the shRNA transgene, it is incorporated into the genome, transcribed, exported to cytoplasm and engaged by the endogenous RNAi machinery to drive sequence specific cleavage of the mRNA target [41].
One possible method to generate transgenic animals with lentiviral particles is the subzonal injection where the viral particles are injected into the perivitelline space of one-cell zygote [44]. It is independent on the localization or visualization of the nucleus, furthermore it does not disrupt the cytoplasmic or the nuclear membrane. An other advantage of lentiviral transduction is that only a single copy of the transgene integrates into the genome, which is more similar in structure to endogenous genes, and may therefore be less prone to epigenetic silencing (methylation, histone deacetylation) [45;46].

I.2 AIMS

The overall aim of my work was to generate TRPV1 knockdown mice and rats. Several steps were required for this:

1. testing the effectiveness and specificity of previously published anti-TRPV1 siRNAs, *in vitro*.
2. building several shRNA and miRNA constructs and validate them *in vitro*.
3. inserting the best working construct into a lentiviral plasmid system to produce RNAi lentiviruses.
4. microinjection of lentiviral vectors into one-cell embryos
5. identification of transgenic animals then breeding them to establish a transgenic line.
6. characterizing the transgenic animals in different *in vitro* and *in vivo* models.

I.3 METHODS

Experiments with siRNA oligonucleotides

Flow cytometry: ND-C and CHO cells were cotransfected with ratTRPV1eGFP fusion protein expressing vector pZS5 [47] and already published [48] anti TRPV1 siRNA (VsiR1) or VsiR1INV as control. After overnight incubation the amount of ratTRPV1eGFP fusion protein was measured by flow cytometry and the results were compared to transfected cells with pZS5 alone (100%) in percentage.

Microfluorimetry: Primary cultures of TRG neurons were prepared from Wistar rat pups [49]. TRG neurons were incubated with VsiR1 or VsiR1INV siRNA and the next day the cells were stained with 1 μM fluorescent Ca^{2+} indicator dye fura-2-acetoxymethyl ester (AM). Then calcium transients of TRG neurons were examined under a fluorescence microscope where the cells were illuminated by light at alternately 340 and 380 nm. The light emitted at >510 nm was measured and the fluorescence ratio (R=F_{340}/F_{380}) was monitored continuously. To excite TRPV1 receptors 330 nM capsaicin (diluted in ECS) was applied, and TRPA1 receptors were stimulated with 100 μM mustard oil (diluted in ECS). Cells showing a ΔR of >0.1 after agonist treatment were considered to be receptor-expressing cells and the cells reacting to agonist treatment were counted in the samples which were compared to siRNA untreated cells (100%) in percentage.

Generation of knockdown transgenic animals

Building and testing of shRNA and miRNA constructs: 14 shRNA constructs were designed. Six of them (shRNA*a*, and from shRNA*c* to shRNA*g*) has different targeting site on the rat TRPV1 mRNA. The shRNA*a* has the same targeting site as VsiR1 siRNA. Furthermore seven variants of shRNA*a* (shRNA*b*, and from shRNA*i* to shRNA*m*) were designed as well. DNA encoded sequences of shRNAs were built from four oligonucleotides then inserted into the cloning 'base' sequence of pU6Abase plasmid downstream of the human U6 promoter.

miRNA constructs were built the same way as shRNAs and the ready-made miRNAs were inserted into the pDsRedmibase vector between 5' and 3' miRNA regulatory sequences which is coding miRNA155. This whole construct is downstream of the DsRed fluorescent protein which is driven by CMV promoter.

Finally these shRNA and miRNA expression plasmids were cotransfected with ratTRPV1eGFP fusion protein expressing vector pZS5 into ND-C cells and the remained expression of ratTRPV1eGFP fusion protein was measured by flow cytometry. The results were compared to cotransfected cells with pZS5 and pU6Abase or pDsRedmibase (100%) in percentage.

Preparation of lentiviral particles: These experiments were done by Krisztián Kvell in Department of Immunology and Biotechnology, University of Pécs, Hungary.The U6 promoter and shRNA*a* construct were moved into the pWPTS-GFP plasmid at the same orientation as the EF1 driven eGFP transcription unit. Then this pLshRNA*a* lentiviral transfer vector was cotransfected with psPAX2 and pMD2g helper plasmids into HEK293T cells by $CaCl_2$ transfection method. The generated viruses were collected and filtered from the supernatant of cells. The titer of the viral solution was determined by transducing HeLa cells where the percentage of GFP expressing cells was measured by flow-cytometry. Finally, viral solution was concentrated in 20% sucrose solution by ultracentrifuge and the final biological titers of concentrated viral particles exceeded 10^8 TU/ml.

Introduction of lentiviral vectors: These experiments were done by Balázs Bender and Zsuzsa Bősze in Agricultural Biological Center, Gödöllő, Hungary.

In Sprague Dawley female rats and FVB/Nhsd female mice superovulation was induced and after fertilization the single-cell embryos were removed from oviducts. Then lentiviral particles were injected based on the modified perivitelline space injection method [50]. The tip of the injection pipette was pushed through the *zona pellucida* into the perivitelline space and gently applied some positive pressure, so that the viral concentrate was discharged slowly from the injection pipette. Finally, early-stage embryos with an intact *zona pellucida* were transferred to the oviducts of timed pseudopregnant females.

Determination and characterization of transgenic animals

Genotyping of transgenic animals: DNA was extracted from tail tissue followed by isopropanol precipitation. The existence of the transgene was detected by genomic PCR using GFP-specific (forward: 5'-CTCGTGACCACCCTGACCTAC-3', reverse: 5'-CATGATATAGACGTTGTGGCTGTT-3') and shRNA-specific (forward: 5'-GAGGGCCTATTTCCCATGAT-3', reverse: 5'-TAAAGGTACCTCGCGAATGC-3') primers.

Determination of the integration site of transgene by ligation-mediated-PCR (LM-PCR): The integration site of the transgene was determined by LM-PCR according to [51]. Genomic DNA was digested with *HaeIII* restriction endonuclease. The first PCR was done with transgene specific primer no.1 (5'-CAGGGTACCTTTAAGACCAATGAC-3'), the second PCR was done with Y-primer D and transgene specific primer no.2 (5'-TTTGCTTGTACTGGGTCTCTCTGG-3'), and the third PCR was done with Y-primer G and transgene specific primer no.3 (5'-TCAAGTAGTGTGTGCCCGTCTG-3'). The dominant fragment of LM-PCR (~500 bp) was sequenced with the no.3 and Y-primer G primers. Based on the obtained internation on flanking chromosomal DNA the integration site was determined using BLAST. Then the whole integrated provirus of about 4.5 Kb with the flanking chromosomal DNA was amplified by integration site specific primers Chr3A (5'-GCTTTAAATGCCTTCCTTGTTAAA-3') and Chr3B (5'-TAACTGAGAAGCAAGGTTTTGTTG-3'). Finally the junctions were directly sequenced with the same primers.

Quantitative PCR (qPCR): TRG neurons were isolated from 4-week-old mice and total RNA was isolated then was reverse-transcribed using oligo-dT primers. The TRPV1, TRPA1 and TRPV3 mRNAs were quantified using TaqMan assays Mm01246301_m1, Mm00625268_m1 and Mm00454996_m1, respectively. In each sample were normalized against housekeeping genes hypoxanthine phosphoribosyltransferase 1 and β2-microglobulin to get ΔC_t values.

Measurement of TRPV1 activity with microfluorimetry on TRG cells: Preparation of primary cultures of TRG neurons from mouse pups, the measurement, excitation of the receptors with capsaicin or mustard oil and the evaluation were done the same way as be described in the case of siRNA. The cells reacting to agonist treatment were counted in the samples which were compared to untreated cells in percentage.

Eye-wiping test: Capsaicin (10 μg/ml) was instilled into the eyes of mice and the number of protective eye-wiping movements with the forelegs was counted.

Nocifensive response on the paw: Capsaicin (20 μl, 100 μg/ml) or just its solvent was injected into the sole of the left hindpaw of mice. Nocifensive behavior was quantified as a pain score by summing the duration of paw licking in seconds and the number of flinches.

Measurement of acute neurogenic oedema formation in the ear: 30 μl of 2.5% capsaicin dissolved in 96% ethanol or 3% mustard oil dissolved in paraffin oil was smeared topically onto both sides of the ears of mice and the diameter of the ear was measured with an engineer's micrometer every hour during the 5-h examination. Swelling was expressed as percent relative to the initial control values and was compared to solvent treated mice.

RTX-induced hypothermia: Body temperature of mice was measured using a digital thermometer in the rectum before i.p. injection of 20 μg/kg RTX and at 7.5, 15, 30 and 60 min after injection.

RTX-induced peripheral vasodilatation: Female transgenic, non transgenic, and *Trpv1* KO mice were anaesthetized and injected i.p. with 20 μg/kg RTX. Blood flow in

the tail of the mice was measured with a high-resolution PIM-2 laser Doppler perfusion imager.

Environmental thermopreference: Mice were put into an apparatus where they could choose between two ambient temperatures (30°C vs. 35°C) by locomotion. The time spent in each compartment was recorded over a period of 40 min at 10-min intervals and the results were given as percent. Rectal temperature was measured before and after the exposure.

I.4 RESULTS

Experiments with siRNA oligonucleotides

Effects of siRNAs in ND-C and CHO cotransfected cells: Compared to transfection with pZS5 plasmid alone (100%) a significant decrease in GFP fluorescence was observed when the plasmid was cotransfected with the VsiR1 siRNA, the remaining expression levels were 15.4 ± 3.5% in ND-C cells and were 25.3 ± 6.7% in CHO cells. As expected, TRPV1 knockdown activity was not observed or was only moderate with the control VsiR1INV siRNA where the expression was 102.4 ± 9.4% in ND-C cells and 85.1 ± 6.8% in CHO cells.

Effects of siRNAs on native TRPV1 expressing TRG neurons: In the untreated sample, 49.5% of the TRG cells (47 out of 95) reacted ($\Delta R_{F340/380}$ >0.1) to 330nM capsaicin. A significant decrease was observed after VsiR1 siRNA treatment, as only 6% of cells (8 out of 134) responded to capsaicin administration. As expected, after treatment with the control VsiR1INV siRNA, the proportion of cells responding to capsaicin remained at 48% (38 out of 79).
To confirm the selectivity of VsiR1 siRNA and to exclude nonspecific inhibition of receptor expression, mustard oil-induced TRPA1 receptor activation was also evaluated. In the untreated sample, 23% of neurons (14 out of 60) reacted with Ca^{2+}-influx to mustard oil. VsiR1 treatment had no effect on TRPA1 activation, as the percentage of mustard oil-sensitive cell remained at 23% (15 out of 66). These results confirmed the potent and selective TRPV1 knockdown effect of the VsiR1 siRNA.

Generation of knockdown transgenic animals

In vitro tests of different shRNA and miRNA constructs: The relative expression values of different shRNA constructs in relation to pU6Abase control vector are shown in *Table 1*.
Construct pU6shRNA*a*, which was designed based on the VsiR1 siRNA, almost completely inhibited the expression of ratTRPV1eGFP. A large degree of inhibition was also observed in the case of pU6shRNA*c* and pU6shRNA*e*, but just a moderate difference was detected in case of pU6shRNA*f*. In contrast, pU6shRNA*d* and pU6shRNA*g* did not influence the expression of ratTRPV1eGFP. Interestingly, pU6shRNA*h* which was designed as a scrambled control shRNA also showed a small, but significant decrease in expression. The reason for this effect is not clear and was not investigated further.

Table 1.

Name	Target site (start:length)	shRNA sequence (sense-loop-antisense)	TRPV1eGFP expression (mean±SD%)
pshRNA*a*	1373:21	5'GCGCAUCUUCUACUUCAACUU$^{C}_{G}$ UUCGCGUAGAAGAUGAAGUUGAA$_{A}^{A}$	2±1 ***
pshRNA*c*	1726:21	5'GGACCAACAUGCUCUACUAUA$^{G}_{A}$ UUCCUGGUUGUACGAGAUGAUAU$_{A}^{G}$	11±2 ***
pshRNA*d*	1946:20	5'GCCAGGUAACUCUUACAACA$^{C}_{G}$ UUCGGUCCAUUGAGAAUGUUGU$_{A}^{A}$	100±5
pshRNA*e*	1000:21	5'GCAUGUACAACGAGAUCUUGA$^{A}_{A}$ UUCGUACAUGUUGCUCUAGAACU$_{G}^{C}$	8±3 ***
pshRNA*f*	2471:20	5'GGAUGCAAGCACUCGAGAUA$^{A}_{A}$ UUCCUACGUUCGUGAGCUCUAU$_{G}^{C}$	43±6 ***
pshRNA*g*	802:20	5'GGAGGCCUGGCUUCUACUUU$^{A}_{A}$ UUCCUCCGGACCGAAGAUGAAA$_{G}^{C}$	98±5
pshRNA*h*	scrambled control	5'GCACGAUCAUCGUCUACAAUA$^{G}_{A}$ UUCGUGCUAGUAGCAGAUGUUAU$_{A}^{G}$	79±4 ***
pshRNA*b*	opposite loop	5'GAAGUUGAAGUAGAAGAUGCGC$^{C}_{G}$ UUCUUCAACUUCAUCUUCUACGCG$_{A}^{A}$	37±9 ***
pshRNA*i*	mismatch	5'GCGCA**a**CUUCUACUUCAACUU$^{C}_{G}$ UUCGCGU**u**GAAGAUGAAGUUGAA$_{A}^{A}$	9±1 ***
pshRNA*j*	mismatch	5'GCGC**ua**CUUCUACUUCAACUU$^{C}_{G}$ UUCGCG**au**GAAGAUGAAGUUGAA$_{A}^{A}$	40±3 ***
pshRNA*k*	mismatch	5'GCGCA**a**CUUCUA**g**UUCAACUU$^{C}_{G}$ UUCGCGU**u**GAAGAU**c**AAGUUGAA$_{A}^{A}$	81±3 ***
pshRNA*l*	big loop	5'GCGCAUCUUCUACUUCAAC$^{UUAG}_{C}$ UUCGCGUAGAAGAUGAAGUUG$_{GUCA}$	4±2 ***
pshRNA*m*	deletion	5'GCGCA-CUUCUACUUCAAC$^{UUAG}_{C}$ UUCGCGUAGAAGAUGAAGUUG$_{GUCA}$	15±2 ***
pshRNA*n*	inverted control	5'GCAACUUCAUCUUCUACGCGUU$^{C}_{G}$ UUCGUUGAAGUAGAAGAUGCGCAA$_{A}^{A}$	101±3

Moreover, we tested the efficiency of seven variants of shRNA*a* (shRNA*b*, shRNA*i*-shRNA*n*). pU6shRNA*b* has an antisense-loop-sense configuration and an extra G at the beginning and were not able to decrease ratTRPV1eGFP expression as much as pU6shRNA*a.* The single mismatch variant pU6shRNA*i* was still very effective, the tandem mismatch variant pU6shRNA*j* decreased the expression moderately, while the separate double mismatch construct pU6shRNA*k* decreased expression the least. pU6shRNA*l*, a nine-base loop variant worked equally well as pU6shRNA*a*, and a single nucleotide deletion in the sense strand of pU6shRNA*m* decreased the efficiency of the construct, but not a large degree of inhibition was observed. Finally, the pU6shRNA*n*, which mimics the previously tested VsiR1INV control siRNA was ineffective, as expected.

Furthermore, two miRNAs were designed targeting the same sites on the rat TRPV1 mRNA as the two most effective shRNAs. pDsRedmiRNA*a* could inhibit the ratTRPV1eGFP expression less (24 ± 10%) than the corresponding pU6shRNA*a*. The other miRNA construct, pDsRedmiRNA*e* was similarly less effective (38 ± 15%) than pU6shRNA*e*.

shRNA*a* was the most efficient in these *in vitro* tests, so it was further used as a tool to generate TRPV1 knockdown animals. Since shRNA*a* targets a region that is

conserved between rat and mouse so it can be used to generate gene knockdown in these species.

Lentiviral transgenesis: From 96 lentivirus-injected rat embryos, 24 newborn animals were obtained, but none of them showed visible GFP expression under 455-495 nm wavelength blue light and none was positive in GFP-specific genomic PCR tests.
From 60 injected mouse embryos, 43 newborn mice were obtained, of which 5 gave a positive GFP genomic PCR result and two had visible GFP expression. Two of the five GFP-PCR-positive founder animals (#4 and #11) produced transgenic offspring when crossed with wild-type FVB/Nhsd mice.
GFP-PCR-positive offsprings of founder animals were tested in capsaicin-induced eye-wiping test. In wild type mice and in animals expressing GFP alone the numbers of eye-wiping movements were 14.2 ± 0.9 and 15 ± 0.9, respectively. In contrast, *Trpv1* KO animals responded with zero or one eye-wiping movement. Most of our transgenic offsprings displayed phenotype between these two extremes, responding to similar treatment with fewer than five wiping movements.
Based on these results, the most insensitive animal #10 was selected for establishing the transgenic mouse line for further experiments. Integration of the lentiviral vector was determined by LM-PCR and found to be a single copy in chromosome 3 at nucleotide 154300070 right behind the crystalline, zeta (GeneID 12972) gene.
After weaning, every single offspring of animal #10 were tested by genomic PCR and capsaicin sensitivity in the eye-wiping test. Based on the results, the animals were classified as having the *transgene (tg+)* or *lacking the transgene (tg-)*. Tg- littermates were used as control animals in further experiments.

Characterizations of transgenic animals
Analysis of TRPV1 expression in TRG neurons of mice: TaqMan qPCR assays were used to determine TRPV1, TRPV3 and TRPA1 mRNA levels in TRG neurons of transgenic animals. The levels of TRPV3 mRNA were below the detection limit in both tg+ and tg- animals. There was significant difference in TRPV1 mRNA levels between tg- (ΔC_t = 4.4) and tg+ (ΔC_t = 8.1) animals. This $\Delta\Delta C_t$=3.7 difference indicates that compared to tg- mice the TRPV1 mRNA expression was reduced 13-fold, to ~8% in tg+ animals. On the other hand, there was no significant difference in TRPA1 mRNA expression levels between the tg+ and tg- animals. These results indicate that the shRNA*a* transgene significantly and selectively reduced TRPV1 expression in the TRG neurons of tg+ animals.
The presence of functional TRPV1 and TRPA1 receptors in TRG neurons of tg+ mice was also analyzed by fura-2 microfluorimetry. 63% of TRG neurons (77 out of 123) isolated from tg- animals reacted to capsaicin (ΔR=1.11 ± 0.16). None of the TRG neurons (0 out of 134) from tg+ animals responded with a detectable fluorescent ratio change above the background noise (ΔR< 0.1) to a similar capsaicin administration. Furthermore, in tg- mice, 23% of neurons (20 out of 85) (ΔR=0.65 ± 0.36), while in tg+ animals similarly 21% of neurons (17 out of 81) (ΔR=0.59 ± 0.35) reacted to mustard oil. These results indicate that the anti-TRPV1 shRNA transgene practically eliminated functional TRPV1 receptors, while had no effect on TRPA1 receptor expression.

Nocifensive response on the paw in mice: In tg- mice the pain score was 20.2 ± 22.0 after intraplantar injection of solvent which increased significantly (84.5 ± 33.2) after capsaicin injection. In contrast, the reaction to the same capsaicin treatment was

markedly reduced in tg+ animals, 24.4 ± 26.2. Furthermore, there was no significant difference between capsaicin and solvent treatment (12.0 ± 12.7) in tg+ mice indicating that very little residual TRPV1 activity remained in the polymodal sensory neurons of the paw of tg+ animals.

Acute neurogenic oedema formation in the ears of mice: In tg- mice the swelling of the capsaicin treated ear was most pronounced 2 h after treatment (42.6 ± 5.9%) and could be seen at all measurement time points. In contrast, oedema did not develop in the ears of tg+ animals, as no significant increase in ear thickness was observed compared to solvent-treated animals.
In both tg- and tg+ animals, after mustard oil treatment the largest increase in ear thickness was observed 2 h after treatment and the increments were 28.2 ± 5.5 and 31.4 ± 7.5%, respectively. The solvent treatment did not cause significant change in ear thickness in any of the animals.
These results indicate that TRPA1 function was not impaired in tg+ animals and that not just the pain perception, but neurogenic inflammation was inhibited selectively in our transgenic animals.

Effect of RTX on body temperature and peripheral blood flow in mice: After i.p. injection of RTX the body temperature decreased significantly at all measurement time points in tg- mice, the lowest body temperature was measured 30 min after injection (33.3 ± 0.1°C, where the initial value was 37.2 ± 0.2°C). In tg+ mice the body temperature was only slightly, but not significantly lower. The lowest body temperature was measured 30 min after injection, the values were 36.2 ± 0.2°C. These results also support that in tg+ mice the activity of TRPV1 is lower significantly, but it is not eliminated totally.
The blood flow in the tail of tg-, tg+ and *Trpv1* KO mice was monitored after i.p. injection of RTX by a Laser Doppler perfusion imager. The treatment induced a significant increase in blood flow in the tail of tg- mice which was most pronounced at 15 min (75.8 ± 25.9% compared to the initial value). Only a modest change was observed in blood flow in tg+ and *Trpv1* KO animals, the maximum increases reaching 35.5 ± 2% and 21.9 ± 18.5%, respectively, at 27 min. These results indicate that in the tail of tg+ animals vasodilatation following i.p. injection of RTX was markedly inhibited.

Environmental temperature selection of mice: There was no statistically significant difference between tg- and tg+ female mice in temperature preference or rectal temperature. Tg+ male mice, however, spent slightly more time at 35°C than tg- male controls, but the difference was statistically significant only for the second interval (tg- , 21.1 ± 2.0%; tg+, 29.0 ± 2.7%). Surprisingly, although tg+ male mice spent slightly more time in the warmer environment than the tg- animals, elevation of their rectal temperature after the exposure was significantly lower.

I.5 DISCUSSION

In the first part of my study I described how we could generate and characterize TRPV1 receptor knockdown animals.
First, we were able to effectively and selectively inhibit TRPV1 expression with siRNAs in transfected cells and isolated rat TRG sensory neurons. Since our further

aim was to generate transgenic TRPV1 knockdown mice and rats, therefore we had to create an efficient anti-TRPV1 shRNA construct.
At first, several potential shRNAs were designed to target different sites on the rat TRPV1 mRNA. The most effective shRNA (shRNA*a*) was based on the previously *in vitro* tested VsiR1 siRNA. We did not search for the causes why some shRNAs could not work effectively, but our experience agrees with other's perceptions that several interfering RNA candidates need to be generated and tested for every target gene. Furthermore, we designed different variants of the most effective shRNA*a*. Neither the 9 nt-loop variant (shRNA*l*), nor the antisense-loop-sense variant (shRNA*b*) were better than shRNA*a*. In addition, our results show that a single mismatch was tolerated and caused minimal decrease in effectiveness in agreement with other's results [52], but the tandem and mainly the separate double mismatch resulted in a considerable reduction in effectiveness. In addition to shRNAs, the use of artificial miRNAs has become an alternative approach to achieve knockdown phenotype. Because artificial miRNAs more naturally resemble endogenous RNAi substrates therefore they might be more effective [53]. However, others found that pol III driven shRNAs are more potent because they are expressed at considerably higher levels than pol II transcribed miRNAs [54]. Our results are in agreemant with this later observation, because our miRNAs were less effective than the corresponding shRNAs.
After prevalidation we used the most suitable shRNA (shRNA*a*) construct in lentiviral transgenesis.
Despite several studies which could demonstrate successful transgenesis by subzonal injection of lentiviruses in one-cell embryos in rats [55;56] unfortunatelly our efforts to create TRPV1 knockdown rats by this technique were unsuccessful.
On the other hand, as the target site of shRNA*a* on the TRPV1 mRNA is conserved in rats and mice, therefore the same virus can be used in mice. In our case the transgenic efficiency (~12%) was behind reported values (80%; [45]), but was still several times higher compared to pronuclear injection (~2%; [57]). 5 founder mice showed positive GFP genomic PCR, but among them only 2 had visible GFP fluorescence (founder #4 and #11). The cause of non visible GFP fluorescence can be that the proviruses intengrated in non-active chromatin domains. Among the offsprings of founder animals (F1 generation) there were several transgenic animals which were GFP-PCR-positive, showed GFP fluorescence and had strongly reduced capsaicin sensitivity in eye-wiping test. This confirmed that the lentivirally delivered shRNA*a* construct was effectively built in the grem line and it can be inherited. Furthermore, the least capsaicin sensitive progeny (#10) from F1 generation was chosen to establish the transgenic mouse line (tg+). We determined by LM-PCR that a single copy of the lentiviral vector is integrated into the genome of this line.
Tg+ mice showed reduced sensitivity (1-5 wiping movements) to capsaicin in eye-wiping test. This means that some mRNAs could escape from the RNAi pathway resulting in a modest residual receptor expression. We determined the level of remaining TRPV1 mRNA by qPCR, which was about 8% in tg+ mice compared to tg- littermates. We could not determine directly the remaining small amount of residual receptor protein, but based on the results of Ca^{2+}-influx measurements, we estimated that the receptor level was less than 5%. Despite the possibility of the presence of minimal residual receptors in our tg+ mice, a TRPV1 receptor defficient phenotype was observed both *in vitro* where not a single TRG neuron responded to capsaicin, and also *in vivo* where diminished capsaicin-sensitivity in plantar nocifensive test and total lack of capsaicin-induced neurogenic inflammation was observed.

The thermoregulatory behavior of TRPV1 knockdown mice was also tested. Here we showed that in tg+ mice the hypothermia and tail vasodilatation induced by i.p. injection of RTX was markedly inhibited which indicates a robust loss of TRPV1 channels. Furthermore, similarly to *Trpv1* KO mice [26;58], no hyperthermia was observed in tg+ mice at room temperature or after heat exposure. These results with TRPV1 knockdown mice do not support the hypothesis that TRPV1 receptors have a putative tonic, predominant function in body temperature regulation with an abdominal preference [26;59].
We tried to determine the TRPV3 mRNA level from TRG [60] by qPCR, but it was below the detection limit both in tg- and tg+ animals. This result argues with a previous observation of increased TRPV3 mRNA level in tg+ mice [61].
In our tg+ mice we also investigated the expression and function of TRPA1 receptor on isolated TRG neurons and in mustard oil-evoked inflammation model. Since neither the level of TRPA1 mRNA, nor the numbers of mustard oil responsive TRG neurons and nor the sensitivity of mustard oil in inflammation model were altered in our tg+ mice we could establish that effect of TRPV1 knockdown is selective and does not influence TRPA1. At the same time, it has been shown that the mustard oil-gated current is larger in the sensory neurons collected from wild type mice than from *Trpv1* KO mice, and the number of mustard oil responsive cells was lower in the absence of TRPV1 [62;63]. So it is possible that there is a translational or post-translational regulation between TRPV1 and TRPA1 receptors, and in TRPV1 knockdown mice the slight residual amount of TRPV1 channels might be enough to stabilize or regulate TRPA1 receptors on the plasma membrane.
Unfortunatelly, we have never been able to get homozygous tg+ pups only tg- or heterozygous tg+ animals. It has been shown that overexpression of U6-driven shRNA could interfere with cellular miRNA expression, leading to lethality in mice [64]. Therefore we suspect that two copies of U6-shRNA construct in homozygotes produce too much shRNAs resulting nonviable embryos.
In this chapter of my thesis I presented how we generated a well working TRPV1 knockdown transgenic mouse line with lentiviral transgenesis. We plan further studies to find answers for several unsettled issues related to this mouse line and the function of TRPV1 cation channel.

II. EFFECTS OF LIPID RAFT DISRUPTION ON ACTIVITY OF TRPV1 AND TRPA1 CHANNELS

II.1 INTRODUCTION

Structure of lipid rafts. Membranes of cells are not uniform, as it was thought according to Singer-Nicholson model, but clusters of lipids in a more ordered state (termed as lipid rafts) exist in it [65]. Lipid rafts are enriched in cholesterol, sphingolipids (glycosphingolipids (GSLs) and sphingomyelin (SM)) and saturated glycerophospholipids (GPLs) in comparison with non-lipid raft regions. The disposition of these components is asymmetric in the bilayer of lipid rafts. Sphingolipids are mainly located in the outer leaflet, while in the inner leaflet more saturated GPLs are found. Cholesterol is present in both leaflets and fills the space under the head groups of sphingolipids or extends the interdigitating fatty acyl chains

in the opposing leaflet. Furthermore, lipid rafts contain proteins attached to the outer or inner leaflet, or associating through their transmembrane domains [65;66].

Lipid rafts in receptor research. There are several options to investigate the role of lipid rafts in a receptor's function: *methyl-β-cyclodextrin ($M_\beta CD$)* enhances the solubility of cholesterol and removes it from membranes [67], SM can be hydrolyzed by *sphyngomyelinase (SMase)* [68], the SM biosynthesis can be inhibited by *myriocin* [69], and decreased GSLs synthesis can be achieved by *D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP)* [67].
So far only few attempts were made to study the role of lipid raft in the function of TRPV1 channels, with controversial results. Depletion of cholesterol by incubation with $M_\beta CD$ resulted in no change in heat-activated currents, while the amplitude of capsaicin-activated currents was significantly reduced in DRG neurons [70]. In contrast, it has been reported previously that TRPV1 receptors are not modulated by cholesterol depletion in rat C6 glioma cells [71].

II.2 AIMS

We planned to determine whether changing the lipid raft can modulate the activation of TRPV1 and TRPA1 channels:

1. We wanted to examine the rise in intracellular Ca^{2+}-influx in TRPV1-transfected cell line when various vanilloid and nonvanilloid activators of the TRPV1 channel were applied after depletion of cholesterol, SM or GSLs.
2. We also wanted to determine the TRPV1 activity in native sensory neurons under the same conditions.
3. Finally, we wanted to investigate the TRPA1 activity in native sensory neurons when different TRPA1 agonists were used after SM depletion.

II.3 METHODS

Experiments with TRPV1-expressing CHO cells: CHO cells stably expressing the TRPV1 receptor were pretreated with $M_\beta CD$ for 30 min, with SMase for 60 min, and with myriocin or D-PDMP overnight. The control cells were similarly pretreated with the respective diluted solvent of chemicals. Then the cells were washed with calcium free Hank's solution (pH 7.4) and were incubated with the desired amount of agonist (100 nM capsaicin, 3 nM RTX, 10 μM AEA or 10 μM OLDA), and 200 μCi/ml $^{45}Ca^{2+}$ isotope. When the receptor activation was induced by pH 5.5, first pH 7.4, then pH 2.05 Hank's solution was dropped onto the cell containing plates to reach the desired pH. After activation the cells were washed with ECS more times, the residual buffer was evaporated, the retained isotope was collected in 0.1% SDS and the radioactivity was measured in scintillation liquid in a scintillation counter. The values of $^{45}Ca^{2+}$ uptake in percent were compared to solvent controls (100%).

Experiments with primary cultures of TRG neurons: Preparation of the primary cultures of TRG neurons and the measurement of activity of native TRPV1 or TRPA1 receptors with fura-2 microfluorimetry were prepared as described in section I/3.
TRG neurons were incubated with different concentrations of $M_\beta CD$ for 30 min, SMase, ceramide and sphingosine for 60 min, myriocin or D-PDMP overnight, under standard cell culture conditions. In case of controls the plates were pretreated with only the respective solvent of the chemicals.

After fura-2-AM staining 10-s pulse of capsaicin (330 nM), or RTX (3 nM) were applied to excite TRPV1 receptors. TRPA1 receptors were stimulated with mustard oil (200 µM), or formalin (0.01%) for 30 s. To release Ca^{2+} from inner stores thapsigargin (200 nM) was used for 30 s. Finally, to excite voltage gated channels KCl (50 mM) was applied for 3 s.
Cells showing a ΔR of >0.1 after activation were considered to be receptor-expressing cells and the percentage ratio of responsive and non-responsive cells was compared to the solvent controls.

II.4 RESULTS

Effect of cholesterol depletion on TRPV1 receptor
In TRPV1-expressing CHO cells after 1, 3 or 10 mM $M_\beta CD$ pretreatment the capsaicin responses were diminished dose dependently to 81.7 ± 8.8, 72.7 ± 19.4 or 57.5 ± 16.6%, respectively. Similarly, significant decreases were also obtained after activation by OLDA (56.9 ± 17.6% and 56 ± 22.7% in case of 3 and 10 mM $M_\beta CD$, respectively). Surprisingly, the $^{45}Ca^{2+}$ uptake evoked by other investigated TRPV1 agonists such as RTX, the endogenous ligand AEA and low pH of 5.5 remained unchanged under the same experimental condition.
In the untreated control the ratio of capsaicin sensitive TRG neurons was 62% (58 out of 93) which decreased to 39% (15 out of 38) and 10.5% (8 out of 76), after 3 and 10 mM $M_\beta CD$ pretreatment, respectively. Moreover, in case of RTX this value decreased from 57% (40 out of 70) to 22% (17 out of 77) and 15% (12 out of 80), respectively.

Effect of SM depletion on TRPV1 and TRPA1 channels
Changed TRPV1 activation after SM depletion: In TRPV1-expressing CHO cells the pretreatment with 1 and 10 mUN SMase diminished the capsaicin-induced $^{45}Ca^{2+}$ uptake significantly to 42.3 ± 15.8 and 19 ± 7.7%, respectively. However, the same pretreatments did not change the RTX-induced Ca^{2+} signal. In addition, capsaicin induced $^{45}Ca^{2+}$ uptake was 51.3 and 23.5% of the control values after 10 and 20 µM D-PDMP pretreatments, respectively. These pretreatments also reduced the RTX-evoked responses to 68 and 38%, respectively. Similar results were obtained using myriocin, where 5 and 50 nM concentrations significantly decreased the response to capsaicin (51 and 22%, respectively). Furthermore 50 nM myriocin also reduced the response to RTX to 47%.
In case of TRG neurons the percentage of responsive neurons after capsaicin excitation was 65% (101 out of 155) which slightly decreased to 56% (42 out of 75) after 10 mUN SMase and significant reduction to 48% (36 out of 75) after 30 mUN SMase pretreatment. In case of RTX the ratio of activated neurons from 60.3% (70 out of 116) decreased to 45% (15 out of 33) and 34% (11 out of 32) after 10 and 30 mUN SMase pretreatments, respectively. Pretreatment with the higher concentration of D-PDMP (50 µM) caused significant decrement to both capsaicin (22%; 12 out of 55) and RTX (11%; 5 out of 47) as well. Finally, after incubation with the higher concentration of myriocin (200 nM) the decrement of responsive cells was similarly significant, namely 30% (17 out of 56) in case of capsaicin and 40% (24 out of 60) in case of RTX.

The effect of SMase pretreatment on TRPA1 activation: Without SMase pretreatment the percentage of responsive TRG neurons after mustard oil exposure was 26.8%

(98 out of 379). After 10 and 30 mUN SMase incubation this proportion was significantly decreased resulting in 16.9 (25 out of 148) and 4.1% (4 out of 97) responsive cells, respectively. In case of formalin, the ratio of activated neurons in control plates was 39.7% (35 out of 88) and this value was altered significantly to 26.3% (21 out of 80) after 30 mUN SMase, but not after 10 mUN SMase pretreatment.

SMase pretreatment did not influence the inner cell membranes and voltage gated Ca^{2+} channels: In control plates the percentage of responsive TRG neurons after thapsigargin exposure, which can induce rapid Ca^{2+} release from inner stores [72], was 48.1% (74 out of 154). This value did not change significantly on SMase pretreated plates, 44.3 (85 out of 192) and 44.9% (48 out of 107) in case of 10 and 30 mUN SMase, respectively, indicating that SMase could not have an effect on the inner membranes.
In control plates the percent of TRG neurons reacting to KCl was 96% (73 out of 76) which did not change after 30 mUN SMase pretreatment (95.2%; 79 out of 83). Likewise SMase did not influence the peak of the mean fluorescence responses after KCl exposure, resulting ΔR=1.107 ± 0.496 on control and ΔR=1.143 ± 0.506 on treated plates. In contrast, after exciting TRPV1 with capsaicin and TRPA1 with mustard oil this value diminished significantly because of SMase pretreatment from 1.066 ± 0.618 and 0.714 ± 0.316 to 0.403 ± 0.229 and 0.313 ± 0.120, respectively.

Neither ceramide nor sphingosine influenced the activity of TRPV1 receptor: TRPV1-expressing CHO cells were pretreated with ceramide or sphingosine as products of SMase enzyme (1 and 10 μM in both cases) and TRPV1 was activated with capsaicin. We did not detect decreased receptor activity neither after ceramide pretreatment (1 μM: 120 ± 31.2, and 10 μM: 126 ± 33.9%) nor sphingosine pretreatment (1 μM: 117 ± 29.8, and 10 μM: 115 ± 36.7%).

Combined application of $M_\beta CD$ and SMase changed the activition of TRPV1 receptor

Low, non-efficient concentration of $M_\beta CD$ (1 mM) and SMase (0.1 mUN) were administrated together to TRG neurons and they did not inhibit the response of capsaicin or RTX significantly.
Surprisingly, on the TRPV1-expressing CHO cells the combined pretreatment resulted in a pronounced inhibition where the values were 14.5 and 18.7% in case of capsaicin and RTX, respectively.

II.5 DISCUSSION

In this part of my thesis we investigated the effect of disruption of lipid rafts by depleting its cardinal constituents of cholesterol, SM or GSLs on the opening properties of the TRPV1 and TRPA1 cation channels.
On TRPV1-expressing CHO cells depletion of cholesterol by $M_\beta CD$ inhibited the calcium response evoked by capsaicin or OLDA, but not by RTX, AEA or low pH. Glu 648 and Glu 600 amino acids, which are situated at the external part of the pore loop, are responsible for proton mediated activation and channel opening [73]. Thus, it is not surprising that decomposition of the lipid raft could not influence their role in gating function. Furthermore, the difference between the gating effect of capsaicin and RTX after cholesterol, and SM depletion can be explained by the different

allosteric binding sites of these agonists. These agonists can bind intracellularly, and H-bonding ability of vanilloid moiety is inevitable for the action of capsaicin, but not for RTX. Probably the large hydrophobic skeleton of the diterpene with a 3α-keto substitution is critical for the action of RTX [74] and it is sufficient for binding in an overexpressed cell line even when the lipid raft is decomposed by cholesterol or SM depletion. In contrast, in native TRG cells the effect of capsaicin and RTX were similarly inhibited by cholesterol or SM depletion. These results might be partly due to differences between the lipid raft surrounding the ion channel in the TRPV1-expressing cell line and native sensory neurons. Nevertheless, the results of combined application of $M_{\beta}CD$ and SMase, where attenuation was observed only on TRPV1 overexpressed cells, but not on TRG neurons, can be explaned by the interaction between TRPV1 and other TRP channels [62].
The disturbed lipid rafts by SMase caused reduced receptor activity with both TRPA1 agonists. At the same time this decrement was observed neither on voltage-gated Ca^{2+} channels, nor after thapsigargin exposure. And after ceramide or sphingosine pretreatments there was no reduced TRPV1 activity. Together these results indicate that composition of the cell membrane inside lipid rafts can play a more important role in the activition of ligand-gated TRP channels than it was thought before. Moreover, the reliability of SMase pretreatment as a good pharmacological tool is confirmed.
Further experiments are needed to answer the questions whether differences between the effects of SMase and SM and GSLs biosynthesis inhibitors are related to RTX binding or to the longer exposure of cells to the latter compounds which might elicite a more profound alteration in the lipid raft.
It can be concluded, that lipid rafts may play a more important role in drug action at the TRP channels than it was believed.

SUMMARY

In this thesis, I presented new approaches to increase our knowledge about the much investigated TRPV1 receptor.
In the first part of my thesis, I described how we generated transgenic TRPV1 knockdown animals by lentiviral transgenesis.
We designed several shRNA and miRNA constructs and tested them on cell cultures. The most effective shRNA construct was inserted into a lentiviral vector, virus particles were prepared and introduced into rat and mouse one-cell embryos by subzonal injection. In mice we were able to prove several ways that the transgenesis was successful and the TRPV1 receptor knockdown phenotype is a permanent and heritable attribute. So based on our results, we could confirm the effectiveness of lentiviral transgenesis and we support the view that transgenic RNAi is a useful method which can play an important role in pain research such as validation and characterization of new potential targets. But keep in mind that the absence of the same protein can result different side-effects depending on the used technique which is demonstrated well with the changed or unchanged TRPA1 expression levels in TRPV1 knockout or knockdown mice, respectively. That's why my opinion is that these techniques can not substitute for each other, but together they can give a more accurate picture.
In the second part of my thesis, we used lipid raft depletion as a new pharmacological tool to investigate not only the receptor as single protein, but as a

member of a complex membrane structure. We could demonstrate that lipid rafts influence activity of TRPV1 and TRPA1 receptors by changing the access of activators. Moreover, these changes can be different depending on the used lipid raft modifying agents and the used receptor expressing systems. These results give several evidences that this approach can be a new tool in pharmaceutical research.

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