Potassium channels represent the largest superfamily of ion channels. They are expressed practically in every cell, primarily in the plasma membrane and they have fundamental impact on the membrane potential. K^+ channels play a major role in various cellular processes, including neuronal activity, action potential characteristics, muscular excitability, exocrine and endocrine secretion, immune response, cell volume regulation, etc. K^+ channels can be grouped into three major families; these can be distinguished both by characteristic structural properties and functional/regulatory similarities. The voltage-gated and/or calcium-activated channels are characterized by a general molecular architecture of six transmembrane segments and one pore domain in each subunit (6TMS/1P). These channels possess a voltage sensor, a charged transmembrane helical segment and they activate upon depolarization or in response to a calcium signal. The second major family, the inwardly rectifying channels possess two transmembrane segments and one pore domain (2TMS/1P). They close upon depolarization; the effect is due to the block of the channel by intracellular Mg^{2+} or by polycations. To attain the functional channel structure of the pore, members of the aforementioned two families assemble as tetramers. Members of the third family, the K2P channels possess four transmembrane segments and two pore forming domains in each of their subunits (4TMS/2P) accordingly they dimerize. They conduct leak or background K^+ current i.e. their activity is not (or in some cases only moderately) influenced by the membrane potential. They are responsible for maintaining the highly negative resting membrane potential in different excitable cell types. Their regulation by voltage-independent mechanisms is of importance, since K2P channels generally determine the responsiveness of the cell to different excitatory stimuli and can also efficiently counter massive depolarization. The fifteen members of the K2P family are regulated by diverse mechanisms conferring temperature-, mechano-, or pH-sensitivity in the physiological range and modulated by a great variety of signaling pathways and protein interactions. The different excitable cell types typically express a specific subset of these channels to meet the regulatory requirements for their functions. TRESK (TWIK-related spinal cord K^+ channel, K2P18.1) is the last discovered member of the family. Dominant negative mutation of human TRESK was reported to be linked to a rare form of familial migraine, and further reports suggesting a role for the channel in pain disorders. Regulation and function of K2P channels has been the major interest of our group with special interest on TRESK. We were the first to describe the calcium mediated activation of the channel (what is unique among the K2P channels) and the signal transduction pathway what mediates the effect of the calcium signal: the dephosphorylation by calcineurin.

In the frame of the present project we analyzed further aspects of the regulation of TRESK including intracellular molecular (protein-protein) interactions. We also found useful compounds which can be used to study the physiological/pathophysiological significance of the channel. TRESK is predominantly expressed in sensory neurons (dorsal root ganglia (DRG), trigeminal ganglia). In addition to TRESK, these neurons express predominantly leak K^+ channels of the TREK family; this urged us also to find distinctive pharmacological tools and TREK channel interactions.

1/ Under basal condition TRESK is constitutively phosphorylated what provides low channel activity. In response to a calcium signal TRESK is dephosphorylated (and thereby activated) by calcineurin. We have previously shown that calcineurin has to bind to the channel at a non-catalytic interacting site (PQIVID in the mouse and PQIIIS in the human channel) in order to exert its effect (the sequence is homologous to that of the famous target of calcineurin: nuclear factor of activated T cells (NFAT)). While mutation of the mouse NFAT-like sequence completely abolished the activation, deterioration of the human motif (PQIIIS) only partially prevented dephosphorylation and failed to abrogate completely activation. We demonstrated that human TRESK possesses another important binding motif for calcineurin (LQLP), which

is another major determinant of the interaction with the phosphatase. According to our binding studies calcineurin is anchored to PQIIIS under resting conditions. When the cytoplasmic [Ca^{2+}] is elevated, calcineurin is forced to dock to the LQLP motif and becomes adequately positioned for the dephosphorylation of the regulatory serines of the channel (Fig. 1.). As this second interaction is also calcium dependent, it can fundamentally determine the calcium-sensitivity of TRESK activation. Our results also indicated that several aspects of the kinase and phosphatase reactions (regulating TRESK) are different between the human and mouse orthologs, and care must be taken if data are extrapolated from rodent experimental models to the physiology of human channel. These results were published in the Journal of Biological Chemistry 2014; 289(43):29506-18.; PMID:25202008: *The LQLP calcineurin docking site is a major determinant of the calcium-dependent activation of human TRESK background K+ channel.* An additional aspect of our published data is (beyond the mechanism of TRESK regulation) that calcineurin may establish similar interactions with its other substrates as indicated by citations of our paper also from the field of immunology.

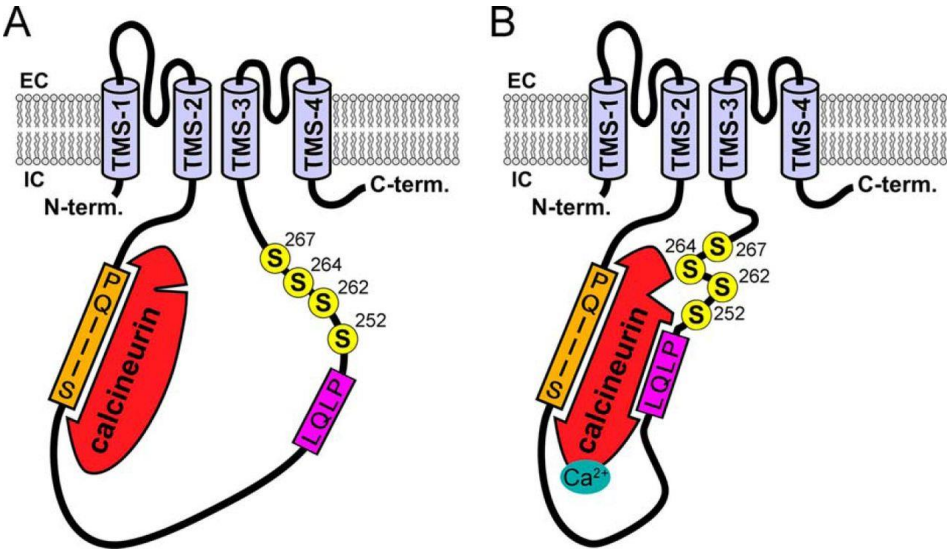

Fig. 1. Binding sites on the intracellular loop of TRESK for calcineurin

2/ Several members of the K2P channel family are regulated by protein-protein interactions. Some of these were shown to influence targeting of the particular channel to the plasma membrane (or cause retention in the endoplasmic reticulum). We have previously shown that 14-3-3 proteins are important negative regulators of TRESK; instead of affecting intracellular colocalisation they influence the phosphorylation/dephosphorylation i.e. the major acute regulatory process of the channel. With the aim of identifying further interacting partner/s we applied an affinity chromatography approach: using the immobilized intracellular loop of the TRESK channel as a bait we purified an interacting protein from mouse brain cytosol. With MALDI TOF mass spectrometry this partner protein was identified as tubulin. This interaction appeared to be particularly interesting in the light of our earlier results, indicating that TRESK is regulated by MARK (microtubule-affinity regulating) kinases. By engineering several mutant constructs we demonstrated that there are multiple contact points between the TRESK channel and tubulin. The major determinant of the interaction was constrained to a fragment of the C-terminal part of the intracellular loop (amino acids 256–271 in human TRESK). This sequence includes the previously identified regulatory serines (S262 and S264) which can be phoshorylated by MARK kinases. S252 of the human channel, which, if phosphorylated, can bind 14-3-3 proteins, is also adjacent to the identified tubulin-binding sequence. We have also

demonstrated that tubulin competes with 14-3-3 for the binding to TRESK; these results were published in PLoS One (2014, 15;9(5):e97854; PMID: 24830385: *Tubulin binds to the cytoplasmic loop of TRESK background K^+ channel in vitro*). Although we tried several experimental approaches to obtain direct evidence for the functional relevance of the interaction, it turned out to be a challenging task, and still remains to be examined.

3/ The evaluation of the contribution of a given K2P channel to the background current of an excitable cell is substantially hampered by the lack of specific and efficient pharmacological tools, which could be applied to selectively modify the activity of a particular channel type. The K_{2P} channels possess a peculiar structural element, the extracellular cap domain, which restricts access to the channel pore (this is the mode of action of high affinity specific toxins in the case voltage dependent K^+ channels). Local (lidocaine, bupivacaine, benzocaine) and volatile (halothane, isoflurane, sevoflurane) anesthetics can modulate TRESK, however, other K_{2P} channels are also sensitive to these compounds. In addition, available antibodies often fail to provide reliable information about the channel repertoire, because of their limited specificity.
As an observation in a high-throughput study, the anti-amoebic drug cloxyquin was described as an activator of TRESK, but its specificity within the K_{2P} channel family has not been addressed. Although the reported degree of activation was moderate we decided to examine whether it acts directly on the channel (and not through the modulation of signaling pathways) and whether its effect is specific for TRESK. We tested the effect of cloxyquin in our *Xenopus laevis* oocyte heterologous expression system. The results were very promising; a 4.4-fold stimulation was obtained with an EC50 of 26.4 μM. The effect was entirely specific among the K2P channels (Fig. 2.)

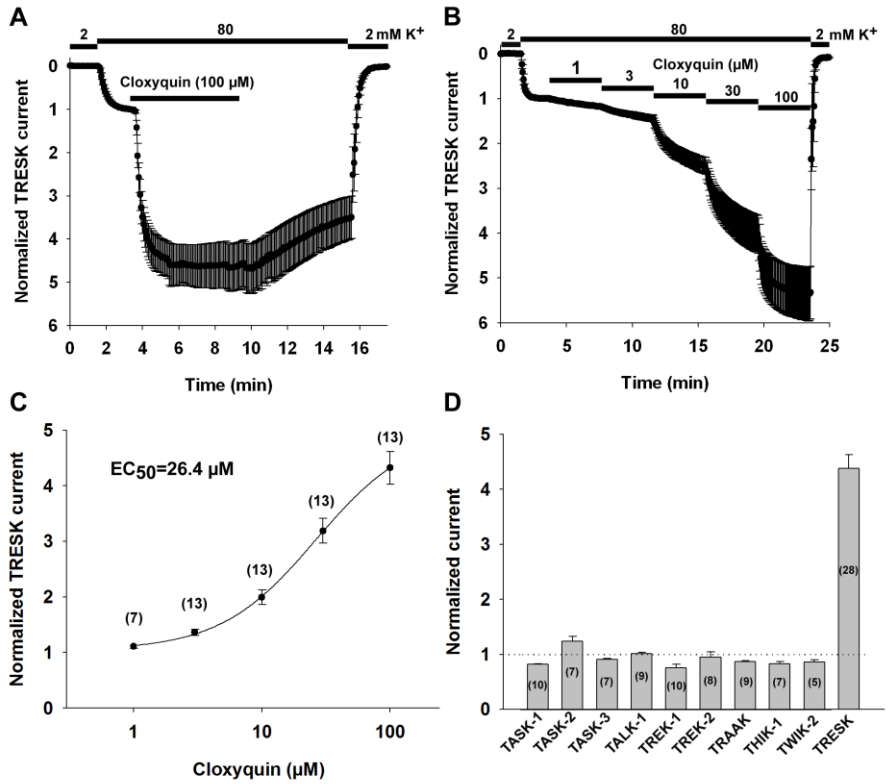

Fig. 2. Cloxyquin specifically and concentration dependently activates TRESK

Cloxyquin remained efficient when the cytoplasmic Ca^{2+} concentration was buffered at low concentration (10-100 nM level), suggesting that the effect of cloxyquin is not mediated

calcineurin activation. This suggestion was confirmed as cloxyquin stimulated TRESK constructs in which the calcineurin docking motif was mutated, mimicking the basal/phosphorylated state. Still the efficacy of cloxyquin reflected the activation state of the channel. The current through the unstimulated (phosphorylated) channel was increased 4-5-fold by cloxyquin. On the other hand, when the drug was added after the application of a calcium mobilizing agonist or the calcium ionophore, ionomycin (what led to dephosphorylation and activation by the calcium/calmodulin-dependent protein phosphatase calcineurin) TRESK channels were not affected by cloxyquin. Similarly, mutants, mimicking the dephosphorylated (activated) state of the channel were not affected by cloxyquin (the drug accordingly is a state dependent activator of the channel; Fig. 3.).

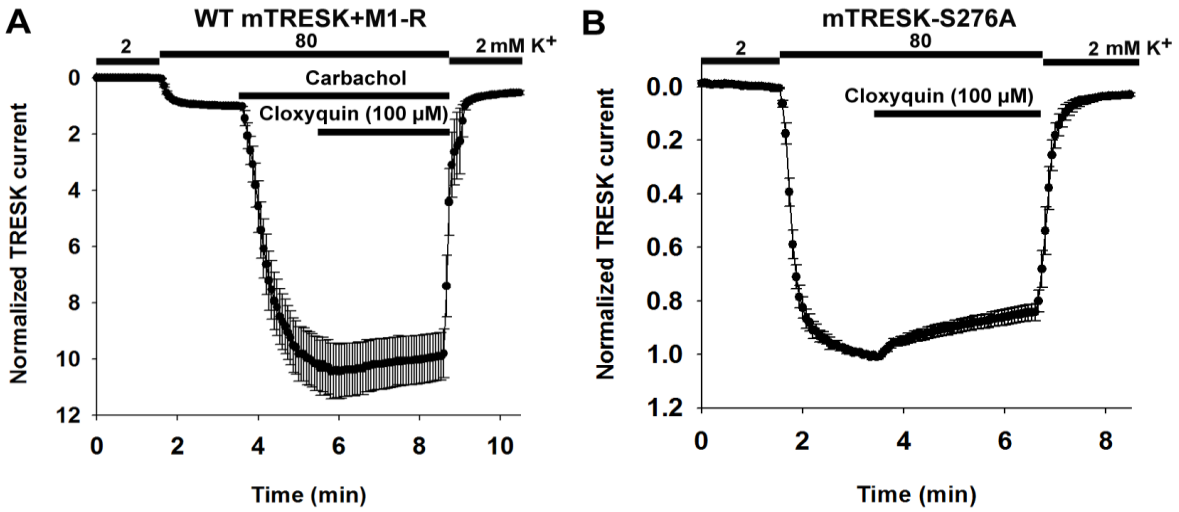

Fig. 3. Cloxyquin is a state dependent stimulator of TRESK; it does neither activates the carbachol-stimulated channel nor the dephosphorylated form mimicking mutant

The results were promising regarding the applicability of TRESK for identifying TRESK in native tissues. Indeed, in a population of isolated DRG cells cloxyquin increased the leak K^+ current (Fig. 4.). The paper was accepted for publication (British Journal of Pharmacology 2017 Jul;174(13):2102-2113: *Selective and state-dependent activation of TRESK (K_{2P} 18.1) background potassium channel by cloxyquin.*).

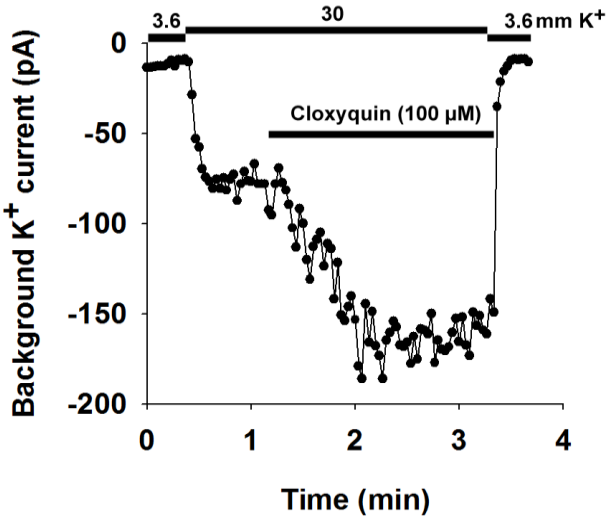

Fig. 4. Cloxyquin increases the background K+ current in a population of isolated DRG neurons.

4/ While the specificity of cloxyquin was impressive, with the aim to obtain a TRESK stimulator with an improved potency we have produced 28 chemically modified analogues of cloxyquin (in collaboration with Professor Péter Mátyus) and tested their effect on TRESK channels. Some of these derivatives activated TRESK with similar efficacy and potency as the parent compound, however, their specificity (within K_{2P} channels) was lower. On the other hand, unexpectedly, we obtained several (11) compounds with inhibitory properties. Based on the pilot screening of the compounds, the most promising inhibitors of mTRESK, A2764 and A2793 were chosen for detailed analysis. The compounds were tested for selectivity and for inhibitory characteristics. Similarly to the effect of cloxyquin, the efficacy of the inhibitors was also state dependent: the basal TRESK current was inhibited by 42.8% while the activated channel (7.3 fold by ionomycin-) was inhibited much stronger (76.8%) (Fig. 5.). The potency of the inhibitor was also slightly better towards the activated channel.
The specificity of the inhibitor was also tested for other K2P channels; while it was not entirely specific, only TALK1 and TREK1 were inhibited by 20%. The inhibitor was also tested in native cells (in DRG neurons) to characterize their background K^+ conductance. In these experiments another inhibitor of TREK2, ruthenium red (RR) and our TRESK deficient mice strain was also utilized (see below).

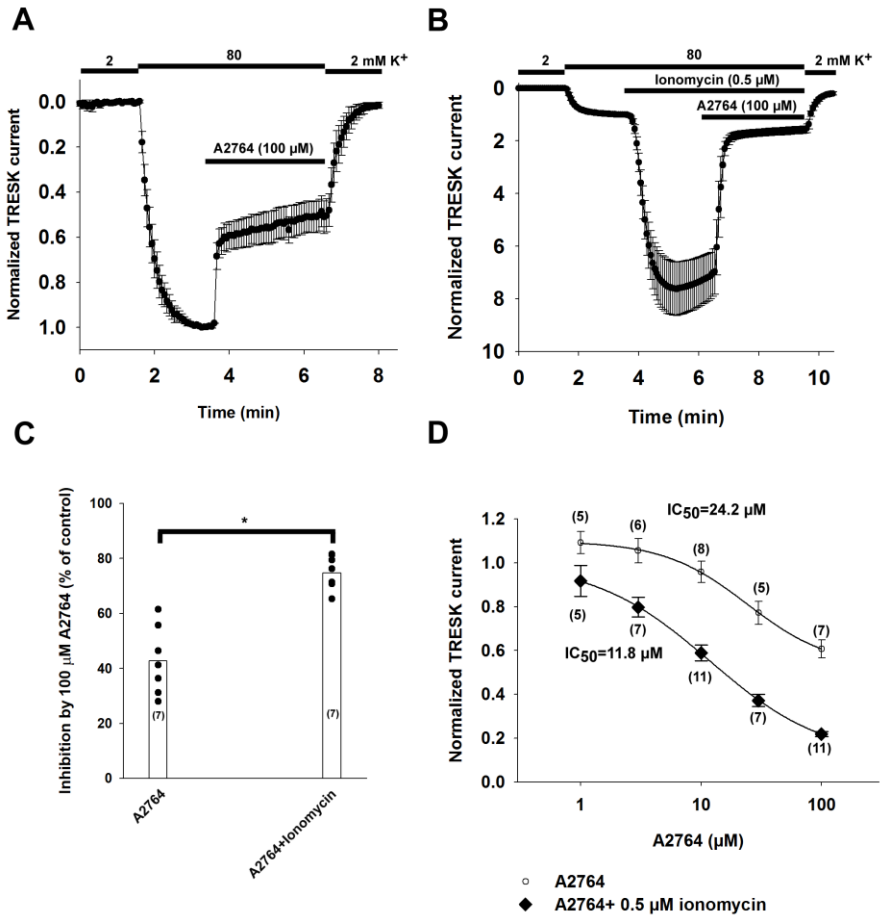

Fig. 5. The cloxyquin analogue A2764 inhibits more efficiently and with higher potency the activated channel

5/ While the activity of the K2P channels is not influenced by the membrane potential (this is the basis of their leak conductance characteristics), a large variety of physicochemical factors and intracellular signaling pathways influence their gating. The majority of these effects converge on the intracellular C-terminus of the channels, resulting in the modification of the gating at the selectivity filter. A distinguishing structural feature of TRESK is its long intracellular loop between the 2nd and 3rd transmembrane segments which is the target of the regulatory phosphorylation/dephosphorylation. An earlier report on the KCNK0 *Drosophila* K2P channel indicated that the regulation of this channel by protein kinases involves conformational changes in the selectivity filter similar to the C-type inactivation described in K_v channels. This raised the possibility that also in the case of TRESK this other gating mechanism, might be affected and lead us to examine channel gating at the helix bundle crossing region. Ba^{2+} was applied to the intracellular side of excised membrane patches and the characteristics of the channel block were determined. We compared the kinetics of the development of Ba^{2+} block when the channels were phosphorylated (inhibited) or dephosphorylated (activated) and also in different mutants mimicking the two functional states. Neither the phosphorylation/dephosphorylation nor the point mutations influenced the development of Ba^{2+} block, indicating that the conformational changes at the bundle crossing region do not contribute to the phosphorylation-dependent gating of TRESK. These results were published in PLoS One. 2018;13(5):e0197622 PMID: 29763475: *TRESK background*

potassium channel is not gated at the helix bundle crossing near the cytoplasmic end of the pore.

6/ In addition to TRESK, mostly members of the TREK K^+ channel family are expressed in the DRG sensory neurons and contribute to their background potassium conductance. This result derived from pain stacking excised patch experiments in which single channel current characteristics were measured and evaluated. This was necessary because of the lack of specific inhibitors also for TREK channel family members. We have previously shown that TASK-3 is inhibited while TASK-1 is not influenced by micromolar concentrations of the polycationc dye ruthenium red (RR). This observation proved to be useful because of the remarkably selective action of RR distinguishing between the two closely related TASK channels, despite the limited overall specificity of the compound. In a present study we performed a more comprehensive analysis and evaluated the effect of RR on all the K2P channels.
According to our results the closely related TREK-1 and TREK-2 channels are differently influenced by RR. While TREK-2 was efficiently inhibited by the compound (IC50=0.2 μM) TREK-1 current was not affected at all (Fig. 6.).

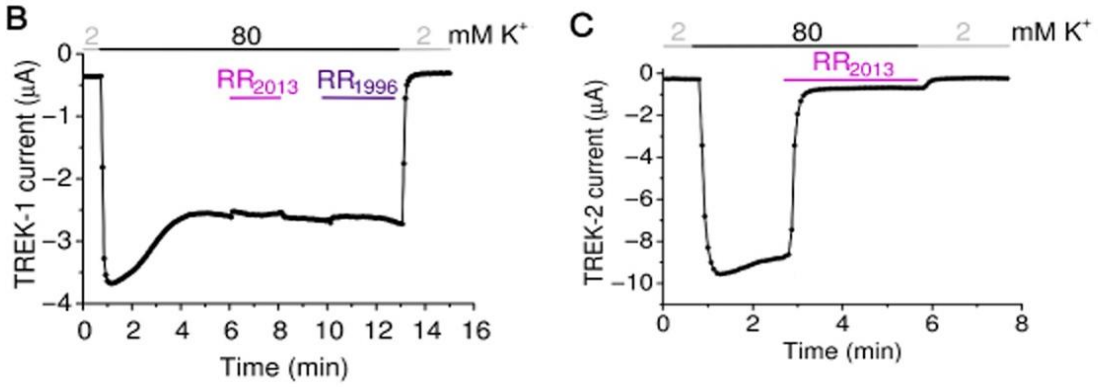

Fig.6. Ruthenium red inhibits TREK2 but fails to influence TREK1 current.

By mutating the amino acids located extracellularly and having negatively charged side chains, we also localized the site of the interaction of RR with TREK-2 within the extracellular ion pathway, close to the outer entrance of the pore. Among the other K2P channels RR inhibited TASK3 (in agreement with our earlier report) and TRAAK but in this case the IC50 was an order of magnitude higher (2 μM). During the course of the experiments the inhibitory effect of different RR batches differed significantly which led to the assumption that TRAAK inhibition could be related to a contamination in the RR preparation. We have purified this compound and identified it as ruthenium violet. Indeed, its inhibitory potency toward TRAAK was much higher ((IC50=0.1 μM) and it also turned out that RV binds at a different location to TRAAK: at the outer surface of the extracellular ion pathway. Accordingly, ruthenium compounds appeared to be useful tools for identification of specific background potassium channels also in native tissues. Indeed in a population of DRG neurons the leak potassium current was significantly inhibited by RR (published in: Br. J. Pharmacol. 2015; 172(7):1728-38. *Differential sensitivity of TREK-1, TREK-2 and TRAAK background potassium channels to the polycationic dye ruthenium red.*).

6/ Functional potassium channels are formed from subunits by tetramerization or in the case of K2P channels by dimerization. This may provide an even larger diversity of the final channels than the actual number of their coding genes if heteromerization is allowed. While this phenomenon is widespread among voltage dependent channels and inward rectifiers, in the K2P channel family we reported the first (and before our recent results the only) example for heteromerisation between TASK1 and TASK3. TREK-1, TREK-2 and TRAAK are the three members of a K2P channel subfamily; they show relatively large sequence similarity/identity, still their regulation, e.g. by extracellular pH, different drugs (eg. ruthenium red, RR) or bioactive molecules (eg. spadin) and the biophysical properties (e.g. their single channel conductance) differ significantly. The expression profile of these subunits in different tissues is partially overlapping thus the tissue distribution of the members of the TREK subfamily is consistent with the hypothesis that these K2P channels may constitute heterodimers in vivo.

In order to test whether the assumption about the heterodimerization of TREK channels is feasible, we engineered a concatamer (tandem) construct and examined its properties. We proved that the connected subunits of the tandem construct form a functional channel when this single polypeptide is expressed in *Xenopus* oocytes. Based on the characteristics of this tandem heterodimer (in which the interaction was forced by the covalent linkage of the subunits) we designed experiments to investigate whether the individually expressed subunits also form heterodimers. The heterodimers of individually expressed subunits were identified on the basis of their special pharmacological properties (characteristic spadin, pH and RR sensitivity), which had been found to be different from those of the TREK homodimers in the case of the tandem construct. The heteromerization of TREK-1 and TREK-2 subunits was verified also by single channel measurements and by coimmunoprecipitation. Relevant features of the heterodimer channel are its sensitivity to pH (extracellular acidification is inhibitory) and ruthenium red, its characteristic single channel conductance and its sensitivity to spadin (Fig. 7.).

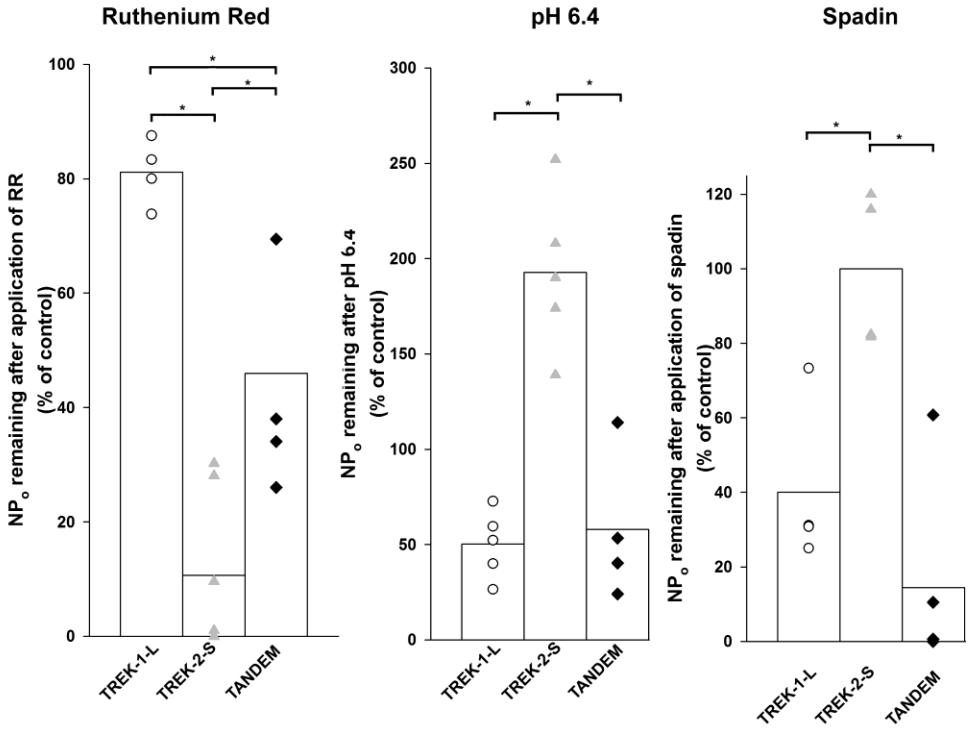

Fig. 7. Inhibitory effect of Ruthenium red, acidification and spadin on TREK1, TREK2 and TREK1/TREK2 tandem channel respectively

Heteromerization was confirmed also in a mammalian expression system and finally (based on the characterized pharmacological fingerprint) we demonstrated that in sensory DRG neurons heterodimers contribute to the background K^+ conductance of the cell (Fig. 8.). These results were published in The Journal of Biological Chemistry (2016 Jun 24;291(26):13649-61: *Formation of Functional Heterodimers by TREK-1 and TREK-2 Two-pore Domain Potassium Channel Subunits*.)

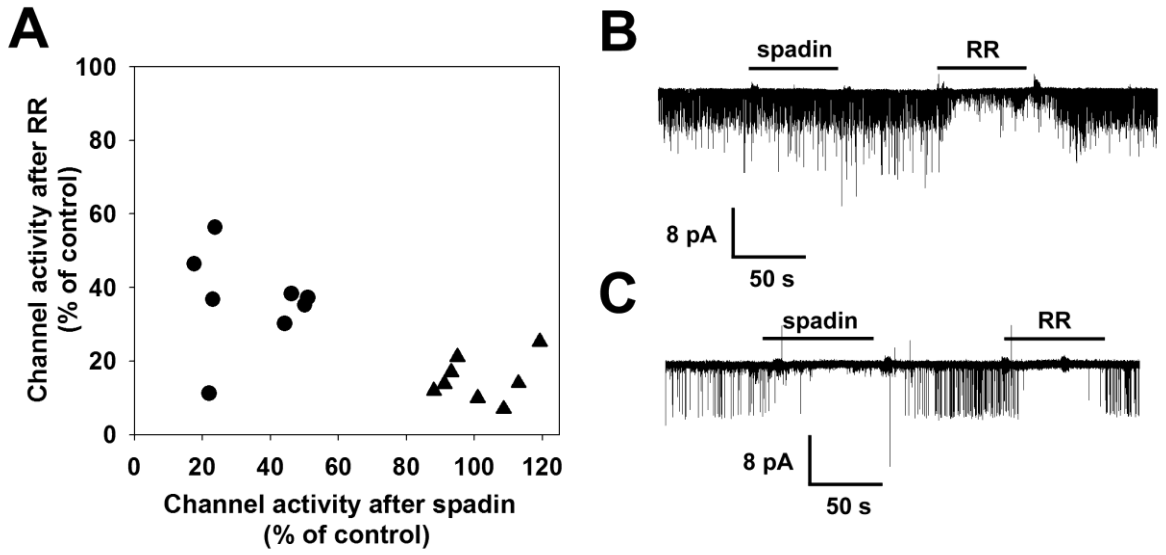

Fig. 8. Demonstration of TREK2 channels (being sensitive only to RR; triangles) and TREK1/TREK2 heterodimer channels (being sensitive to both spadin and RR; circles) in isolated DRG neurons with single channel patch clamping.

7/ Creation of TRESK gene deficient mouse strain: We had/have double aim with the modification of the TRESK gene in the mouse. First (although with cautious reservations regarding the results) we may gain insight into the significance of the channel when its gene is ablated. While the long term absence of TRESK may be compensated by overexpression of related K2P channels, the availability of the KO animal together with specific pharmacological tools, both inhibitors and activators, promised a valuable toolset for these studies. The other aim was to generate a knock-in mouse strain with tagged TRESK which otherwise does not differ from the wild type channel. This could have solved the lack of badly needed good quality specific antibodies (a general problem in the K2P channel field).
We engineered TALEN (mammalian expression) plasmid constructs which were first tested in a cell culture system and which efficiently modified (disrupted) the first exon of the TRESK gene. The TALEN constructs were subcloned into appropriate plasmids and *in vitro* cRNA was synthesized. We initiated a project (in collaboration with the Medical Gene Technological Unit of KOKI) in order to inject and implant mouse oocytes with the TALEN cRNA pair. We followed the event of desired gene modification by applying a modified surveyor mutation detection assay. The assay indicated a difference between the TRESK alleles in several offspring. Thus the homologous recombination event in TRESK gene has been successfully induced in these mice heterozygous TRESK mutations. We established a mouse strain in which a 27 base pair deletion coincided with the appearance of a premature stop codon, this was the founder of our KO strain. Then we engineered an AU1 epitope tagged TRESK channel construct. We verified in a heterologous (*Xenopus* oocyte) expression system that the tag (which

is inserted in the N-terminal intracellular domain) does not affect the activity and the regulation of the channel. The tagged channel was also tested and proved to be detectable with monoclonal AU1 antibodies even at high antibody dilutions. We inserted the relevant AU1 coding sequence into a genomic construct (which contained the first exon of TRESK with additional 5' and 3' flanking genomic regions) designed for homologous recombination in order to produce TRESK AU1-tag knock-in mice. In spite of strenuous efforts, no homologous recombination was detected in different trials although different approaches (using shorter synthetized donor molecules, changing DNA targeting approach to CRISPR/Cas method etc.) The final conclusion was that (possibly as a consequence of short base repeats in the targeted region) the N-terminal domain is not suitable for generating the knock-in construct. Therefore, we decided to tag the channel at another domain. Tagging the extracellular loop between the 1st and 2nd TMS resulted only in functionally impaired channels. Therefore finally the C terminal was chosen. After the necessary functional and immunostaining control experiment we started gene modification with the CRISPR/Cas method recently.

Taking advantage of the pharmacological repertoire for TRESK and TREK channels and also the TRESK deficient mouse strain we could analyze the contribution of TRESK and RR-sensitive conductances to the background K^+ conductance in a mixed sensory DRG neuron preparation. The results indicate at these two channel types are responsible for the majority of the current and complement each other. There was a set of neurons in which TRESK current represented the majority (up to 80-90% of the total) Fig. 9.. In these cells the TRESK inhibitor A2764 not only inhibited the current but, under current clamp measurements depolarized the membrane potential by 6.6 mV and significantly reduced the control from 615 ± 132 to 486 ± 138 pA. The inhibitor had no effect on these parameters in cells prepared from KO animals. These results (together with the cloxyquin analogue studies) are submitted for publication.

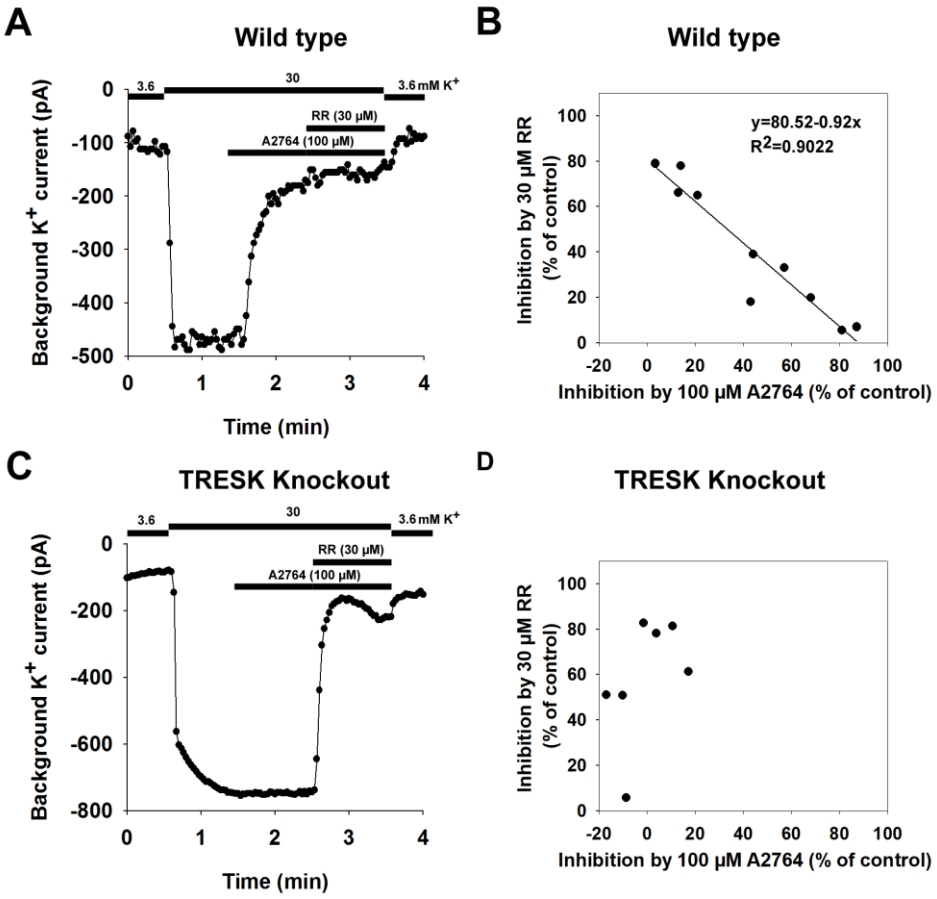

Figure 9. A2764 and ruthenium red inhibit the background K^+ current in isolated DRG neurons

The TRESK KO mice do not show any apparent phenotype under „everyday" conditions. Therefore, we initiated experiments in which the consequence of impaired function (or absence) of the channel is addressed under pathological or disease conditions. In collaboration with Mária Dux (Department of Physiology, Szeged University, an *ex vivo* mouse migraine model is assembled: In the course of migraine attacks liberation of CGRP (calcitonin gene related peptide) from trigeminal sensory neurons innervating the dura mater contribute to the vascular events. According to our preliminary results the absence or inhibition of TRESK does not influence the basal release of CGRP from dura preparations, however, A2764 augments the release induced by submaximal concentrations of the TRPV1 agonist capsaicin. In another experimental inflammatory reaction model, we plan to examine the contribution of TRESK to the painful response in an arthritis model in which the inflammation evoked by K/BxN serum (the project is a collaboration with the group of Attila Mócsai, in our department). Unfortunately, in the FVB-Ant mouse strain (in which the TRESK deletion was induced) is resistant to K/BxN-serum induced immune response, therefore we are in the progress of outcrossing the mutant TRESK gene into the Black6 strain; we are already waiting to the homozygous offsprings.