ABSTRACT BOOK

International Kv7 Channels Symposium 2019

Kv7 INTERNATIONAL Kv7 CHANNELS SYMPOSIUM

12 - 14 September 2019
Naples · Italy

www.kv7channels2019naples.org

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Oral Abstracts

KEYNOTE SPEAKER

[O1] NEURONAL KV7 M-CHANNELS: PROPERTIES AND REGULATION

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I joined the band of future Kv7 afficionados 40 years ago, when we discovered the M-current in bullfrog sympathetic neurons (Brown & Adams, 1979: ***Soc. Neurosci. Abstr***. 5:585; 1980:Nature, 283;673). This was seen as a voltage-dependent, subthreshold, non-inactivating potassium current that had a dramatic braking action on repetitive action potential discharges. It was dubbed "M-current" because it was inhibited by muscarine, acting on muscarinic acetylcholine receptors (mAChRs), thereby increasing action potential discharges. M-current is an imperfect descriptor because the current can also be inhibited by activating other Gq-type G protein-coupled receptors (GPCRs) and muscarine itself only works if the cell has M1, M3 or M5 receptors, not M2 or M4 (Robbins ***et al.***, 1991: ***Eur J Neurosci.*** 3: 820). The molecular composition of the M-channel was revealed some two decades later by David McKinnon and his colleagues as a combination of the ***KCNQ2*** and ***KCNQ3*** gene products Kv7.2 and Kv7.3 (Wang ***et al***., 1998: ***Science***. 282: 1890), probably as a 2+2 heteromer (Hadley ***et al***, 2003: ***J Neurosci.*** 23: 5012). Though gated by voltage, the channels require the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP2) to open (Zhang ***et al***., 2003: ***Neuron.*** 37: 963). Activation of M1 muscarinic receptors closes the channels by reducing the availability of PIP2 following its hydrolysis by Gq-activated phospholipase Cβ (PLCβ) (Suh & Hille, 2002: . ***Neuron.*** 35: 507; Winks ***et al***, 2005: ***J. Neurosci***. 25: 3400). This is facilitated by protein kinase C (PKC)-mediated phosphorylation of Kv7.2 induced by diacylglycerol (DAG), a product of PIP2 hydrolysis (Hoshi ***et al***, 2003: ***Nat Neurosci.*** 6: 564); PKC-phosphorylation lowers the "affinity" of the channel for PIP2 (Kosenko ***et al***., 2013: ***EMBO J.*** 31:3147). Channel closure following activation of some other receptors, such as bradykinin B2 receptors, results primarily from the effect of another product of PIP2 hydrolysis, inositol-1,4,5 trisphosphate (IP3), in releasing Ca^{2+} from internal stores (Cruzblanca et al, 1998: ***Proc Natl Acad Sci U S A.*** 95: 7151); this closes M-channels (Selyanko ***et al***., 1996: ***Neuron.*** 16:151) by interacting with cell-attached calmodulin (CaM; Gamper & Shapiro, 2003: ***J. Gen. Physiol***. 122: 17) and again reducing channel sensitivity to PIP2 (Kosenko ***et al***, ***op cit***). These regulatory processes involving PIP2, Ca^{2+}-CaM, PKC and A-kinase anchoring protein (AKAP 79/150, needed for PKC action), operate via overlapping sites on the Kv7 channel C-terminus.

[O2] OF MICE AND KCNQ2/3 CHANNELS

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Background: KCNQ2 and KCNQ3 potassium channels are now recognized as essential for normal brain function. Highlighting their importance, a growing number of loss-of-function (LOF) and gain-of-function (GOF) variants in KCNQ2 and KCNQ3 have been reported in patients with severe neonatal and infantile epileptic encephalopathy and more recently autism spectrum disorders. However, the precise role of KCNQ2 and KCNQ3 channels across different cell types and brain regions is not fully known.

Method: To probe the role of KCNQ2 and KCNQ3 channels in neuronal physiology, we use a series of conditional ***Kcnq2 and Kcnq3*** knock out mice as well as ***Kcnq2*** knock-in mice targeting glutamatergic and GABAergic neurons in the brain combined with electrophysiology and mesoscale calcium imaging.

Results: In this presentation I will present our recent efforts to understand the function of KCNQ2 and KCNQ3 channels in the developing brain. In particular, I will (i) discuss how the function of KCNQ2 and KCNQ3 channels is cell-type and brain region dependent and (ii) how cell-type ablation of KCNQ2 and KCNQ3 channels in neonatal brain alters forebrain circuit activity ***ex vivo*** and ***in vivo***.

Conclusion: KCNQ2 and KCNQ3 channels control the activity of multiple cell-types across multiple brain regions including the forebrain, thalamus, and brainstem.

SESSION 1: KV7 CHANNELS IN NEURONAL PHYSIOLOGY

[O3] KV7 CHANNELS AND PAIN: WHAT HAVE WE LEARNED THUS FAR?

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Background: Chronic pain is an unmet clinical problem with hundreds of millions of sufferers worldwide; its impact on individuals, societies and economies is hard to underestimate. Many types of pain cannot be successfully treated with current medications and most efficacious analgesics are, in themselves, a source of serious clinical and societal problems due to tolerance and dependence issues. Pathologic activity of the peripheral damage-sensing (nociceptive) afferent nerves is one of the major drivers of chronic pain. Since the excitability of a neuron is set and controlled by a complement of ion channels it expresses, in order to understand and treat pain, we need to develop a mechanistic insight into the key ion channels controlling excitability within the mammalian pain pathways. Here, I will discuss current data on the expression in pain pathways, functional role and a potential for therapeutic targeting of Kv7 channels, a family of K^+ channels, which is increasingly recognized as one of the important mechanisms controlling nociceptive fibre activity. I will focus on recent developments in the field as well as on the emerging understanding of the sites of algesic efficacy of Kv7 channel potentiating drugs ('openers') within the mammalian nervous system.

SESSION 1: KV7 CHANNELS IN NEURONAL PHYSIOLOGY

[O4] HYPEREXCITABILITY DURING NORMAL AGING AND AMYOTROPHIC LATERAL SCLEROSIS IS GOVERNED BY CHANGES IN M-CURRENT AND H-CURRENT

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Background: Amyotrophic lateral sclerosis (ALS) is the most common form of motor neuron disease (MND) and causes the degeneration of both upper and lower motor neurons. In patients and in animal models of MND it has been observed that there is a change in the properties of motor neurons, termed neuronal hyperexcitability, which is an exaggerated response of the neurons to a stimulus. Previous studies suggested neuronal hyperexcitability is one of the leading causes for neuronal loss. However, the factors that instigate excitability changes in neurons over the course of disease onset and progression are not well understood, as previous studies have looked mainly at embryonic or early postnatal stages (pre-symptomatic). As hyperexcitability is not a static phenomenon, the aim of this study was to assess the overall excitability of upper motor neurons during disease progression, specifically focusing on their oscillatory behavior and capabilities to fire repetitively.

Method: We assessed the electrophysiological properties of layer 5 motor neurons in the M1 motor cortex in pre-symptomatic (1-2 months) and symptomatic animals (5-7 months) and compared them to their wild-type littermate controls at the same ages, in order to assess the changes in excitability in normal aging and in ALS.

Results: Our results showed that increases in the intrinsic excitability of motor neurons are a global phenomenon of aging, however the cellular mechanisms that underlie this hyperexcitability are distinct in SOD1G93A ALS mice compared with wild-type controls. The ionic mechanisms driving increased excitability involved alterations of the expression levels of HCN and KCNQ channel genes leading to a complex dynamic of H-current and M-current activation. Moreover, we found a negative correlation between the disease onset and disease progression, which correlated with a decrease in the expression level of HCN and KCNQ channels.

Conclusion: These findings provide a potential explanation for the increased vulnerability of motor neurons to ALS with aging and highlight the central role of M-current and H-current in driving neuronal hyperexcitability in ALS.

SESSION 1: KV7 CHANNELS IN NEURONAL PHYSIOLOGY

[O5] KV7.4 CHANNELS AND MODULATION OF MONOAMINE NEURON EXCITABILITY

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Background: Monoamine, including dopamine and serotonin (5-HT) neurons in CNS are involved in human behavior including attention, mood and memory, and are implicated in the pathophysiology of many diseases and are the targets of many commonly used drugs. Firing pattern of these monoamine neurons are the key determinant for their function. A recent emerging development in the mechanism of major depression is the finding that alteration in neuronal excitability of VTA dopamine (DA) neurons in the midbrain is directed linked with the development of animal depression-like behavior. Based on these new developments, it is suggested that targeting the VTA DA neuron excitability is a potential new strategy for treatment of depression. For this, potassium channels which have been implicated in the altered excitability of VTA DA neurons in animal depression state, are interesting candidates for this new strategy.

Method: *in vitro* and *In vivo* electrophysiology techniques including whole-cell and loose-attached patch clamp, and extracellular recodings were used to recode the Kv7.4 currents, membrane potential and firing frequency and pattern of the action potentials from VTA dopaminergic neurons and from DRN 5-HT neurons; immunofluorescence and single cell PCR were used to detect the expression of Kv7.4; gene knockout and pharmacological tools were used to assess the role of Kv7.4.

Results: We found Kv7.4 is more selectively expressed in VTA DA and DRN 5-HT neurons. Using a newly identified selective Kv7.4 activator and Kv7.4 knock-out mice we demonstrate that Kv7.4 is a dominant modulator of neuronal excitability and targeting Kv7.4 in these neurons can alleviate the depression-like behavior in animal model of depression.

Conclusion: Kv7.4 play important role in modulation of excitability of monoamine neurons including VTA dopamine and DRN 5-HT neurons; Kv7.4 is a potential target for treatment of major depression.

SESSION 2: KV7 CHANNELS IN SMOOTH MUSCLE AND CARDIAC PHYSIOLOGY

[O6] KV7 CHANNELS IN SMOOTH MUSCLE: PROVIDING NOVEL INSIGHTS INTO CHANNEL TRAFFICKING

Thomas Jepps[1]

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Background: In the last decade, Kv7 channels have been established as important regulators of contractility in vascular and non-vascular smooth muscle. ***KCNQ*** transcript and protein expression have been identified in an array of vascular and non-vascular smooth muscle where Kv7.1, 7.4 and 7.5 predominate. The use of various Kv7 channel activators and blockers has allowed a functional role of the Kv7 channels to be determined in multiple smooth muscle tissues, including the vasculature, uterus, bronchi, gastrointestinal tract, bladder and penis, where they contribute to setting the resting membrane potential and are functional end-points for several intracellular signalling molecules.

Method: A multitude of techniques have been used to elucidate the expression and functional role for Kv7 channels in smooth muscle. Our latest findings on Kv7 channels in vascular smooth muscle were obtained with isometric tension recordings, immunocytochemistry, proximity ligation assays and in vivo myography.

Results: Much of our overall understanding of how Kv7 channels are trafficked and regulated comes from research outside the smooth muscle field. However, recently we have shown, for the first time that the microtubule network plays an important role in the regulation of Kv7 channels, with the disruption of the microtubule network increasing the membrane expression and functional contribution of Kv7.4 channels in vascular smooth muscle cells. Furthermore, we have found the microtubule motor protein, dynein, to be a critical regulator of Kv7.4 channel membrane expression and identified critical dynein-binding sites in the c-terminus of the Kv7.4 channel protein, which are conserved throughout the Kv7 channel family.

Conclusion: Almost 25 years after the KCNQ genes were discovered, the importance of Kv7 channels is established in multiple physiological systems, and we are still constantly discovering new roles for these channels and new ways in which they are regulated. The novel microtubule-dependent regulation of Kv7 channels that we have discovered in vascular smooth muscle has implications not only in other smooth muscle systems but also throughout Kv7 channel physiology.

[07] RATIONAL DESIGN OF FATTY ACID ANALOGUES TARGETING THE CARDIAC KV7.1 CHANNEL

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Background: Polyunsaturated fatty acids (PUFAs) and their analogues affect many ion channels and have been proposed to have anti-epileptic and anti-arrhythmic effects in the brain and the heart. The molecular mechanism behind these effects are not completely understood. The overall aim of this project was to determine the molecular mechanism of how PUFA analogues activate the cardiac Kv7.1 channel and how to utilize this understanding to rationally design PUFA analogues with improved effect.

Method: Electrophysiological techniques were used to test the effect of PUFAs and PUFA analogues on the human $K_V7.1$ channel (expressed in ***Xenopus*** oocytes), cardiomyocytes, and isolated guinea pig hearts. Computer simulations and electrophysiology were used to study the mechanism of action of PUFA analogues on the $K_V7.1$ channel.

Results: We observe that PUFA analogues activate the $K_V7.1$ channel by shifting the voltage dependence of channel opening towards more negative voltages and increasing the maximal conductance. The shift in channel voltage dependence is caused by the negatively charged head group of the PUFA analogue interacting with a positively charged arginine in the voltage-sensing domain of the channel. The increase in maximal conductance is caused by the negatively charged head group of the PUFA analogue interacting with a positively charged lysine in the pore domain. The chemical structure of the head group and the lipid tail determines how effective PUFA analogues are to activate the $K_V7.1$ channel. Specific PUFA analogues act anti-arrhythmically in cardiomyocytes and guinea pig hearts.

Conclusion: We anticipate that the molecular understanding of how PUFA analogues activate the $K_V7.1$ channel will allow for future development of more effective anti-arrhythmic drugs based on PUFA analogues.

SESSION 2: KV7 CHANNELS IN SMOOTH MUSCLE AND CARDIAC PHYSIOLOGY

[O8] AN IPSC-BASED SYSTEM TO STUDY KV7.1/KCNE1 IN HUMAN CARDIOMYOCYTES

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Background: The heart allows for coordinated cardiac function whereas the physiological acute stress reaction is associated with positive chronotropic and inotropic effects on heart muscle cells mediated through β-adrenoceptor activation. Increased systolic calcium is required to enable stronger heart contractions whereas elevated potassium currents are to limit the duration of the action potentials and prevent arrhythmia. The latter effect is accomplished by an increased functional activity of the K_v7.1 channel encoded by the ***KCNQ1 gene***.

Method: We have studied K_v7.1/KCNE1 channel pharmacology in wt iPSC cardiomoycytes.

Results: Because **c**urrent iPSC-based cardiac technology is limited in the study of K_v7.1/KCNE1 channel due to weak expression of the channel, we have successfully developed an inducible genetic ***KCNQ1*** complementation approach in ***KCNQ1***-deficient human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) to control the expression level of K_v7.1/KCNE1 channels.

Conclusion: This novel iPSC cell line is well suited to study physiological as well as pharmacological features of the channel in a human iPSC cardiomyocytes.

SESSION 2: KV7 CHANNELS IN SMOOTH MUSCLE AND CARDIAC PHYSIOLOGY

[O9] ORGANOTYPIC EXPRESSION OF VASCULAR SMOOTH MUSCLE KV7.1 CHANNELS - POTENTIAL FOR RENOPROTECTION?

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Background: Blood flow regulation is controlled largely by vascular smooth muscle voltage-gated potassium (Kv) channels, especially Kv7.4 channels. Kv7.1 channels are also expressed in vascular smooth muscle but at different levels compared to Kv7.4 channels in different vessels. This study addressed the hypothesis that Kv7.1 channels expressed at a similar level as Kv7.4 channels contribute to the regulation of arterial contractility.

Method: Mesenteric, coronary, skeletal muscle and renal segmental arteries of Wistar rats were studied using real-time qPCR and isometric myography. Intact kidneys were explored using constant flow perfusion.

Results: A functional impact of Kv7.1 channels was postulated when the effect of specific Kv7.1 channel openers (R-L3, ML277) on methoxamine (MX)-induced contraction was antagonized by the specific blocker HMR1556. In skeletal muscle arteries, the expression level of Kv7.1 channels was much smaller than that of Kv7.4 channels and no functional impact of Kv7.1 channels was observed. A similar expression level of Kv7.1 and Kv7.4 channels was found in coronary, mesenteric and renal arteries. Only in mesenteric and renal, but not in coronary arteries a functional impact of Kv7.1 channels was detected. In all vessels, HMR1556 alone was without effect on MX-induced contraction. Further, in renal arteries HMR1556 did not affect the anticontractile effect of the cGMP-coupled vasodilator ANP and the cAMP-coupled vasodilator urocortin. Notably, the effect of ANP and urocortin were the same in the absence and the presence of R-L3. In addition, R-L3 reduced methoxamine-induced perfusion pressure in intact perfused kidneys. This effect was attenuated considerably by HMR1556. HMR1556 alone was without effect on methoxamine-induced perfusion pressure. Urocortin reduced methoxamine-induced perfusion pressure. This, effect was the same in the absence and the presence of R-L3.

Conclusion: The results show that Kv7.1 channels are expressed in different rat arteries. Only when expressed at a similar level compared to Kv7.4 channels, pharmacological opening of these channels was able to alter arterial contractility, particularly in renal segmental arteries. In these vessels Kv7.1 channels do not contribute to MX-induced contraction or ANP- and urocortin-induced relaxation. After pharmacological activation, Kv7.1 channels reduce basal tone and basal perfusion pressure, but leave MX-induced contraction as well as ANP- and urocortin-induced relaxation intact. Thus, Kv7.1 channel activation improves renal perfusion without altering vasoconstrictor- or vasodilator-evoked regulation suggesting that these channels may serve as targets for renoprotection.

SESSION 3: KV7 CHANNEL REGULATION

[O10] PHOSPHORYLATION OF KV7.2 SUBUNITS AND ITS ROLE IN PATHOLOGY

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Background: It has been revealed that Kv7.2 subunits anchor several signaling enzymes including kinases. In particular, protein kinase C plays an important role in M-current suppression induced by Gq-coupled receptors. Numerous ***in vitro*** studies demonstrate M-current suppression transiently increase neuronal excitability. However, its role ***in vivo*** is not well understood.

Method: We generated knock-in mice that lack the critical PKC phosphorylation acceptor in Kv7.2 subunit. These Kv7.2 knock-in mice have been tested for several chemoconvulsants. Induced seizures, neuronal damage and epileptogenesis after an initial status epilepticus have been evaluated.

Results: Our Kv7.2 knock-in mice showed milder seizures against chemoconvulsant-induced seizures. Even when equivalent status epilepticus has been delivered by co-administration of XE991 with pilocarpine, Kv7.2 knock-in mice showed minimal neuronal death after initial seizures. In addition, Kv7.2 knock-in mice did not develop spontaneous recurrent seizures after three weeks from initial seizures.

Conclusion: These results suggest that M-current suppression during and after seizures plays important roles for pathophysiology of seizures as well as seizure-induced brain damage.

SESSION 3: KV7 CHANNEL REGULATION

[O11] ACTIVITY-DEPENDENT REGULATION OF NEURONAL KCNQ CHANNEL TRANSCRIPTION: FROM PERIPHERAL GANGLIA TO BRAIN

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We have previously shown KCNQ2 and KCNQ3 channel transcription to be upregulated in sympathetic ganglia, and in hippocampus after a seizure. We have now examined in detail the region-specific up-regulation of M-channel transcription in hippocampus following moderate hyperactivity that does not provoke seizure, and after a dose of chemoconvulsant that does induce seizure in mice. To monitor KCNQ2 transcription, we utilized reporter mice that are transgenic for a bacterial artificial chromosome (RP23-247P15) containing the ***kcnq2*** locus with enhanced green fluorescent protein (EGFP) in the first exon (***(Kcnq2-EGFP)-FW221Gsat,*** GENSAT). In such KCNQ2-mRNA EGFP reporter animals, the transcription of KCNQ2 mRNA occurred concomitant with EGFP protein expression, serving as a probe for KCNQ2 transcription. We also performed Chromophore ***In Situ*** Hybridization (CISH) assays for KCNQ2 and KCNQ3, and brain-slice electrophysiology. We find that induction of moderate or profound hippocampal hyper-excitability increases KCNQ2 RNA after 48 hr in CA1 and CA3, but not in dentate gyrus (DG), which dissipated by 7 days. Brain-slice whole-cell clamp recordings revealed parallel increases in M-current amplitudes and dampened excitability in CA1 pyramidal neurons, but not DG granule cells. The CISH results paralleled the immunofluorescent experiments. In a blunt model of traumatic brain injury (TBI), moderate trauma, which is known to induce hyper-excitability, induced up-regulation of KCNQ2 mRNA in the ipsilateral, but not the contralateral, hemisphere to the injury, including cortical and hippocampal neurons. In such mice, a sub-threshold dose of chemoconvulsant nonetheless provoked increases in KCNQ2, but not KCNQ3, transcription. Acute application of retigabine, post-TBI, enhanced the increase in KCNQ2 expression. We suggest that transient (~7 days) up-regulation of KCNQ2 transcription may serve as a protective mechanism against the prompt deleterious cascade of seizure progression from many causes, which may normally have a temporary time-window, but which could potentially be extended by pharmacological intervention.

[O12] STRUCTURAL DETERMINANTS OF KV7.5 POTASSIUM CHANNELS THAT CONFER CHANGES IN PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE AFFINITY AND SIGNALING SENSITIVITIES IN VASCULAR SMOOTH MUSCLE CELLS

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Background: Kv7 family voltage-dependent potassium channels have well established roles in the regulation of excitability of smooth muscle cells, which commonly express two functional Kv7 channel α-subunits, Kv7.4 and Kv7.5. Previous research suggests that Kv7.4 and Kv7.5 channels have different sensitivities to stimulatory signaling via cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), and via protein kinase C (PKC).

Method: Chimeric channels composed of different components of the Kv7.4 and Kv7.5 α-subunits were expressed in vascular smooth muscle cells to determine which structural elements are essential for cAMP/PKA-dependent enhancement of channel activity, or PKC-dependent suppression of channel activity. Whole cell patch clamp techniques were used to measure Kv7 currents.

Results: Forskolin (1 μM), a direct activator of the cAMP/PKA pathway, enhanced wild-type Kv7.5, but not wild-type Kv7.4 currents. Replacing the amino-terminus of Kv7.4 with the amino-terminus of Kv7.5 conferred partial responsiveness to forskolin. In contrast, swapping the carboxy-terminal domain was without effect on the forskolin response, but conferred responsiveness to arginine-vasopressin (an inhibitory response mediated by PKC). Serine-to-alanine mutation at position 53 of the Kv7.5 amino-terminus abrogated its ability to confer sensitivity of Kv7.4 to forskolin. Using voltage-dependent phosphatase (ci-VSP) to manipulate membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) levels, we found that forskolin treatment reduced the sensitivity of Kv7.5 channels to ci-VSP-induced PIP_2 depletion, whereas direct activation of PKC with phorbol-12-myristate-13-acetate (PMA) (but not with inactive analog 4α-PMA) potentiated the ci-VSP-induced decline in Kv7.5 current amplitude. Under resting conditions, Kv7.4 and Kv7.5 exhibited differential responsiveness to ci-VSP activation and different rates and extent of current rundown in ruptured patch recordings.

Conclusion: Our findings suggest that PKA-dependent phosphorylation of serine 53 on the amino-terminus of Kv7.5 channels increases the affinity of Kv7.5 for PIP_2, while PKC-dependent phosphorylation of the Kv7.5 carboxy-terminus is associated with a reduction in PIP_2 affinity; these changes in PIP_2 affinity have corresponding effects on current amplitude. Transfer of the amino-terminus or the carboxy-terminus of Kv7.5 onto Kv7.4 is sufficient to confer sensitivity of Kv7.4 to PKA- or PKC-dependent regulation, respectively. Resting affinities for PIP_2 also appear to differ for Kv7.4 and Kv7.5.

SESSION 3: KV7 CHANNEL REGULATION

[O13] KV7.1 CHANNELS IN COLORECTAL CANCER AND EPITHELIAL REPAIR

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Background: Kv7.1 has been proposed as a tumour suppressor in colorectal cancer (CRC). Kv7.1 has also been implicated in epithelial repair.

Method: We investigated the molecular mechanisms regulating Kv7.1 expression in CRC and their contribution to epithelial-mesenchymal transition (EMT) of CRC cells. We also investigated differential spatio-temporal relationship between Kv7.1 and Kca3.1 channel expression with β-catenin and EGFR proliferative signalling pathways during CRC epithelial wound closure.

Results: The expression of Kv7.1 channels was lost with increasing mesenchymal phenotype in poorly differentiated CRC cell lines as a consequence of repression of the Kv7.1 promoter by β-catenin:TCF4. In well-differentiated epithelial CRC cell lines, Kv7.1 was located at the plasma membrane in a complex with β-catenin and E-cadherin. ShRNA knock-down of Kv7.1 caused a re-localization of β-catenin from the plasma membrane into the cytosol and nucleus which was associated with disruption of tight junctions and a loss of epithelial phenotype in CRC spheroid 3-D cell cultures. Analysis of human primary CRC tumour patient databases showed a female gender-specific positive correlation between the expression of ***KCNQ1*** and ***KCNE3*** genes with disease-free survival. Moreover, estrogen caused uncoupling of the Kv7.1:KCNE3 channel complex to promote endocytotic recycling of Kv7.1 to capture cytosolic β-catenin for plasmamembrane relocalization.

In wound healing, Kv7.1 expression decreased while Kca3.1 and the Wnt/β-catenin were activated over the intermediate phases of epithelial repair following a scratch wound. Pharmacological inhibitors or gene silencing of Kv7.1 channels reduced cell migration and delayed wound closure. In contrast, inhibition of Kca3.1 channels produced an early increase in cell migration but delayed cell proliferation. During the terminal phase of closure of the wound, Kv7.1 channels were re-activated to enhance the formation of tight junctions and restore epithelial polarity while Kca3.1 expression and activated β-catenin were switched off to terminate cell proliferative signalling pathways. Reverse phase protein micro-array pathway analysis indicated that Kv7.1 channels regulate the initial phases of wound closure, cell protrusion, actin cytoskeleton remodelling, cell-cell adhesion, cell spreading and migration. Kca3.1 channels, on the other hand, activate key intermediates of cell proliferation including Wnt/β-catenin and MAPK pathways to stimulate the proliferative phase of epithelial repair.

Conclusion: We conclude that Kv7.1 is an estrogen-regulated target of the Wnt/β-catenin pathway and Kv7.1 repression leads to CRC cell proliferation, EMT and tumorigenesis. This work has also helped define the spatio-temporal role of Kv7.1 and Kca3.1 channels in the epithelial repair process and their signalling targets modulating cell migration and proliferation.

KEYNOTE SPEAKER

[O14] MULTIFACETED KV7 POTASSIUM CHANNELS: FROM HEART WAVES TO NEURONAL PLASTICITY

Bernard Attali[1]

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The initial discovery of the M-current in the nervous system and the subsequent molecular, structural and physiological characterization of the Kv7/KCNQ new family of voltage-gated K+ channels have opened large avenues in the understanding of the mechanisms by which these membrane proteins play crucial roles in the physiology of many systems and are centrally involved in human diseases. Five Kv7 potassium channel members form a subfamily of voltage-gated K+ channels (Kv) that play important functions in various tissues including brain, heart, kidney, stomach, pancreas or inner ear. In the heart, co-assembly of Kv7.1 with KCNE1 produces the IKS current, which together with IKr (hERG channel) form the main repolarizing currents of the cardiac action potential. In the brain, the complexes formed by Kv7.2/3 and Kv7.3/5 subunits produce the so called "M-current", a slowly activating, non-inactivating K+ current modulated by muscarinic agonists and other Gq protein-coupled receptor agonists. M-current has profound effects on neuronal excitability, as its low voltage-threshold for gating and slow activation act as a brake for repetitive firing. Kv7 channels have a prominent role in human channelopathy diseases and can harbor numerous mutations that produce severe cardiovascular and neurological disorders. For many years, our laboratory focuses on the structural, biophysical and physiological attributes of normal and diseased Kv7 channels. First, I will address the importance of the gating module located in the proximal C-terminus of Kv7 channels, which is engaged in complex interactions with calmodulin and PIP2 and is involved in the integration in time and space into activation and inactivation gating under normal and diseased conditions. I will also describe the nature of Kv1 channel gating, by using a thermodynamic mutant cycle analysis. Finally, I will show how sustained M-channel inhibition triggers at different temporal scales intrinsic and synaptic homeostatic plasticity in hippocampal pyramidal neurons.

SESSION 4: STRUCTURAL ASPECTS OF KV7 CHANNEL FUNCTION

[O15] A CLOSER LOOK AT CALCIUM REGULATION OF KCNQ CHANNELS

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Background: KCNQ channels (Kv7) are regulated by Ca2+ through conformational changes imposed into calmodulin (CaM). Recent cyo-EM images show that four CaM molecules, each bound to helices A and B of the four subunits, are forming a ring just underneath the intracellular pore gate, and directly apposed to the voltage sensor. Helix A is directly connected to the S6 transmembrane segment. The S6 bundle crossing forms the inner gate of the pore, and helix B is connected through helix C to the coiled-coil tetrameric helix D.

Method: To investigate CaM mediated Ca2+ gating of the channel, we used fluorescence and NMR spectroscopy to structurally and dynamically describe the association of helices hA and hB of Kv7.2 with calmodulin (CaM), as a function of Ca2+ concentration.

Results: We found that the distal coiled-coil stabilizes the CaM/AB complex, promoting trans-binding (CaM embracing neighboring subunits) and modulates PIP2 sensitivity, such as the BFNE L619R mutation de-stabilizes the tetramer, weakens CaM binding, and perturbs the influence of CaM on PIP2 sensitivity and voltage-dependence. Our data suggest that hB is always engaged with the N-lobe loaded with Ca2+ and, thus, this lobe does not participate in transducing Ca2+ information to the channel. On the other hand, hA suffers conformational changes and remains engaged to the C-lobe upon Ca2+ loading. The conformational change is observed upon loading just the third of the four Ca2+-biding sites, and consist of a small rotation of the C-lobe around the cylinder that helix A represents, accompanied by a 18º torsion of the angle between helix A and helix B.

Conclusion: The calcium sensor forms a hub that reciprocally influences, through physical contacts with different domains of the channel, voltage-dependence, PIP2 sensitivity and tetramerization and trafficking. Since helix A is directly bound to the internal pore gate conformed by the four S6 bundle crossing, we hypothesize that the 18º rotation of helices A and B is transmitted to the inner gate, regulating the passage of K+ ions in response to Ca2+ intracellular oscillations.

SESSION 4: STRUCTURAL ASPECTS OF KV7 CHANNEL FUNCTION

[O16] HIGH RESOLUTION VIEWS OF KCNQ MODULATION

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Kv7 (KCNQ) voltage-gated potassium channels control excitability in the brain, heart, and ear. Calmodulin (CaM) is crucial for Kv7 function. Our integrated structural biology studies establish a regulatory mechanism for Kv7 CaM modulation based on a common architecture in which a CaM C-lobe calcium-dependent switch releases a shared Apo/CaM clamp conformation. This C-lobe switch inhibits voltage- dependent activation of Kv7.4 and Kv7.5 but facilitates Kv7.1, demonstrating a common mechanism that is shared by Kv7 isoforms despite the different directions of CaM modulation. Our findings provide a unified framework for understanding how CaM controls different Kv7 isoforms and highlight the role of membrane proximal domains for controlling voltage-gated channel function.

SESSION 4: STRUCTURAL ASPECTS OF KV7 CHANNEL FUNCTION

[O17] MECHANISM OF VOLTAGE-DEPENDENT ACTIVATION OF KV7.1

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Background: The voltage-gated Kv7.1 potassium channel is open when the voltage sensor domain (VSD) is activated to either the intermediate state (intermediate open state, IO) or the fully activated state (activated open state, AO). The IO and AO states show different functional properties, such as voltage dependence, ion selection, and pharmacology, which underline the versatile tissue specific phenotypes of Kv7.1 channels.

Method: In this study, with collaboration of several laboratories, we show the structural basis of these VSD activation steps and how individual steps contribute to electromechanical coupling between the VSD and the pore domain to open the channel.

Results: Our results reveal the VSD motion from the intermediate to activated state. Each step of VSD activation evokes a distinctive set of interactions necessary to trigger pore opening. The results suggest that when the S4 segment in the VSD moves to the intermediate state the C-terminus of the VSD-pore linker (S4-S5L) interacts with the pore in the same subunit; when S4 subsequently moves to the activated state the joint between S4 and S4-S5L interacts with the pore of a neighboring subunit to open the pore.

Conclusion: This gating mechanism may broadly apply to other K_V channels.

[O18] STEPWISE ACTIVATION OF TETRAMERIC KCNQ1 CHANNEL COMPLEXES, IN THE PRESENCE AND ABSENCE OF KCNE1

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Background: A potassium current in the heart (I_{Ks}) is important in regulating the heartbeat. The current has an established role in regular cardiac action potential repolarization, as it activates during the late plateau phase of cardiac systole, and provides a repolarization reserve at times of higher heart rate to support increased cardiac output. The underlying tetramers of KCNQ1 co-assemble along with 1-4 KCNE1 accessory subunits and have four voltage-sensitive activating domains and a pore-component, which opens to allow current to pass through. However, how these components together gate the I_{Ks} complex to open the pore is controversial. The number of voltage-sensitive components required to activate in order to allow the pore to open has been debated extensively, and currently, either a concerted movement involving all four subunits of the tetramer, or allosteric regulation of open probability through voltage-dependent subunit activation are thought to precede opening.

Method: By using the locking E160R mutation in KCNQ1 or the F57W mutation in KCNE1 to prevent or impede, respectively, voltage sensors from moving into activated conformations, we demonstrate that a concerted transition of all four subunits after voltage sensor activation is not an obligatory step preceding pore opening in KCNQ1 or I_{Ks} channels. E160R subunits incorporate normally into KCNQ1 channel complexes as determined by introduced extracellular TEA+ sensitivity via V319Y residues in the same subunits. As well, tracking voltage sensor movement, via MTSET modification and fluorescence recordings, shows that E160R-containing voltage sensors do not translocate to be detected externally upon depolarization.

Results: E160R, when expressed in all four KCNQ1 subunits, produces non-conducting channel complexes, even when expressed with KCNE1, but if one, two, or three voltage sensors contain the E160R mutation, whole cell and single channel currents are still observed in both the presence and absence of KCNE1, and average conductance is reduced proportionally to the number of E160R voltage sensors. A model of independent voltage sensor movement, incorporating intermediate and activated voltage sensor states directly coupled to open states can simulate experimental changes in I_{Ks} current kinetics, including the non-linear depolarization of the G-V, and tail current acceleration as the number of non-activatable E160R subunits is increased.

Conclusion: The data suggest that KCNQ1 + KCNE1 channels gate like KCNQ1 alone, with movement of each component resulting in increasingly more current. This makes for a uniquely flexible channel complex, tunable for a broad range of responses.

SESSION 5: NOVEL INTERACTIONS AND ASSOCIATIONS

[O19] DIGGING DEEPER: G PROTEIN BETA GAMMA SUBUNIT REGULATION OF CARDIOVASCULAR KV7 CHANNELS

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Background: The Kv7 family of voltage gated potassium channels have important roles in the cardiovascular system where Kv7.1 channels contribute to the cardiac action potential and Kv7.4 and Kv7.5 channels are important in vascular control mechanisms. Recently it was shown that Kv7.4 channels and native vascular Kv7 channels are positively regulated by G protein βγ subunits (Gβγ), and that these subunits are essential for the basal activity of these channels. However, there are 5 different Gβ and 12 Gγ subunits and it is increasingly clear that in the regulation of other Gβγ effectors individual isoform combinations can perform specific functions. Additionally, the role of Gβγ in regulation of Kv7.1 channels is unclear. Kv7.1 channels are most commonly found in cardiac myocytes complexed with an auxiliary subunit – KCNE1. The presence of this subunit alters the biophysical properties of the channel and is important in transducing regulatory actions on Kv7.1, such as by the A-kinase Anchoring Protein.

Method: Gβγ effects on Kv7.1 and Kv7.4 were studied by a combination of electrophysiology on heterologously expressed channels in Chinese Hamster Ovary cells, proximity ligation assay in overexpressed cells and native vascular myocytes, and morpholino directed knockdown of specific Gβ subunits in rat renal arteries.

Results: Strikingly, we now show that Gβγ negatively regulate Kv7.1 and Kv7.1/E1 channels which has profound implications on cardiac function and regulation. For Kv7.4 channels we show that only specific Gβ subunits (Gβ 1, 3 and 5) positively regulate channels, adding a further layer of complexity to this mechanism of regulation. We further show that it is the Gβ3 subunit which is responsible for the basal activity of Kv7 channel in vascular smooth muscle (renal artery), whereas the Gβ1 subunit appears to contribute to Kv7.4 protein synthesis or stability.

Conclusion: These findings demonstrate the critical role of Gβγ in the regulation of cardiovascular Kv7 channels and are significant in revealing the fundamental importance of Gβ specificity in ion channel regulation.

SESSION 5: NOVEL INTERACTIONS AND ASSOCIATIONS

[O20] KV7 CHANSPORTER COMPLEXES

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Background: Voltage-gated potassium (Kv) channels open in response to depolarization of the cell membrane, permitting diffusion of K^+ ions through the channel pore, down their electrochemical gradient. Members of the Kv7 (KCNQ) family of Kv channels serve a wide variety of roles in the nervous system, vasculature, heart and many epithelia. Sodium-coupled solute transporters such as the SMITs exploit the downhill sodium gradient to transport molecules such as ions, sugars and neurotransmitters into the cell.

Method: We utilized mouse genetics, cellular electrophysiology techniques, tritiated *myo*-inositol uptake assays, immunofluorescence and protein biochemistry to discover and characterize the functional and physical assocation between Kv7 channels and specific sodium-coupled solute transporters.

Results: We originally discovered that Kv7.1 channels form reciprocally regulating complexes with sodium-coupled *myo*-inositol transporters (SMITs) in the choroid plexus epithelium. We subsequently found that Kv7.2/7.3 channels also form complexes with SMITs, in the nervous system and when heterologously co-expressed. The complexes form via the channel pore module and physical association of the transporter alters Kv7 channel gating and ion selectivity. Our latest findings uncover novel types of crosstalk within chansporter complexes, including how ion channels act as biosensors for various types of sodium-coupled transporters.

Conclusion: Coupling with Kv7 channels appears to facilitate optimal activity of SMITs and other sodium-coupled transporters, while also enabling regulatory feedback. In addition to our work, other groups have recently reported functional and/or physical association of Kv7 channels with a variety of sodium-coupled transporters. Together with additional studies describing analogous complex formation between other classes of channels and transporters, the new findings reveal a previously largely overlooked mechanism for functional crosstalk and a new type of cellular signaling hub, which we refer to as “chansporter complexes”.

SESSION 5: NOVEL INTERACTIONS AND ASSOCIATIONS

[O21] KCNQ1 AN UNEXPECTED MEDIATOR OF COLD AVOIDANCE

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Background: Menthol, a topical analgesic substance, as well as low temperatures evoke cold sensation by activating transient receptor potential channels (TRPM8, TRPA1) in peripheral cold receptor neurons. However, block of potassium channels by menthol and cold might be an additional mechanism of cold sensation which was investigated in the current study.

Method: We employed a combination of whole-cell electrophysiology, computational modelling, immunocytochemistry, immunohistochemistry, RT-PCR, thermal preference assay and nociceptor recordings, to study the action of menthol and temperature on neuronal different potassium channels.

Results: Here, we found that menthol is an open channel blocker of KCNQ1 channels as it interacts with the residues within the central cavity of the channel. Surprisingly, KCNQ1 channels are expressed in skin and dorsal root ganglion neurons and show highest temperature sensitivity in mild cold temperatures between 25 and 35°C. Strikingly, KCNQ1$^{-/-}$ mice were not able to avoid cold as efficient as the wild type mice and nociceptors of KCNQ1$^{-/-}$ mice had smaller cold response.

Conclusion: We describe for the first time a functional expression of KCNQ1 in the peripheral nervous system. Due to the its functional expression pattern in peripheral nervous system and menthol- and cold-sensitivity, we propose that the KCNQ1 channel may act as a modulator of cold sensation and provide a binding mechanism for menthol.

SESSION 5: NOVEL INTERACTIONS AND ASSOCIATIONS

[O22] VERSATILE MODULATION OF KV7-MEDIATED K+ CURRENTS THROUGH (SILENT) MODIFIER KV CHANNEL SUBUNITS

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Background: Functional voltage-gated potassium (K_v) channels are composed as homotetramers of four individual subunits, but may also be heterotetramers formed by members within the same family. Of the twelve K_v channel families known (K_v1-K_v12), members of four families (K_v5, K_v6, K_v8, K_v9) are particular exceptions in two respects: Firstly, they do not form functional homotetrameric channels, i.e. when expressed alone, these channels are electrically silent (termed K_vS). Secondly, these K_vS subunits assemble with co-expressed K_v2 subunits into heteromeric K_v2/K_vS channel complexes with unique properties and thus are physiologically-important modifiers of K_v2 channels. This K_v2-K_vS interaction may constitute the only conclusive example yet known for heteromeristation of subunits of distinct K_v channel families.

Method: Phylogenetic analyses indicated somewhat closer relationship of K_v7 channels to K_vS and K_v2 than to the other K_v families. We thus hypothesized that K_vS may also constitute modifiers of K_v7 family members, and analyzed properties of recombinant K_v7 channels co-expressed with K_vS. We also generated K_vS-knock-out mice to study potential physiological relevance of such modulation in native tissue and living animals.

Results: We found that in Chinese hamster ovary (CHO) cells co-expression of certain K_v5, K_v6, K_v8 or K_v9 subunits significantly affected the voltage dependence of K_v7.2 and K_v7.4 subunits as well as voltage-dependent steady-state currents through these K_v7 isoforms. In line with the observed changes of whole cell current amplitudes, co-expression of certain K_vS modulated membrane expression levels of K_v7.2 in ***Xenopus laevis*** oocytes. Proximity ligation assays (PLA) and ***co-immunoprecipitation*** (Co-IP) indicated that K_vS and K_v7 subunits were located in very close proximity and closely interacted in the employed expression systems. To further analyze this unknown interaction, we generated K_vS-knock-out mice. Careful analysis of neurons natively co-expressing K_vS and K_v7 subunits will unravel potential (neuro)physiological relevance of K_vS-K_v7 heteromeric channels in-vivo and in-vitro.

Conclusion: Taken together, our results demonstrated that silent K_v subunits modified the properties of neuronal K_v7 channel subunits and provided strong evidence for functional K_v channel heteromers formed by members of distinct K_v channel families.

[O23] IDENTIFICATION OF NOVEL KV7.2/KV7.3 OPENERS USING ADVANCED HIGH-THROUGHPUT SCREENING TOOLS

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Background: The voltage-gate potassium Kv7.2/Kv7.3 channels generate M-currents that control the subthreshold excitability of the cell membrane. Therefore, drugs that stabilize the open state of Kv7.2/Kv7.3 could be used in a broad range of CNS diseases that are characterized by neuronal over-activity, including pain, stress, anxiety and epilepsy. In this respect, retigabine and flupirtine were the first therapeutically used openers of Kv7 channels as a first-in-class antiepileptic drug and analgesic agent, respectively. Despite the withdrawal of both drugs not related to on-target effects, Kv7.2/Kv7.3 channel is still validated molecular target, thus calling for the development of new therapeutic treatments with the same mechanism of action.

On these bases, High-Throughput Screening (HTS) and chemoinformatics approaches were applied to the identification of novel Kv7.2/Kv7.3 openers.

Method: HTS was performed on a proprietary Subset Library of 35.000 compounds, representing the chemical space covered by a more exhaustive Full Library (160.000 compounds). The library of ~ 190000 compounds was designed to represent several chemotypes collecting enough analogues.

The primary screening was carried out in single dose (30 µM) on a thallium transport FLIPR-based assay and the results were exploited for the selection of active molecules. In the "Hit confirmation" phase the putative hits together with their analogues, recovered from the Subset and Full Libraries according to scaffold-based approach, were evaluated in triplicate both in the primary assay for activity confirmation and in a mock cell-based counter-screening to identify false positive binders. The confirmed hits were selected and pursued to EC_{50} determination.

Results: From the HTS, 315 compounds with Kv7.2/Kv7.3 activation percent higher than 15% were classified as actives and advanced with their recovered analogues to the "Hit confirmation" phase. This effort provided 580 compounds with specific activity on Kv7.2/Kv7.3 and positive channel modulation potency higher than 15%. Next, 320 out of the 580 hits were selected for EC_{50} determination. Interestingly, 230 compounds showed a good dose-response curve and 150 out of them exhibited EC_{50} values in the micromolar range.

Conclusion: The HTS campaign afforded interesting Kv7.2/Kv7.3 openers, whose electrophysiological effect is currently under evaluation. Based on the obtained ***in vitro*** pharmacological profile, the most promising chemotypes will be pursued to a further hit optimization program.

SESSION 6: PERSPECTIVES FROM THE INDUSTRY

[O24] LU AA41178: A NOVEL, BRAIN PENETRANT, PAN-SELECTIVE KCNQ/KV7 POTASSIUM CHANNEL OPENER WITH EFFICACY IN PRECLINICAL MODELS OF NEUROLOGICAL AND PSYCHIATRIC DISORDERS

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Background: The voltage-gated Kv7.2-Kv7.5 channels are broadly expressed in the central nervous system, where they, among other functions, control the excitability of neurons by acting as a 'brake' on repeated action potential discharges. Thus, pharmacological activation of neuronal Kv7 channels has proven to be a successful therapeutic strategy within partial-onset seizures by use of the Kv7 activator retigabine (Gunthorpe et al, 2012). Interestingly, activation of Kv7 channels also holds promise as a therapeutic strategy within psychiatric disorders such as schizophrenia (Sotty et al, 2009) and depression (Friedman et al, 2016).

Method: In the present study we have characterized the pharmacological profile of the novel, pan-selective Kv7.2-7.5 opener Lu AA41178, using both ***in vitro*** assays and a broad range of ***in vivo*** assays related to epilepsy, schizophrenia, and depression.

Results: Using two-electrode voltage clamp in ***Xenopus*** oocytes expressing human Kv7.2-Kv7.5 Lu AA41178 was confirmed to be a pan-selective activator of Kv7 channels. When applying a voltage ramp protocol the activation threshold of Kv7.2+Kv7.3, Kv7.4 and Kv7.5 was significantly left-shifted in the presence of increasing concentrations of Lu AA41178 (0.3-30 µM). Additionally, the IV curve of Kv7.2+Kv7.3 was left-shifted in the presence of Lu AA41178. Importantly, Lu AA41178 did not display inhibitory effects on human Kv7.1 channels stably expressed in CHO cells, suggesting no impact on cardiac repolarization safety.

Next, we tested Lu AA41178 in preclinical models of neurological and psychiatric disorders. In the maximum electroshock threshold test (MEST) subcutaneous pre-treatment of mice with Lu AA41178 significantly increased the electroconvulsive shock threshold, thus demonstrating an anticonvulsant effect. In the mouse forced swim test, a model of behavioral despair with antidepressant predictive validity, Lu AA41178 significantly reduced immobility time to the same extent as the positive control, imipramine. Additionally, in ventral tegmental area (VTA) brain slices from mice subjected to chronic social defeat stress (CSDS) we tested the impact of Lu AA41178 on neuronal firing as assessed with whole-cell patch clamping. Here, the increased activity of VTA dopamine neurons of CSDS animals could be reversed by Lu AA41178. Behavioral testing of Lu AA41178 was accompanied by plasma and brain exposure sampling, revealing minimum effective plasma levels below 1000 ng/ml.

Conclusion: In summary, Lu AA41178 is a potent activator of neuronal Kv7 channels demonstrating efficacy in animal models of epilepsy, schizophrenia and depression. Thus, it may serve as a valuable tool for exploring the role of Kv7 channels in both neurological and psychiatric disorders.

SESSION 6: PERSPECTIVES FROM THE INDUSTRY

[O25] THE RE-EMERGENCE OF KV7 DRUG DISCOVERY

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Background: After the identification and cloning of the five Kv7 genes in the mid-1990s, several pharmaceutical drug discovery teams initiated medicinal chemistry programs to discover and develop novel modulators of these Kv7 ion channels for a range of therapeutic indications. Retigabine, a compound originally reported as a $GABA_A$ modulator, effective in mouse models of epilepsy, and later discovered to be a pan-Kv7 activator, was approved in 2011 as an add-on therapy for refractory adult focal seizures. Only a few other Kv7 molecules moved from preclinical discovery into clinical development, but unfortunately none were approved for human use. Over the past two decades, there has been an increased understanding of the important role this ion channel family plays in controlling cell physiology and excitability. In addition, the greater knowledge of Kv7 genetic mutations associated with diseases (long QT syndrome, benign neonatal familial convulsions (BNFC) and KCNQ2-neonatal epileptic encephalopathy, and deafness) has further illuminated the critical function of these channels. In pursuit of new therapies, there is a resurgence of interest in this structural class of ion channels for treatment of 'hyperexcitable' disease states as well as genetically defined diseases in which the modulation of selective Kv7 channels could be beneficial.

Method: Engineered cell lines expressing Kv7.2/7.3, Kv7.4, Kv7.3/7.5 and Kv7.1/KCNE1 are used in a screening format to characterize compound activity. A thallium flux assay is the primary screen to generate EC_{50} values for Kv7.2/7.3 activity and to rank order compounds. Active compounds are then counter screened against the other Kv7 cell lines. The QPatch HTx automated patch clamp system is a confirmatory functional assay to further characterize the electrophysiology properties of these novel compounds.

Results: During the course of our Kv7 drug discovery program, we have discovered molecules with a wide range of in vitro Kv7 activity profiles that may have the potential to treat various CNS and smooth muscle indications. In particular, Kv7.2/7.3 and Kv7.4 modulators with ranges of channel activation and varying degrees of inhibition have been identified.

Conclusion: While the majority of these compounds will not be developed as therapeutics, they remain important pharmacological tool compounds to probe a large number of questions on how selectively modulating Kv7 channel subtypes may deliver targeted treatments for different diseases with added potential for an improved side effect profile.

SESSION 6: PERSPECTIVES FROM THE INDUSTRY

[O26] RATIONAL DESIGN OF HIGHLY POTENT & SELECTIVE KV7.2/3 AGONISTS AS POTENTIAL ANALGESIC DRUGS

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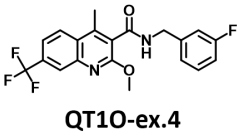
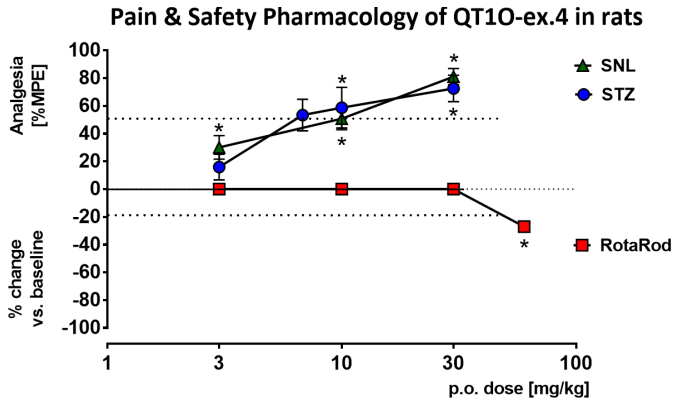


QT1O-ex.4

Kv7.1	10µM	no effect
Kv7.2/3	ED_{50}	**0.068µM**
Kv7.2/3	relEFF (RTG)	**120%**
Kv7.4	10µM	no effect
Kv7.5	10µM	no effect



Background: Heterotetramers of Kv7.2/3 subunits underlie the M-Current in neurons. Openers of this voltage-gated K^+ channel shift the cell resting potential towards hyperpolarization and decrease (hyper-)excitability. Therefore agonists are assumed to act as analgesic drugs. Limited availability of Kv7.2/3 subtype selective compounds triggered our research on identification of such and their characterization in pain pharmacology.

Method: A voltage-sensitive fluorescence dye assay with human Kv7.2/3 expressing CHO-K1s cells was used to characterize literature known (tool) compounds and to run a High Throughput Screening (HTS) of ~300k compounds. Starting from this initial dataset, rational drug design was applied to identify novel motifs and establish a Structure Activity Relationship. Optimized agonists were evaluated on selectivity against Kv7.1, Kv7.4 and Kv7.5. Most promising compounds after further in-vitro and pharmacokinetic profiling were progressed to rodent pain pharmacology models and in-vivo antagonism experiments. From ED_{50} results of a drug candidate in rat Spinal Nerve Ligation (SNL) and rat RotaRod, a first therapeutic window was derived and compared to those of main competitive compounds.

Results: In our assay Kv7.2/3 activity of numerous references (Retigabine, ICA-27243, BMS-568274) was confirmed, whilst others were inactive (Diclofenac, NH6, Flindokalner). Besides several hit series the HTS campaign also delivered a singleton hit, NICO-ex.89 with an EC_{50} of 1.6 µM and 129% efficacy relative to Retigabine (relEFF). Through rational design including structural information from published agonists, potency was increased by 20 times as shown for lead compound QAT1-ex.30 (EC_{50} 0.082 µM). Further optimization, especially of metabolic stability lead to drug candidate QT1O-ex.4 (EC_{50} 0.068 µM, 120% relEFF), which was void of any effect on Kv7.1, Kv7.4 and Kv7.5 at 10µM, corresponding to >147-fold subtype selectivity. Solubility at pH7.4 was limited to 20 µmol/L (kinetic) and 0.3 g/L (thermodynamic). When applied orally in a streptozotocin (STZ) induced model of diabetic polyneuropathy in rats, the agonist showed an ED_{50} of 7.2 mg/kg. This effect was partially reversed by pan-Kv7 antagonist XE-991 (1mg/kg i.p.). Further profiling of QT1O-ex.4 in a rat SNL model (ED_{50} 8.3 mg/kg p.o.) and on motor coordination in rat RotaRod (NOEL 30 mg/kg p.o.) revealed an improved pre-clinical therapeutic window compared to Retigabine, Pregabalin and Morphine.

Conclusion: A subtype selective, highly potent and orally available Kv7.2/3 agonist was discovered, revealing excellent potency and efficacy in pain pharmacology models. Despite poor solubility hampered progressing of QT1O-ex.4, it could serve as important tool for Kv7 research and for pre-clinical evaluation of other indications.

[O27] SCREENING OF NEGATIVE CHARGES BY CA2+ IN THE TURRET REGION CONTROLS KV7.1 INACTIVATION GATING

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Background: Inactivation is an intrinsic property of numerous voltage-gated K^+ (Kv) channels and can occur by N-type or/and C-type mechanisms. While fast N-type inactivation involves the inner pore occlusion by N-terminal peptide domains of α and β subunits, C-type inactivation is suggested to involve structural rearrangements in the outer pore leading to a loss of K^+ coordination sites in the selectivity filter. In Kv7.1 channels, inactivation is invisible macroscopically and does not exhibit the hallmarks of N- and C-type mechanisms. However, Kv7.1 inactivation is revealed by hooked tail currents, which reflect the recovery from an inactivation state.

Method: Whole-cell patch-clamp recordings were used in transfected CHO cells.

Results: We show that removal of external Ca^{2+} increased the activation kinetics and produced a large voltage-dependent inactivation of Kv7.1 channels. Increasing external Ca^{2+} suppresses inactivation gating with an EC_{50} of 1.5 µM. While Sr^{2+} and Cd^{2+} mimicked the effects of Ca^{2+}, other divalent cations like Mg^{2+} and Mn^{2+} were ineffective. External K^+ (50 mM) did not prevent the inactivation evoked in Ca^{2+}-free external solutions suggesting a mechanism different from C-type inactivation. Introduction in the pipet solution of calcified calmodulin or PIP2 slowed down the activation kinetics and precluded inactivation gating, evoked in Ca^{2+}-free external solutions. Mutagenesis studies and structural modeling suggest that external Ca^{2+} ions act to screen the negative charges of neighboring glutamate and aspartate residues located respectively, in the turret and filter entrance of the channel pore. In external free Ca^{2+} solutions, D317 and E295 are well hydrated, non-protonated and remain negatively charged. Under these conditions, D317 is quite flexible and cannot be efficiently engaged in H-bonding interaction with W304, which destabilizes the selectivity filter and leads to inactivation. In external Ca^{2+} solutions, Ca^{2+} ions can screen the negative charges of E295 and D317, which strengthens the interaction between D317 and W304 and stabilizes the open conformation of the selectivity filter. External acidification, which protonates E295 and D317 prevents Kv7.1 inactivation. Experimental data and kinetic modeling indicated that Kv7.1 channels exhibit two distinct inactivation states.

Conclusion: Our results reveal a new mechanism whereby external Ca^{2+} exquisitely controls inactivation gating of a Kv channel that is allosterically modulated by PIP2 and calcified calmodulin at the inner face of the channel transmembrane core.

[O28] PHOSPHORYLATION-DEPENDENT REGULATION OF HELIX B IN KV7.2 C-TERMINUS ALTERS KV7 CHANNEL FUNCTION AND EXPRESSION ON NEURONAL AXON

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Background: Neuronal M-channels containing $K_v7.2$ and $K_v7.3$ subunits are highly enriched at the axonal plasma membrane and generate sub-threshold, slow-activating outward M-currents that potently prevent repetitive and burst firing of action potentials. Suppression of M-channels upon G_q-protein coupled receptor activation increases action potential firing rates. Consistently, mutations in $K_v7.2$ and $K_v7.3$ are associated with early-onset epilepsy including benign familial neonatal epilepsy and epileptic encephalopathy, although the underlying pathogenic mechanisms remain elusive. Our novel statistical algorithm has recently revealed that epileptic encephalopathy mutations are highly concentrated at helix B and helix B-C linker in $K_v7.2$ where two serine residues (S523, S530) are suggested to be the targets of PKC phosphorylation crucial for G_q-induced M-current suppression. However, it remains unclear how phosphorylation of these serine residues affects channel expression and function in neuronal axon.

Method: We hypothesize that phosphorylation-dependent regulation of three conserved serine residues (S511, S523, S530) in helix B and helix B-C linker regulates M-channel function and expression at the axonal surface of pyramidal excitatory neurons whereas epilepsy mutations disrupt such regulation. In this study, we will test this hypothesis using interdisciplinary approach including molecular and biochemical tools, electrophysiology, and imaging of M-channel trafficking and neuronal activity.

Results: We generated phosphomimetic (aspartate/D) and dephosphomimetic (alanine/A) point mutations of these serine residues and demonstrated that these mutations variably affect calmodulin binding and surface and total expression of M-channels at the axons. Interestingly, we discovered that helix B and helix B-C linker are the sites for PIP_2 regulation of M-channels and epileptic encephalopathy K524T and R525L mutations located immediately downstream of S523 disrupt PIP_2-sensitivity of M-channels. Using phosphorylation site-specific antibodies and imaging tools, we are currently dissecting the effects of K524T and R525L mutations on the phosphorylation of three serine residues and subsequent calmodulin- and PIP_2 -regulated function and expression of M-channels at the axonal surface.

Conclusion: Taken together, our findings suggest that axonal expression of M-channels is regulated by coordinated actions of CaM, PIP_2, and helix B phosphorylation. Our on-going studies on this project would provide mechanistic insights into the axonal expression of M-channels, which is critical for their proper function to reduce neuronal excitability.

[O29] VENOM MOLECULES AS NOVEL THERAPEUTIC AGENTS TO TREAT KV7.2/7.3 RELATED DISORDERS

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The heteromeric Kv7.2/7.3 channel is implicated in disorders such as benign familial neonatal epilepsy, epileptic encephalopathy and peripheral nerve hyperexcitability. My work focuses on the electrophysiological behaviour and the pharmacology of the Kv7.2/7.3 channel. For this I predominantly use venoms as a natural source of bioactive molecules that can be screened for novel tool and therapeutic agents. Our lab has an extensive collection of >700 arachnoid venoms from mainly spiders and scorpions. Venomous animals rely on their toxic cocktail for their survival, and for millions of years these arachnoids have fine-tuned their toxins to affect the nervous system of animals for predation and defence. Each venom can contain up to a thousand active molecules with peptides frequently being the largest and most important toxin class present. I will attempt to identify venom molecules capable of selectively modulating the electrophysiological behaviour of the Kv7.2/7.3 channel. These molecules will subsequently be utilised to develop new insights into Kv7.2/7.3 disease mechanisms and potentially provide novel therapeutic agents.

[O30] IDENTIFICATION AND PHARMACOLOGICAL CHARACTERIZATION OF NOVEL INDOLE DERIVATIVES ACTING AS POTENT KV7 CHANNEL ACTIVATORS

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Background: Despite optimal treatment with modern antiepileptic drugs, about one third of epileptic patients still continue to have seizures, and drug-induced side effects are common. For these reasons, development of novel and safer antiepileptic drugs is needed. Kv7 channels are attractive targets for antiepileptic treatment because of their critical involvement in controlling neuronal excitability; among Kv7 activators, retigabine (N-(2-amino-4-(4-fluorobenzylamino)phenyl)carbamic acid ethyl ester) is the only compound approved for human use. Unfortunately, its major limitations (scarce selectivity among Kv7 channels of different subunit composition, poor pharmacokinetics, side-effects related to chemical instability) have recently led to retigabine market withdrawal. To possibly overcome some of these limitations, we have designed and synthesized a small library of 42 novel conformationally-restricted retigabine derivatives, and characterized some of these compounds with respect to: selectivity, chemical stability, and in vitro pharmacokinetics.

Method: The pharmacological properties of the novel retigabine derivatives were evaluated in CHO cells transiently expressing Kv7.2, Kv7.3, Kv7.2+Kv7.3 or Kv7.4 channels using the whole-cell configuration of the patch-clamp technique.

Results: Blocking the labile N-4 and removing the N-2 of retigabine, indoline, indole and tetrahydronaphthalene analogues of the 2-amino-4-((4-fluorobenzyl)amino)phenyl scaffold were designed and synthesized. All were investigated for their ability to enhance Kv7.2 currents at a single concentration (10 μM). When tested on homomeric Kv7.2 channels, two indole-based derivatives (defined as 23a and 24a) showed higher efficacy than retigabine. When compared to retigabine (0.93±0.43 μM), the EC_{50}s for Kv7.2 current enhancement by 23a (0.08±0.04 μM) was lower, whereas no change in potency was observed for 24a (0.63±0.07 μM). When compared to retigabine, 23a and 24a also showed higher potency in activating heteromeric Kv7.2/Kv7.3 and homomeric Kv7.4 channels. Substitution of a tryptophan at position 236, located within the pore region of the channel, with a smaller and less hydrophobic leucine largely prevents the ability of retigabine and 23a to activate Kv7.2 channels, suggesting that the indole-based derivatives and retigabine recognize the same hydrophobic pocket located in the Kv7.2 pore. Finally, 23a and 24a displayed an improved chemical stability over retigabine after 6 hours of exposure to UV/visible light.

Conclusion: The N5-alkylamidoindole moiety of derivatives 23a and 24a provides a suitable pharmacophoric scaffold for the design of a new, chemically-stable, highly-potent and subunit-selective selective Kv7 agonists.

[O31] MAPPING KV7.3 BINDING SITE BY IN SILICO DRIVEN STRUCTURE BASED APPROACH

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Background: Over the years Kv7 channels have gained increasing attention as potential targets for different diseases, such as central nervous system diseases, respiratory system diseases, genitourinary system diseases (bladder) and cardiovascular diseases. Retigabine represents the prototypical K_v7 activator; the drug recognizes a hydrophobic pocket located in the cytoplasmic parts of the S5 and the S6 transmembrane domains in the open channel configuration. Within this cavity, H-bonds are established between the carbamate group of retigabine and the aromatic side chain of a tryptophan present at the intracellular end of the S5 helix. Besides this specific interaction, the architecture of the retigabine binding site, the drug-target interaction network and the resulting drug binding mode remain puzzling. For these reasons we decided to attempt the mapping of retigabine binding site in Kv7.3 channel using an ***in silico*** driven structure-based approach.

Method: A new computational model of Kv7.3 channel was generated integrating the existing homology models with a three-dimensional pharmacophore model previously built from our research group. Site specific mutagenesis assays were performed to validate the model. A set of variously substituted N-(2-amino-4-(benzylamino)phenyl)amides as Kv7.3 agonists was designed and synthesized. The pharmacological properties were evaluated in stable CHO cell lines expressing Kv7.2, Kv7.2+Kv7.3, Kv7.3 A315T (a mutant channel that increased current density by 10-fold) or Kv7.4 using a fluorescence-based assay that uses Thallium (Tl^+) as a surrogate of K^+ ions and a fluorescent Tl^+-sensitive dye (FluxOR).

Results: Results obtained confirmed the predictivity of the in silico model and led to the identification of specific channel residues involved in the interaction with the agonists. New and interesting structure-activity relationship clues, modulating potency and selectivity of synthesized Kv7 agonists, were also evidenced. In particular, molecular interaction with A315, S342, F343, F344 and P347 seems to drive the binding mode and/or the potency of investigated Kv7.3 modulators. The most potent derivatives showed low submicromolar potencies and a remarkable leftward shift in the current–voltage relationship when compared to retigabine. Moreover, structure-based subtype selectivity towards Kv channel was evidenced.

Conclusion: These findings seem highly promising for the rational development of a new class of Kv7 channel modulators and to disclose the molecular mechanism at the basis of Kv7 channel activation.

[O32] PLATELET-DERIVED GROWTH FACTOR ACTIVATES NOCICEPTIVE NEURONS BY INHIBITING KV7/M-CURRENTS AND PLAYS A ROLE IN INFLAMMATORY PAIN

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Background: Endogenous inflammatory mediators contribute to the pathogenesis of pain by acting on nociceptors, specialized sensory neurons that detect noxious stimuli. The activity of these inflammatory mediators leads to dramatic alterations in somatosensory function. Identification of specific inflammatory mediators and the characterization of their mechanisms is important for developing effective approaches for therapeutic targeting of inflammatory pain. Platelet-derived growth factor (PDGF) has been previously shown to be have a crucial role in inflammatory process, being secreted after tissue injury. Together with data showing that PDGF receptors are expressed in nociceptive-like dorsal root ganglion (DRG) neurons and overexpressed after injury, we hypothesized that PDGF may also modulate the activity of peripheral sensory neurons, thus contributing to inflammatory pain.

Method: In this study we have integrated in vivo behavioral tests, current- and voltage- clamp recordings, immunohistochemistry and calcium imaging techniques along with pharmacological approaches.

Results: Using electrophysiological recordings, we show that PDGF-BB applied ***in vitro*** causes repetitive action potential firing in dissociated nociceptor-like rat DRG neurons and decreases their threshold for action potential generation. Moreover, injection of PDGF-BB into the paw produces nocifensive behavior in rats and leads to thermal and mechanical pain hypersensitivity. We show that PDGF-BB–mediated inhibition of Kv7/M-currents is a key mechanism underlying the PDGF-BB–induced increase in nociceptive excitability. Finally, we demonstrate that sequestration of PDGF or inhibition of the PDGFR with the clinically used PDGR receptor inhibitor imatinib during tissue inflammation attenuates formalin-induced acute inflammatory pain ***in vivo***.

Conclusion: Although a well know inflammatory factor, here we describe an unknown role for PDGF-BB in activating peripheral nociceptive neurons and in mediating inflammatory pain. Our findings suggest that PDGF-BB elicits action potential firing in nociceptors and produces sensitization to painful stimuli in vivo by inhibiting Kv7/M-currents through a PI3K-mediated mechanism. Our discovery of a new pain-facilitating proinflammatory mediator enhances our understanding of inflammatory pain pathophysiology and may have important clinical implications for the treatment of pain.

[O33] TRANSCRIPTOME PROFILING OF THE KCNQ2 PYRAMIDAL NEURON NULL FOREBRAIN REVEALS UPREGULATION OF NEUROPEPTIDES AND INFLAMMATORY GENES

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Background: Several forms of pediatric epilepsy disorders are caused by ***KCNQ2*** variants. ***KCNQ2*** encodes a ubiquitously expressed voltage-gated potassium channel in the brain. KCNQ2-related disorders range in severity; for some, seizures occur early in life and are self-limiting whereas, for others, seizures persist resulting in poor outcomes. Consequently, understanding the changes that occur in the brain as a result of KCNQ2 loss of function is important for the development of new therapeutics.

Method: Here, we carried out RNA sequencing on our previously published ***Kcnq2*** conditional knockout mice, which have seizures and start to die within less than three weeks of life, to test whether loss of Kcnq2 from pyramidal neurons leads to changes in gene expression in the hippocampus and neocortex. Additionally, we performed RNA sequencing of the hippocampus and cortex of mice expressing nonfunctional ***Scn1a*** (Nav1.1) in interneurons, which also have seizures and die within less than three weeks of life. Comparing the RNA transcriptome between the two mouse models allowed us to test whether the mechanism of seizure initiation—pyramidal neuron hyperexcitability vs disinhibition—leads to the same or different transcriptome changes. We confirmed our findings using fluorescence in-situ hybridization (FISH) and immunostaining.

Results: We found that several inflammatory genes, including those for glial fibrillary acidic protein (Gfap), and vimentin (Vim), were upregulated in ***Kcnq2*** conditional knockout mice. Additionally, we found several genes for neuropeptides such as galanin (Gal), proenkephalin (Penk) and thyrotropin-releasing hormone (Trh) were upregulated by between four and ten-fold. Surprisingly, RNA-sequencing revealed ***Scn1a*** mutant mice showed only modest upregulation of inflammatory genes. Additionally, Scn1a LOF from interneurons led to upregulation of Gal, Penk and Trh but to a lesser extent (two to four-fold change). Importantly, FISH for these neuropeptides showed that their upregulation occurs in different subfields of the hippocampus between the Kcnq2 and Scn1a mice.

Conclusion: These results suggest that loss of Kcnq2 from pyramidal neurons leads to unique changes in the RNA transcriptome. Additionally, the striking increase in neuropeptide levels points toward the possibility of using neuropeptide receptors as new therapeutic targets for KCNQ2 encephalopathies.

[O34] LOSS-OF-FUNCTION MISSENSE VARIANTS IN THE KCNQ5 GENE ARE ASSOCIATED WITH GENETIC GENERALIZED EPILEPSIES

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Background: ***KCNQ5*** encodes the subthreshold voltage-gated potassium channel $K_V7.5$, which generates M-type currents. It is mainly expressed in the brain and in skeletal muscle. Recently, ***de novo*** missense variants in ***KCNQ5*** were described as a cause of intellectual disability (ID) and/or developmental and epileptic encephalopathy (DEE). Here, we identified four new missense variants in patients with genetic generalized epilepsy (GGE).

Method: Patients and families diagnosed with GGE were referred for whole exome sequencing. Bioinformatic analysis revealed four clinically relevant missense variants in ***KCNQ5***. Sanger sequencing was performed to confirm co-segregation in affected families and validate patients' variants. To functionally assess the variants, mutations were inserted into a human ***KCNQ5*** cDNA for transient expression in mammalian cells. Chinese hamster ovary (CHO) cells were transiently transfected and channel properties were assessed by patch-clamp recording. ***KCNQ5*** expression was confirmed by Western blot and immunohistochemistry.

Results: Four missense variants were identified in KCNQ5. The first variant segregates in a family with an affected mother and three affected children diagnosed with either childhood absence epilepsy (CAE) or juvenile myoclonic epilepsy (JME). The second variant was found in a patient who developed pharmaco-resistant epilepsy with generalized tonic-clonic seizures (GTCS) and generalized spike and wave discharges on EEG at age 44. Analysis of family history revealed a half-brother diagnosed with GTCS, altogether suggesting a diagnosis of GGE despite the late onset. The third variant was identified in a patient with CAE, and GTCS during puberty, migraine without aura, and depression. Family history was reported positive for epilepsy. The last variant was discovered in a consanguineous family with two affected brothers with JME. Co-segregation identified the mother as a carrier. Noticeably, all variants were located in the C-terminus which contains many interaction sites not only for the correct assembly of the channel itself, but also for its activation. Functional analysis via patch-clamp recordings in transiently transfected CHO cells revealed a consistent total loss-of-function effect across all four variants.

Conclusion: We propose that ***KCNQ5*** loss-of-function variants can play a causative role in the development of GGE in some patients and families.

[O35] A NOVEL GAIN-OF-FUNCTION VARIANT IN KCNQ5 IN A PATIENT WITH NEURODEVELOPMENTAL DELAY AND DRUG-RESISTANT EPILEPSY

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Background: The M current (IKM) is a slowly-activating and deactivating, and non-inactivating potassium current which critically regulates neuronal excitability. Although heteromeric channels composed of KCNQ2 and KCNQ3 subunits are believed to provide a major contribution to IKM in adult neurons, morphological and functional data in experimental animals also suggest that KCNQ5 subunits, possibly together with KCNQ3, may contribute to IKM molecular heterogeneity (Tzingounis et al., 2010; Fidzinski et al., 2015). De novo variants in the KCNQ5 gene have been found in four patients with intellectual disability and/or epileptic encephalopathy (Lehman et al., 2017). In the present work, we report the identification of a novel variant in KCNQ5 in a 7.5-year-old male proband with neurodevelopmental delay and drug-resistant focal hypertonic seizures with onset at 15 months, and describe the changes triggered by the mutation on channel function.

Method: Genetic analysis was performed by next-generation sequencing on a 119-gene panel for Mendelian epileptic disorders. Mutations were engineered by QuickChange mutagenesis; channel subunits were expressed in CHO cells by transient transfection, and currents were recorded 24 h later, using the whole-cell configuration of the patch-clamp technique.

Results: A novel KCNQ5 de novo variant (Chr6:73821040G>A, c.1039G>A, NM_001160133.1) was found in the proband; this variant leads to the G347S substitution, therefore affecting the bottom of the S6 transmembrane domain, a critical region for channel gating. Patch-clamp recordings revealed that, when compared to KCNQ5, homomeric KCNQ5 G347S channels carried currents >10 times larger in size, also showing a marked (>15 mV) hyperpolarization shift in $V_{1/2}$, the midpoint potential of activation, and slower deactivation kinetics; all these biophysical properties indicate a strong gain-of-function in vitro phenotype. Qualitatively similar results were obtained when KCNQ5 G347S mutant subunits were co-expresed together with KCNQ3 subunits; in fact, when compared to KCNQ3+KCNQ5 channels, KCNQ3+KCNQ5 G347S channels displayed a >2-fold increase in current size and a hyperpolarizing shift in $V_{1/2}$ of about 8 mV.

Conclusion: The present data show a strong GoF in vitro phenotype caused by the novel G347S variant in the pore of the KCNQ5 subunit, suggesting its crucial pathogenic role and highlighting this position as critical for regulating pore opening probability, a result consistent with recent cryoelectron microscopy data obtained in highly-homologous KCNQ1 channels (Sun and MacKinnon, 2017). The precise molecular mechanisms by which KCNQ5 GoF mutations result in the characteristic clinical phenotype of the patient is still under investigation.

[O36] KV7 CHANNELS INTERACT WITH THE MYO-INOSITOL TRANSPORTER SMIT1 IN ARTERIAL SMOOTH MUSCLE CELLS

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Background: Kv7 channels regulate the contractile state of vascular smooth muscle at rest and in response to vasorelaxants. Arterial smooth muscle cells mainly express Kv7.1, Kv7.4 and Kv7.5, along with the modulatory subunit KCNE4. Recently, Kv7.1, Kv7.2 and Kv7.3 have been shown to interact in the nervous system with the sodium-coupled transporter SMIT1 (SLC5A3), which transport myo-inositol that regulates cell osmolality and is a substrate for the synthesis of phosphatidylinositol 4,5-bisphosphate (PIP2), a main modulator of Kv7 channels function. Formation of these protein complexes results in reciprocal regulation of the ion channel and transporter. The aim of this study was to evaluate whether SMIT1 was expressed in the vasculature and modulated Kv7 channels activity.

Method: Arteries from adult male Wistar rats were used for isometric tension recording. Vascular smooth muscle cells (VSMCs) were isolated and used for immunofluorescence experiments and Proximity Ligation Assay (PLA). Gene-silencing was obtained by transfecting arteries with morpholino antisense oligonucleotides for 48 hours.

Results: Immunofluorescence experiments revealed a peripheral, plasma-membrane-like staining of SMIT1 in renal and mesenteric VSMCs. Isometric tension recordings showed that incubation of renal arteries with raffinose and myo-inositol (that increase SMIT1 levels) reduced the contractile responses to methoxamine (30nM-30μM). Such reduced vasoconstriction was abolished when the Kv7 blocker linopirdine (10μM) was incubated before methoxamine stimulation. Silencing of SMIT1 increased the contraction of renal arteries segments induced by methoxamine when compared to controls, and impaired the response to the vasorelaxant effects of the Kv7.2-Kv7.5 activator ML213. In contrast, SMIT1-silencing did not interfere with the relaxant responses induced by the Kv7.1 selective-activator RL-3 (3μM) and the BK potassium channel enhancer NS1619 (3μM). PLAs showed that SMIT1 interacted with Kv7.1, Kv7.4 and Kv7.5 channels, as well as the ancillary subunits KCNE4 in both renal and mesenteric VSMCs. Knock-down of either KCNQ4 or KCNQ5 reduced the interaction of SMIT1 with Kv7.4 and Kv7.5, suggesting that SMIT1 interacted with Kv7.4-Kv7.5 heteromers. PLA puncta were also observed for SMIT1 and Kv7 subunits in CHO (Chinese Hamster Ovary) cells overexpressing Kv7 subunits. Interestingly, PLA puncta generated by the interaction between SMIT1 and Kv7.4 were observed in CHO cells only when KCNQ4 and KCNQ5 were co-expressed.

Conclusion: These data show for the first time that SMIT1 protein is expressed in VSMCs, where it regulates arterial contractility and interacts with all vascular Kv7 channels as well as KCNE4. Such modulation is partly Kv7-dependent and likely to the mediated by Kv7.4-Kv7.5 heteromers.

[O37] KCNQ2-ENCEPHALOPATHY IN A DISH: MECHANISTIC INSIGHT THROUGH 2D AND 3D NEURONAL MODELS

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Background: Heterozygous pathogenic variants in the gene KCNQ2 are associated with both severe KCNQ2-encephalopathy (KCNQ2-E), characterized by neonatal seizures and developmental delay, and a self-limiting epilepsy syndrome called benign Familial Neonatal Epilepsy (KCNQ2-BFNE), where development is completely normal. Pathogenic variants leading to KCNQ2-E have a dominant negative (DN) or gain-of-function (GOF) effect, whereas haploinsufficiency results in KCNQ2-BFNE. KCNQ2 encodes for a subunit of the M-channel, which generates a current that is very important for regulation of the resting membrane potential and control of neuronal excitability. While the role of KCNQ2 in epilepsy is straightforward, as dysfunction of the M-channel affects neuronal excitability, its role in neurodevelopment is less well understood. Currently used therapies for KCNQ2-E consists of symptomatic treatment, mainly directed at the (often difficult to treat) seizure activity. So far, no therapies influencing the developmental outcome of KCNQ2-E exist. To develop such a therapy, it is of utmost importance to increase our understanding of the mechanism underlying the neurodevelopmental delay observed in these patients.

Method: To understand how KCNQ2 affects neuronal development, we are generating 2D and 3D neuronal cultures (brain organoids) derived from human induced pluripotent stem cells (hiPSC). Both models will be characterized electrophysiologically and morphologically using Microelectrode Arrays and calcium imaging, and (high content) microscopy, respectively.

Results: So far, we developed hiPSC lines from 1 GOF and 3 DN KCNQ2-E variants, as well as 2 KNCQ2-BFNE variants, and 2 control individuals. By studying 2D neuronal co-cultures of excitatory and inhibitory neurons, generated via a fast overexpression protocol, we aim to develop a read-out system for both the epileptic and the neurodevelopmental features of KCNQ2-E, and to develop a platform that can be used for future drug screening. The brain organoids cultures are used to study the effect of the mutations in a more heterogeneous and complex neuronal network and to identify the affected cell types. Preliminary staining data reveal structural differences (i.e., abnormal morphology and expanded ventricular lumen) in KCNQ2-GOF vs. WT derived brain organoids, 28 days post differentiation.

[O38] ROLE OF KV7 CHANNELS AND KCNE ANCILLARY SUBUNITS IN THE PULMONARY VASCULATURE: IMPLICATION IN PULMONARY HYPERTENSION

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Background: Pulmonary arterial hypertension (PAH) is characterised by vasoconstriction, ***in situ*** thrombosis and vascular remodelling in pulmonary arteries (PA). K^+ channels play a fundamental role regulating membrane potential (Em) of PA smooth muscle cells (PASMCs) and their impairment is a common feature in PAH. In recent years, a key role of Kv7 channels (***Kcnq1-5***) and KCNE ancillary subunits (***Kcne1-5***) in the control of vascular tone has been demonstrated. Moreover, reduced in Kv7 channel activity has been observed in different cardiovascular pathologies such as diabetes, hypertension or long QT syndrome. Therefore, our objective was to study the role of Kv7 channels/KCNE subunits in the pulmonary vasculature and their possible alteration in PAH.

Method: Kv7 channel activity was analyzed in PASMCs from control or PAH (hypoxia + sugen) rats using the whole cell configuration of the patch-clamp technique. Vascular reactivity was assessed in PA mounted in a wire myograph. The characterization of Kv7 channels was performed using a blocker (XE-991) and selective enhancers (ML214, ML277, retigabine and S1) of these channels. The expression of Kv7 channels and KCNE subunits was analyzed by qRT-PCR and Western blot in lungs from PAH model. Cell localization studies were analyzed using immunocytochemistry and proximity ligation assay technics.

Results: In PA from control rats, the selective enhancers of Kv7 channels (ML277, ML213, S1 or Retigabine) produce a negligible relaxation. These data were supported by the cellular localization study, which showed that the expression of Kv7 channels and KCNEs subunits in PA was essentially cytosolic. Interestingly, the vascular reactivity data showed an enhanced response to Kv7 channel modulators, XE-991 and Retigabine, in PA from PAH rats. The electrophysiological data showed a decrease in non-inactivating K^+ current and a more depolarized Em in PAH-PASMCs compared to controls. Despite this, the contribution of Kv7 channels to the total current (% of blockade by XE-991) was higher in PAH-PASMCs than in controls. Finally, we also found an altered expression of Kv7α and KCNE subunits in PAH-lungs; with a decrease in Kv7.4α and KCNE3 and an increase in KCNE4 subunit.

Conclusion: In conclusion, there is a downregulation of Kv7.4 channels in PAH; but paradoxically, this seems to be associated with a higher contribution of Kv7 channels and a higher response to Kv7 channel modulators. Our data suggest that the ionic remodeling produced in the PAH pathophysiology makes Kv7 channels more suitable for pharmacological intervention.

[O39] DESTABILIZATION OF KV7.2 CHANNELS ACTIVATED CONFIGURATION AS A PHATOGENETIC MECHANISM FOR EPILEPTIC ENCEPHALOPATY

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Background: The ***KCNQ2 and KCNQ3*** genes code for Kv7.2/Kv7.3 channel subunits underlying I_{KM}, a K^+-selective current crucially involved in the regulation of neuronal excitability (Wang ***et al***., 1998): mutations in both genes are responsible for epileptic diseases, characterized by a heterogeneous clinical severity (www.rikee.org) ranging from Benign Familial Neonatal Seizures (BFNS) to severe developmental and epileptic encephalopathy (DEE). Among the Kv7.2 voltage sensing domain (VSD) variants identified in DEE patients, most are clustered in S_4 and modify voltage-dependent gating. In the present study, we provide clinical and genetic data on a 29-months old male proband affected with neonatal-onset epilepsy and developmental delay, in which a novel KCNQ2 variant was found. In addition, we report on the functional and pharmacological properties of the channels incorporating subunits carrying the newly-found KCNQ2 variant.

Method: Gene variants were identified by next generation sequencing of two panels of about 400 overlapping genes linked to epilepsy or brain malformations performed in proband and parental samples. Mutations were engineered by site-directed mutagenesis; transiently transfected CHO cells were used for patch-clamp recordings in the whole-cell configuration. Structural inference were obtained by homology modelling.

Results: Genetic analysis revealed the presence of a possibly-pathogenic de novo (c.418G>C; p.E140Q) variant in KCNQ2 in the proband; this novel variant neutralized a highly-conserved negatively-charged residue in the S_2 transmembrane segment within the VSD. Homomeric expression of Kv7.2 E140Q subunits generated channels with marked (>50mV) depolarizing shift in their activation gating. Similar, although less prominent, changes were observed in heteromeric configuration with KCNQ2 and/or KCNQ3 subunits. The Kv7-activator retigabine (Miceli et al., 2018) was able to restore wild-type current levels, thus counteracting mutation-induced loss-of-function (LoF) effects. KCNQ2 homology modelling suggested that the E140 residue, together with other negatively-charged residues in S_2 (E130) or S_3 (D172), mainly contribute to the stabilization of the VSD activated state through electrostatic interactions with the R207 residue in S_4. Functional experiments on mutant Kv7.2 subunits carrying coupled charge reversion or cysteine-substitutions at these positions provided strong support for this hypothesis.

Conclusion: Destabilization of the VSD activated configuration is a pathogenetic mechanism underlying Kv7.2-related DEE, and can be caused by variants located outside the previously described S_4 hotspot. In addition, the ability of retigabine to counteract mutation-induced functional effects reinforces the rationale for the use of Kv7 activators in the management of DEE-affected patients carrying Kv7.2 LoF-mutations.

KEYNOTE SPEAKER

[O40] GENOTYPE TO PHENOTYPE CORRELATIONS OF KCNQ1 MUTATIONS ASSOCIATED WITH TYPE 1 LONG QT SYNDROME

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Background: Type 1 Long QT Syndrome (LQT1) is a rather common inheritable arrhythmogenic disorder that predisposes affected individuals to the development of life-threatening arrhythmias (LAE) that manifest clinically as unexplained syncopal spells, or even cardiac arrest and sudden death. LQT1 is secondary to autosomal dominant loss-of-function mutations on the KCNQ1 gene, encoding the alpha-subunit of potassium channel Kv7.1, one of the key players in determining the repolarization of ventricular myocytes. Genotype to phenotype correlations and genotype-specific risk stratification in patients with LQT1 are limited.
In this work, we aimed to assess whether the localization of different mutations over different regions of the KCNQ1 gene influences the severity of phenotype in LQT1 patients, that was defined as the "duration of QT interval" and the occurrence of LAE over the lifespan of affected patients.

Methods: We gathered data pertinent to the phenotype and outcome of 963 patients with the diagnosis of LQT1 (436 probands and their 527 affected family members). The KCNQ1 protein was divided into 5 functional regions: the N-terminus (NT), the voltage sensor (VS, including transmembrane segments S1 to S4), the cytoplasmic loops (CL), the pore (PO, including the transmembrane segments S5 and S6 and the S5-S6 extracellular linker), the C-terminus (CT).

Results: The study population included 963 LQT1 patients: 518 (54%) of them were females; the average age at diagnosis was 20±17 years; the mean QTc at the baseline ECG was 465±38 ms. The average follow-up was 8.3±7 years. During their life, 172/963 (18%) experienced arrhythmic manifestations of LQT1: 31/963 (3%) experienced one or more LAE, while 141/963 (15%) experienced only one or more syncopal spells. In the whole population, we identified a total of 188 different mutations on the KCNQ1 gene. The distribution of the mutations along the structure of the KCNQ1 gene was as follows: 15 (9%) in the NT, 33 (18%) in the VS, 27 (15%) in the CL, 43 (23%) in the PO, 70 (36%) in the CT. The concentration of pathogenic variants per number of amino acids (a.a.) was higher in the CL region, as compared to the other domains (1 mutation every 1.44 a.a). The distribution of the 963 patients in the different functional regions was as follows: 4% in the NT, 15% in the VS, 20% in the CL, 28% in the PO, 33% in the CT.The duration of QTc interval was significantly longer for patients with mutations in the PO region (473±40 ms) and in the CL region (468±38 ms) as compared to the other regions ($p<0.01$). Also, patients with PO and CL mutations had a significantly higher probability of showing high-risk values of the QTc interval (i.e. >500 ms), as compared to patients with mutations in the remaining regions of the gene (PO 18%; CL 16%; vs. 8% all the others, $p<0.001$).

On the clinical side, the majority of LAE and syncopal spells occurred in patients with mutations in the PO and the CL regions (30% and 19% of the total, respectively). Patients with PO and CL mutations had a higher risk of LAE and syncopal spells as compared to patients with mutations in the other functional domains of the protein (HR 2.89, 95% CI 1.95 to 4.29, $p<0.019$ and HR 1.61, 95% CI 1.00 to 2.49, $p=0.05$, respectively). Among 272 patients with mutations in the PO region, we found that those with mutations in the GYGD sequence that is responsible for the channel selectivity are associated with a higher risk of experiencing LAE (HR 19.32, 95% CI 1.02 to 364.74, $p=0.048$). Interestingly, the risk was independent of the duration of the QT interval and gender.

Conclusions: We found that KCNQ1 loss-of-function mutations affecting the pore region and the cytoplasmic loops of the Kv7.1 potassium channel are associated with a more severe form of LQT1. Among the pore mutations, the most significant arrhythmic risk is associated with mutations affecting the GYGD selectivity filter. Our findings provide for the first time a site-specific risk profile for mutations responsible for the most common form of Long QT Syndrome.

SESSION 7: KV7 CHANNELS IN NEUROPSYCHIATRIC DISORDERS

[O41] FROM RIKEE TO ERGENT: ASSESSING NEURONAL KCNQ GENETIC VARIANTS TO ENABLE CLINICAL DIAGNOSIS, PROGNOSTICATION, AND DEVELOPMENT OF PRECISION MEDICINES

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Background: KCNQ variants have been implicated in neurodevelopmental disease, but more standardized procedures for diagnosis are required to enable patient recruitment to trials.

Method: Under an IRB approved protocol, we developed **RIKEE** (**R**ational **I**ntervention for **K**CNQ2 **E**pileptic **E**ncephalopathy), a database on a non-redundant list of patients/pedigrees with variants in ***KCNQ2***, ***KCNQ3***, or ***KCNQ5.*** Based on the findings of RIKEE, we developed a set of criteria predicting high risk of ***KCNQ2/3*** related neonatal-onset epilepsy. We are evaluating these criteria via a prospective cohort observational study, **ERGENT** (**E**arly **R**ecognition of **G**enetic **E**pilepsy in **N**eona**t**es). Neonates are screened for ERGENT study eligibility using an anonymous online questionnaire submitted by referring caregivers. Eligibility requires (1) EEG-proven seizure(s) requiring ongoing seizure medication; (2) early neonatal seizure onset (≤14 days post term); (3) application within 30 days of seizure onset; (4) a negative standard-of-care diagnostic evaluation for acute causes of neonatal seizures including blood tests and brain MRI. Eligible patients, enrolled after parental informed consent, undergo sponsored CLIA-certified next generation sequencing (187 epilepsy-associated genes) and follow-up chart review.

Results: RIKEE has collected over unrelated 640 ***KCNQ2/3/5*** patients. The database includes individuals published in the literature as well as those registered directly by a parent/guardian or enrolled by a treating physician. In the context of broad guidelines for all genetic disorders by the ACMG/AMP (Richards, 2015), we developed a classification procedure for assessing evidence of variant pathogenicity and severity, and have applied this to over 300 variants. As the number of patients has grown, the proportion of those with novel variants has progressively declined. In addition, many variants have undergone functional screening by a combination of patch clamp and biochemical assays. By combining clinical descriptions, recurrence, and functional tests, the proportion of variants of unknown significance has been diminished. Currently, we are developing a higher throughput patch clamp assay as part of an NINDS Channelopathy-Associated Epilepsy Center without Walls, using the Nanion automated planar patch. To date, 21 patient applications have been submitted to ERGENT. Of 11 proband genetic tests completed, 5 (45.4%) showed PVs in KCNQ2 (n=4) or KCNQ3 (n=1). Of these, 2 had the clinico-genetic profile of KCNQ2 Encephalopathy, and 3 had that of Self-limited Familial Neonatal Epilepsy (i.e., BFNE).

Conclusion: Combining the variant classification tools developed by RIKEE with the patient selection strategy of ERGENT can enable early recruitment to trials of candidate treatments for ***KCNQ2*** encephalopathy and other neonatal-onset genetic epilepsies.

SESSION 7: KV7 CHANNELS IN NEUROPSYCHIATRIC DISORDERS

[O42] THE EXPANDING PHENOTYPICAL SPECTRUM OF KCNQ2 AND KCNQ3 ENCEPHALOPATHY

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Background: The genes KCNQ2 and KCNQ3, encoding the Kv7.2 and Kv7.3 potassium channel subunits, are among the first identified epilepsy genes, as loss-of-function variants in these both genes were described in 1998 in the inherited epilepsy syndrome Benign Familial Neonatal Epilepsy (BFNE). Children with this disorder have seizures in the first days of life, which remit after a few days to months. They further show a normal clinical development. In 2012, 8 patients with a heterozygous de novo missense mutation in ***KCNQ2*** and a much more severe phenotype were described, all presenting intractable neonatal seizures, a burst-suppression pattern on EEG, and severe to profound intellectual disability. Since then, de novo variants in KCNQ2 are proven to be the most frequent genetic cause of neonatal epileptic encephalopathies.

Method: The increased use of next generation sequencing techniques in patients with a broad range of neurodevelopmental disorders, led to the identification of ***de novo*** variants in KCNQ2 and KCNQ3 in patients not presenting the classical neonatal epilepsy phenotypes. Collaborative efforts of in-depth phenotyping of patients, and ***in vitro*** functional testing of variants, enable the description of phenotypical entities and the identification of their respective underlying molecular mechanisms.

Results: De novo KCNQ2 variants associated with a ”classical” KCNQ2 encephalopathy phenotype of neonatal onset seizures and developmental delay, appear to be caused by pathogenic variants having a dominant-negative effect on channel function. Gain-of-function KCNQ2 variants on the other hand, lead to developmental delay and infantile or childhood onset seizures. More recently, gain-of-function variants in KCNQ3 were shown to lead to developmental delay with autistic features, and only rarely later onset seizures. In general, the close genotype-phentoype correlations are remarkable, but somatic mosaicism appears to be an important source of phenotypic variability.

Conclusion: Several phenotypic clusters within KCNQ2 and KCNQ3 encephalopathy can be distinguished, which correlate closely with the functional effect of the pathogenic variants. While functional testing of novel variants still aids in variant classification, the recognition of clinical patterns can guide treatment choices and selection of patients for future drug trials.

SESSION 7: KV7 CHANNELS IN NEUROPSYCHIATRIC DISORDERS

[O43] KV7 CHANNELS IN STRESS RESILIENCE: FROM THE PRECLINICAL MODEL TO THE PATIENT

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Background: There is an urgent need for more effective medications for the treatment of major depressive disorder. Less than 50% of depressed patients achieve full remission and many patients are nonresponsive to currently available antidepressants. It is known that prolonged stressful events are a key driver in the development of depression. However, the divergent individual response to stress is intriguing: the majority of people experiencing stressful life events maintain normal psychological functioning (resilience to stress), whereas others develop depression (susceptibility to stress). The neurophysiological basis of resilience in response to stress-induced depression is poorly understood.

Method: In a well-established chronic social defeat stress (CSDS) model for depression, using cell type- and neural circuit-specific electrophysiological, optogenetic and pharmacologic techniques, we investigated the functional role and ion channel mechanism of ventral tegmental area (VTA) dopamine neurons in conferring resilience to social stress.

Results: We reveal that hyperactivity within the VTA dopamine system, observed in susceptible mice, causally links this firing activity to depressive-like behaviors, whereas resilient mice employ a homeostatic mechanism to stabilize dopamine neurons, in order to overcome depression-related behavioral abnormalities. We demonstrate that this crucial homeostatic plasticity seen in the resilient group is established by a new balance between excitatory currents—hyperpolarization-activated cyclic nucleotide-gated (HCN) channel-mediated I_h currents)—and inhibitory currents mediated by voltage-gated potassium channels, including Kv7 (KCNQ) channels. Importantly, promoting naturally occurring resilience by enhancing Kv7 channel function leads to antidepressant effects in both the CSDS rodent model and in human patients.

Conclusion: These studies open a conceptually new avenue for exploring depression treatment, by examining the mechanisms underlying resilience to depression.

SESSION 7: KV7 CHANNELS IN NEUROPSYCHIATRIC DISORDERS

[O44] MODELING KCNQ2 EPILEPTIC ENCEPHALOPATHY USING IPSC-BASED TECHNOLOGIES

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Mutations in KCNQ2, which encodes a pore-forming K+ channel subunit responsible for neuronal M-current, cause neonatal epileptic encephalopathy, a complex disorder presenting with severe early-onset seizures and impaired neurodevelopment. The condition is exceptionally difficult to treat, partially because the effects of KCNQ2 mutations on the development and function of human neurons are unknown. Here, we used induced pluripotent stem cells and gene editing to establish a disease model, and measured the functional properties of patient-derived neurons using electrophysiological and optical approaches. We find that while patient-derived excitatory neurons exhibit reduced M-current early, they develop intrinsic and network hyperexcitability progressively. This hyperexcitability is associated with faster action potential repolarization, larger afterhyperpolarization, and a functional enhancement of Ca2+-activated K+ (BK and SK) channels. These properties facilitate a burst-suppression firing pattern that is reminiscent of the interictal electroencephalography pattern in patients. Importantly, we were able to phenocopy these excitability features in control neurons only by chronic but not acute pharmacological inhibition of M-current. Our findings suggest that dyshomeostatic mechanisms compound KCNQ2 loss-of-function and lead to alterations in the neurodevelopmental trajectory of patient-derived neurons. Our work has therapeutic implications in explaining why KCNQ2 agonists are not beneficial unless started at an early disease stage.

SESSION 8: EMERGING PHARMACOLOGY OF KV7 CHANNELS

[O45] VARIED MECHANISMS AND SITES OF ACTION OF ANTI-EPILEPTIC POTASSIUM CHANNEL ACTIVATORS

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Background: Kv7 (KCNQ) voltage-gated potassium channels are targeted by a variety of activating compounds that shift the voltage-dependence of activation. The underlying pharmacology of these activator compounds is of growing interest for the treatment of epilepsy, pain, and other diseases. Retigabine/flupirtine is the first Kv channel activator approved for human use, but ongoing development of this drug class has revealed a diverse set of structural scaffolds and functional outcomes. The objective of our research program has been to contribute to future development of Kv7 activators by understanding the molecular mechanism(s) of action of these drugs.

Method: We use electrophysiological recording of Kv7 channel mutants and concatenated tetrameric channels with known subunit stoichiometry to investigate the mechanism of action of Kv7 channel activators.

Results: Recent progress draws clear distinctions between at least two subtypes of Kv7 channel activators, based on their site and mechanism of action. Specific amino acids have been identified that selectively abolish sensitivity to either pore- or VSD-targeted activators, indicating they act at separate binding sites. The most widely studied activator, retigabine, targets a pore-delimited binding site present in all non-cardiac Kv7 channel isoforms (Kv7.2-7.5). A single binding site is required for maximal effectiveness of retigabine and likely other drugs of this subtype, and a functional signature of these drugs is that they exhibit a combination of accelerated channel activation, and decelerated deactivation. A second set of voltage-sensor (VSD)-targeted activators appear to bind nearly exclusively to the activated state of the voltage-sensing domain (VSD). These VSD-targeted drugs, including ICA-069673, often discriminate between different Kv7 subtypes and require four drug-sensitive subunits for maximal effectiveness. This leads to a unique functional signature that exclusively affects channel deactivation and varies depending on the number of drug-sensitive subunits in a channel.

Conclusion: Our findings provide a framework for the classification of Kv7 potassium channel activators. Future questions facing development of this drug class are to understand whether different physiological effects arise from these distinct mechanisms of action, whether drug subtypes might synergize for beneficial outcomes, and how this mechanistic understanding can be applied to compound development.

SESSION 8: EMERGING PHARMACOLOGY OF KV7 CHANNELS

[O46] DISCOVERY OF A NOVEL ANTIEPILEPTIC DRUG TARGETING KCNQ CHANNELS

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Background: Epilepsy is one of the most common, serious neurological disorders, affecting about 1% of the world' population and characterized by recurrent seizure attacks. Being a neuronal Kv7 channel activator, retigabine (RTG) has been approved for the treatment of epilepsy. However, the less than ideal distribution of RTG reduces its antiepileptic efficacy and increase potential non-CNS side effects. In addition, skin discoloration on peripheral tissues like nails and/or retinal pigment abnormalities was reported among some patients, presumably due to the chemical instability inherited by RTG's triaminobenzene structure. The supply of RTG has been discontinued in markets since June 2017.

Method: Structure modification; electrophysiology; epilepsy models.

Results: Pynegabine (HN37) was discovered after multiple rounds of structure modification of RTG. Pynegabine displays satisfied chemical stability and improved ratio of brain-to-blood. In addition, pynegabine exhibits an enhanced activating potency on Kv7 channels and a wider therapeutic window than RTG. Pynegabine is currently in Phase I clinical trial.

Conclusion: Pynegabine is an ideal antiepileptic drug candidate for further development

[O47] RESIN ACID DERIVATIVES OPEN THE HKV7.2/7.3 CHANNEL AND PREVENT EPILEPTIC ACTIVITY IN ZEBRAFISH LARVAE

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Background: The voltage-gated potassium channel hKv7.2/7.3 has important functions in setting the resting membrane potential and thereby in regulating excitability of neurons. It is a target for the antiepileptic, channel-opening drug retigabine, which has caused adverse effects and was withdrawn from the market. A major adverse effect of retigabine was its channel-opening effects on the hKv7.4 channel**.** However, retigabine validated opening of the hKv7.2/7.3 channel as an antiepileptic strategy. The aim of this project was to develop hKv7.2/7.3 channel openers with improved ion channel selectivity. To this end, we developed derivatives of naturally occurring resin acids found in resin from pine trees and tested the effect of these compounds ***in vitro*** on different voltage-gated ion channels and ***in vivo*** on a zebrafish larvae epilepsy model.

Method: Human Kv7 channels were expressed in oocytes from the ***Xenopus laevis*** frog and currents were measured using the two-electrode voltage-clamp technique whilst hKv11.1 and hNav1.5 were expressed in cell lines (CHO and HEK-cells respectively) and measured using automated patch clamp. Epileptic seizures in zebrafish larvae were induced by the GABA-A receptor antagonist pentylenetetrazol (PTZ) and the electrical brain activity was recorded with extracellular electrodes.

Results: We found that specific generated resin acid derivatives were as potent hKv7.2/7.3 channel openers as retigabine. Several of our compounds had almost no effect on hKv7.4 and very small structural alterations of the compounds largely altered the effects on hKv7.4. To further investigate potential off-target effects we tested our compounds on the cardiac channels hKv7.1 co-expressed with KCNE1, and on hKv11.1 (hERG). Block of these channels can cause cardiac arrhythmia and subsequent death. None of our investigated compounds blocked any of these channels at 10 µM (estimated highest concentration for clinical effects) and they neither had an effect on the cardiac hNav1.5 channel. To investigate the antiepileptic effect, we induced epileptic seizures in zebrafish larvae. The epileptic seizures were prevented by retigabine (at 10 µM) and our potential lead compounds had similar effects at the same concentration.

Conclusion: Our resin acid derivatives show a potential to develop into a new type of antiepileptic drug by opening the hKv7.2/7.3 channel. Our compounds have a favorable ion channel selectivity profile and prevent epileptic seizures in our ***in vivo*** model.

SESSION 8: EMERGING PHARMACOLOGY OF KV7 CHANNELS

[O48] NEURONAL KV7 CHANNELS AS TARGETS FOR THE ANALGESIC ACTION OF PARACETAMOL

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Background: Sites and mechanisms of action of the most frequently used analgesic, acetaminophen (APAP, paracetamol), remain controversial. Amongst others, known active metabolites of APAP are AM404 (N-arachidonoylaminophenol) and NAPQI (N-acetyl-p-benzoquinone imine), and both molecules have been detected in the central nervous system after systemic APAP administration and were suggested to mediate therapeutic effects. In this study, we investigated whether APAP or its metabolites may exert direct effects on first and second order neurons in the pain pathway.

Method: Primary cultures of dorsal root ganglia (DRG) and spinal dorsal horns (SDH) were prepared from neonatal rats. Electrophysiological recordings were made in voltage and current clamp mode using the perforated patch technique.

Results: In current clamp recordings on SDH and TRPV1-positive DRG neurons, a ten minute application of NAPQI (1 μM) resulted in hyperpolarisation of the membrane potential and a concomitant decrease in action potentials firing in response to depolarizing current injections. On application of linopirdine (30 μM), a blocker of Kv7 channels, NAPQI-induced reduction in excitability and hyperpolarisation were lost in both sets of neurons. The membrane potential was depolarised in the presence of AM404, but remained unaffected by APAP. Excitability of DRG neurons decreased in the presence of AM404, while APAP had no effect. Currents through Kv7 channels of DRG and SDH neurons displayed an irreversible and concentration dependent enhancement by NAPQI, but neither APAP (100 μM) nor AM404 (10 μM) affected these currents. NAPQI (1 μM) also augmented currents through recombinant heteromeric human Kv7.2/7.3 channels. Finally, the inhibitory action of the algogenic mediator bradykinin on currents through Kv7 channels in DRG neurons was rapidly reverted by NAPQI.

Conclusion: The inhibitory effect of NAPQI on membrane excitability in first and second order neurons in the pain pathway involves an activation of Kv7 channels and may contribute to analgesia achieved by APAP.

Poster Abstracts

ASSEMBLY PROPERTIES

[P01] IDENTIFYING PROTEIN INTERACTIONS AND PHOSPHORYLATION SITES OF FOREBRAIN KCNQ2 CHANNELS USING EPITOPE-TAGGED KCNQ2 TARGETED MICE

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Background: Recent human genetic studies have shown that loss or gain of function variants of the voltage-gated potassium channel KCNQ2 causes neonatal epileptic encephalopathy. It is currently assumed that KCNQ2 channels primarily associate with KCNQ3 channels in the brain; however, recent published work has suggested that KCNQ2 channels may also interact with additional transmembrane proteins. Identifying the KCNQ2 membrane complex is necessary in order to understand how KCNQ2 channels dysfunction could lead to epilepsy and to also design better therapeutics. To address this question, we have developed a new epitope tagged mouse line allowing us to analyze KCNQ2 affinity purified complexes from the hippocampus and neocortex using mass spectrometry.

Method: In this study we used the hippocampus and neocortex of one month old mice. Immunoprecipitated proteins using anti-M2 FLAG antibody were isolated on magnetic beads, washed and resuspended in NaCl 450mM wash buffer. Proteins were then digested with trypsin followed by mass spectrometry. Eluted peptides were quantified and analyzed using nanoflow ultra-high performance liquid chromatography coupled to tandem mass spectrometry (nUPLC-MS/MS). Peptide and protein identifications and quantification was performed using MaxQuant v1.6.1.0 searches against Flag-KCNQ2 channels and the Uniprot ***mus musculus*** database. All proteomics results were visualized using Scaffold Q+S (Proteome Software).

Results: Mice engineered with the 3XFLAG-N-KCNQ2 channels are viable and show no obvious abnormalities. Consistent with previous work, epitope tagged KCNQ2 channels are enriched in the axon initial segment and axons across the forebrain suggesting that inclusion of the epitope tag did not alter KCNQ2 subcellular localization. Importantly, analysis of affinity purified KCNQ2 complexes identified multiple phosphorylation sites across KCNQ2 channels. Lastly, besides KCNQ3, we found additional novel KCNQ2 interacting proteins.

Conclusion: The use of epitope tagged KCNQ2 mice may offer a new resource for the KCNQ2 channel field, providing new insights regarding the KCNQ2 channel interactome.

PHARMACOLOGY

[P02] ACTIVATION OF KCNQ CHANNELS BY ANCIENT BOTANICAL MEDICINES

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Background: The KCNQ subfamily of voltage-gated K^+ channels comprises 5 genes, each encoding a 6-transmembrane segment (S) pore-forming (α) subunit with a voltage sensing domain and a pore module. Pharmacological modulation of KCNQ channels has the potential to provide safe, effective therapeutic approaches for treating diseases such as epilepsy and hypertension. Plants have been utilized for medicinal purposes for thousands of years, with records available as far back as ancient Mesopotamia, ancient Egypt and China. However, the molecular basis for the purported multiplex therapeutic actions of many of these ancient remedies remains incompletely understood.

Method: We used ***in silico*** docking, mutagenesis and cellular electrophysiology to elucidate the molecular mechanism of plant extracts commonly used as ancient medicines.

Results: Here, we report the discovery that compounds from plant extracts historically used to treat epilepsy and hypertension share the property of KCNQ channel activation. Furthermore, we have determined the KCNQ channel specificity of these compounds, and the amino acid residues critical for their binding. Finally, using ***ex-vivo*** techniques, we corroborate our ***in-vitro*** findings that the medicinal properties of these ancient botanical medicines are KCNQ channel-specific.

Conclusion: Elucidating the molecular mechanisms underlying therapeutic actions of botanical medicines may open up new therapeutic approaches and novel chemical spaces for the development of KCNQ-specific drug discovery and synthesis.

[P03] COMBINING ENDOCANNABINOIDS WITH RETIGABINE FOR ENHANCED EFFECT ON THE M-CHANNEL AND IMPROVED KV7 SUBTYPE SELECTIVTY

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Background: Epilepsy is a disorder of the nervous system affecting estimated 50 million people in the world. More than 30 % are inadequately helped by available medical treatment, motivating further development of antiepileptic treatment strategies. Retigabine was developed as a unique anticonvulsant by targeting the neuronal M-channel, which is composed of $K_V7.2/K_V7.3$ and contributes to the negative neuronal resting membrane potential. Unfortunately, retigabine caused adverse effects and has been withdrawn. Development of M-channel activators with improved K_V7 subtype selectivity may limit adverse effects.

Endocannabinoids are lipid-based compounds that act as endogenous ligands for cannabinoid receptors. Increasing evidence suggest that endocannabinoids have broader functions caused by interaction with so called non-canonical targets, for instance ion channels. In this project we test whether endocannabinoids act as M-channel activators. We also evaluate the effect of combining endocannabinoids with retigabine.

Method: The M-channel, composed of $hK_V7.2$ and $hK_V7.3$, is expressed in ***Xenopus*** oocytes and currents generated by the channel are measured with the two-electrode voltage clamp technique. The same approach is used to measure currents generated by $hK_V7.1$, $hK_V7.4$, and $hK_V7.5$. The resting membrane potential in cultured dorsal root ganglia (DRG) neurons from mouse is measured using patch clamp electrophysiology.

Results: By screening the effect of 5 endocannabinoids on $hK_V7.2/3$ we identified two compounds, N-arachidonoyl-L-serine (ARA-S) and N-arachidonoyl-γ-aminobutyric acid (NAGABA), that activated $hK_V7.2/3$. In contrast, some of the most commonly known endocannabinoids, e.g. 2-arachidonoylglycerol (2-AG) and ***N***-arachidonoylethanolamine (anandamide), did not activate $hK_V7.2/3$. ARA-S potently activated $hK_V7.2/3$ in ***Xenopus*** oocytes, with significant effects from 0.3 µM. ARA-S activated $hK_V7.2/3$ by shifting the voltage dependence for channel opening towards negative voltages as well as increasing the maximal conductance. ARA-S hyperpolarized the resting membrane potential of DRG neurons that natively express the M-channel. Importantly, we show that ARA-S activates $hK_V7.2/3$ via a different mechanism and display a different K_V7 subtype selectivity compared to retigabine. We demonstrate that co-application of ARA-S and retigabine at low concentrations retain the effect on $hK_V7.2/3$ while limiting the effect on the non-neuronal $K_V7.1$ and $K_V7.4$ channels.

Conclusion: Our findings show that ARA-S, an endocannabinoid with low affinity for the cannabinoid receptor found in mammal brain, activates the M-channel. Combining ARA-S with retigabine limits the effect on non-neuronal K_V7 channels while retaining the effect on the M-channel, suggesting that strategically combining M-channel activators with different subtype selectivity can limit off-target effects.

[P04] POTENCY AND ANTIEPILEPTIC EVALUATION OF A NOVEL KV7/KCNQ CHANNEL OPENER QO-83

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Background: The Kv7 (KCNQ) channel as a target for the treatment of neuronal hyperexcitability diseases such as epilepsy, convulsions, migraine and neuropathic pain has being envaluated for new drug research and development. The Initiative withdrawal of retigabine(RTG) which is the first-in-class drug from market, promotes, not hinders the enthusiasm from looking for KCNQ channel openers with high activity and selectivity. In accordence with this, we designed and synthesized Compound QO-83, a novel opener of KCNQ/M channel.

Method: Compound QO-83 was initially found for its activity of Kv7/KCNQ channel opening among a sreies of comp-onds designed and synthesized based on a core structure of RTG, using ICR8000 and FLIPR as screeing streatagy, verified by electrophysiological patch clamp techniques. Further characterization of compound QO-83 on Kv7/KCNQ channels were further performed on the expressed Kv7/KCNQ isoforms. Anti-epileptic activity of compound QO-83 were envaluated using animal models of maximal electroshock seizure model (MES), metrazol maximal seizure model (MMS) and PTZ-induced kindling model. Also, DSI telemetry, pathological staining, immunohistochemistry, western blot, and LC-MS technology were explored for further assessment of compound QO-83.

Results: Compound QO-83 activates KCNQ2/3 and KCNQ4 with EC_{50} of 0.19-0.38 μM (***VS*** RTG 3.3 μM) and 0.84 μM (***VS*** RTG 1.16 μM), respectively; but did not affect function of Kv7.1/KCNE1. Compound QO-83 prevented MES with a ED_{50} of 1.7 mg/kg (VS RTG 12.5 mg/kg) when injected intraperitoneally, with a similar protection rate to RTG in a does 7 times higher. In the PTZ-induced kindling model, QO-83 at 0.5 mg/kg and 1 mg/kg reduced the Racine score from 4.6 ± 0.2 to 3.5 ± 0.6 and 2.8 ± 0.5, respectively; and the spikes, high-frequency waves and broad waveforms of epi-lepsy EEG induced by PTZ was also improved by QO-83. Pathological examination show compound QO-83 prevented the neuron damages induced by seizure. Compound QO-83 was found to be able to increase the content of GABA in the cortex.

Conclusion: Compound 83 has high activity of opening Kv7/KCNQ channels with modest subtype selectivity, and shows strong anti-epileptic activity.

[P05] KV7.4 CHANNELS: PROMINENT TARGETS IN THE CARDIOVASCULAR EFFECTS OF HYDROGEN SULFIDE-RELEASING DRUGS

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Background: Vascular Kv7.4 channels are expressed in the sarcolemmal membrane of vascular smooth muscle (VSM) cells and are key elements in the fine regulation of vessel contractility and blood pressure (1,2). More recently, Kv7.4 channels were recognized even in cardiac mitochondrial membrane, where they mediate cardioprotective effects against myocardial ischemia/reperfusion (3). Notably, the activation of Kv7.4 channels has been proposed as a key mechanism in the cardiovascular effects of hydrogen sulfide (H_2S) (4). This endogenous gasotransmitter plays key roles in the regulation of the cardiovascular function (5). Indeed, H_2S regulates blood pressure through heterogeneous mechanisms of action. Among these, an important role of Kv7.4 activation in the vasorelaxing effects of H_2S has been clearly demonstrated (4). Endogenous H_2S is also a cardioprotective mediator, although the activation of mithocondrial Kv7.4 channels in the cardioprotective activity of this gasotransmitter has not yet been investigated. In the cardiovascular system, impaired H_2S production has been clearly associated with several disorders, such as increased vulnerability to ischemia/reperfusion injury and hypertension. Consistently, the use of suitable H_2S-releasing agents can be a valuable pharmacological strategy. Currently, several H_2S-releasing chemotypes have been described; some of them are under evaluation in clinical trials as promising drug candidates.

Method: In this work, we aimed at assessing the potential role of Kv7.4 activation in the vasorelaxing and cardioprotective effects of H2S-releasing agents. For this purpose, we selected erucin, a natural compound with long-lasting H2S-releasing properties (6), and tested it in functional pharmacology assays.

Results: Erucin evoked membrane hyperpolarization in VSM cells and caused a concentration-dependent relaxation of rat aortic rings. All these effects were significantly antagonized by the Kv7-blocker XE991. Erucin exhibited also cardioprotective effects against ischemia/reperfusion myocardial injury; moreover, it promoted trans-membrane potassium flows and membrane depolarization in isolated cardiac mitochondria. Again, these effects were significantly antagonized by XE991.

Conclusion: H_2S-releasing agents can be considered as promising drugs for cardiovascular diseases; the activation of Kv7 channels is likely to be one of the most prominent mechanisms responsible for this pharmacological activity.

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[P06] SEX-DEPENDENT DIFFERENCES OF VASCULAR KV7 CHANNELS

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Background: Vascular smooth muscle cells (VSMCs) dictate arterial diameter. As VSMCs contract, blood vessels narrow reducing blood flow and increasing total peripheral resistance. VSMC contraction is achieved by an increase in intracellular calcium. $[Ca^{2+}]_i$ is dictated by VSMC membrane potential and the opening of voltage gated calcium channels (VGCC). Kv7 channels are shown to regulate VSMC membrane potential at rest, their activity mediates VSMCs hyperpolarization deterring VGCC opening and vasoconstriction. Kv7 channels also contribute to an array of receptor mediated responses and in conjunction, Kv7 channels are degraded in hypertension, contributing to a hypertensive phenotype. Despite the known contribution of Kv7 channels to VSMC physiology and pathophysiology, the wealth of current literature focuses predominantly on males.

Method: We have characterized transcript expression patterns of all isoforms of KCNQ and b-auxiliary subunits KCNE via qPCR and demonstrated the efficacy of Kv7 activators (S)-1 and ML277 to relax pre-constricted vessels as well as the contribution of Kv7 channels to thromboxane A2 receptor mediated vasoconstriction via Wire Myography. As a secondary consideration, data collected from female Wistars has been separated according to stages of the oestrous cycle via post-mortem assessment of their vaginal cytology.

Results: Our data indicates a sex-dependent post transcriptional difference in Kv7 regulation, showing a shift in the potency for Kv7 activators to mediate vasorelaxation dependent on the oestrus cycle.

Conclusion: Female sex-hormones appear to regulate Kv7 channels in the vasculature in a non-genomic process.

[P07] RELATIVE SPARING OF INHIBITORY NEURONS IMPROVES THE EFFICACY OF RETIGABINE ON SEIZURES

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Background: Anti-epileptic drug (AED) targets are widely expressed, including by both excitatory and inhibitory neurons, but the relative contributions of excitatory versus inhibitory neurons to AED effects remains unclear. Although Kv7.2 containing channels have been localized immunohistochemically to PV interneurons, very little is known about their functional contribution to excitability in these cells.

Method: PV-Cre mice were crossed with Kcnq2-floxxed mice to remove Kv7.2, a target of the anti-epileptic drug retigabine, from parvalbumin-expressing interneurons (PV-IN). The relative efficacy of retigabine (10 mg/kg ip) in preventing seizures induced by picrotoxin (10 mg/kg ip) or kainic acid (30 mg/kg ip) was tested. Acute hippocampal slices were prepared for patch-clamp experiments at room temperature before and after application of 10 μM retigabine. PV-IN were filled with Alexa 488 and fixed with methanol. Wide field and confocal microscopy was performed after immunohistochemistry with specific antibodies to pan-ankyrin, Kv7.2, and/or Kv7.3.

Results: PV-Kcnq2$^{fl/fl}$ mice had no obvious change in appearance, and body mass, mortality, and EEG spectral power were equal to controls. Confocal imaging showed that immunolabeling for Kv7.2 and Kv7.3 subunits was abolished in the axon initial segment in CA1 stratum pyramidale PV-INs but not neighboring pyramidal cells. Retigabine (10 mg/kg) was ineffective in preventing picrototoxin or kainic-acid seizures in WT mice, but its efficacy significantly improved when ***Kcnq2*** was removed from PV-IN (PV-Kcnq2$^{fl/fl}$). In hippocampal slices, PV-IN of mutant mice showed diminished ability to fire repetitively with high (400 pA > I > 700 pA) current injections compared to controls, and retigabine was less effective in inhibiting action potentials in this range compared to WT mice. Additionally, both the retigabine-induced hyperpolarization of the resting membrane potential and afterhyperpolarization seen in in CA1 PV-IN in PV-Kcnq2$^{+/+}$ was no longer evident in PV-Kcnq2$^{fl/fl}$ mice.

Conclusion: These findings support the hypothesis that anti-epileptic drug efficacy can be improved with relative sparing of inhibitory neurons.

[P08] ROLE OF KCNQ POTASSIUM CHANNELS IN THE CONTROL OF CA2+ SIGNALLING IN VASCULAR SMOOTH MUSCLE CELLS

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Background: Voltage-gated Kv7 (or KCNQ) channels control resting membrane potential and excitability in different cell types, including vascular smooth muscle cells (VSMCs). Generally, activation of KCNQ channels leads to hyperpolarization of the plasma membrane while their suppression results in depolarization; the latter mechanism is believed to be responsible for vasoconstriction of VSMCs. Until now, the effects of KCNQ channel modulators on cytosolic [Ca^{2+}] dynamics in VSMCs have not been fully elucidated. This study aims to investigate potential role of the Kv7 channels in control of intracellular calcium signaling in VSMCs in norm and hypoxia.

Method: We utilised microfluorimetry to investigate the effect of activation and inhibition of Kv7 channels on intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) in fura-2 loaded A7r5 VSMCs and examined how hypoxia (1.0% O_2; 24 hours) or 'chemical hypoxia' due to exposure to HIF-Hydroxylase Inhibitor, DMOG (dimethyloxalylglycine,1 mM for 24 hours) influences $[Ca^{2+}]_i$ and mRNA expression of isoforms of Kv7 and voltage-gated Ca^{2+} channels (VGCCs). The latter was evaluated using reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Specific Kv7 channel inhibitor, XE-991 (10 µM) induced robust calcium oscillations, which were significantly reduced in the presence of a Kv7 channel opener, retigabine (10µM) and calcium channel blockers, nifedipine (L-type channel blocker; 2µM) and NNC 55-0396 (T-type channel blocker; 3µM). Using the inhibitors of IP_3Rs (2-APB, 100 µM), RyRs (tetracaine, 100 µM) and phospholipase C (edelfosine; 10 µM), we found that edelfosine did not reduce XE-991-induced Ca^{2+} oscillations but blunted Ca^{2+} release mediated by IP_3Rs. RT-PCR revealed the expression of K_V 7.1, K_V 7.4, K_V 7.5, Ca_v1.2 (L-type), Ca_v3.1 and 3.2 (T-type) VGCCs in A7r5 cells. Hypoxic treatment significantly reduced expression of KCNQ5 and significantly enhanced XE991-induced Ca^{2+} transients with prolonged and high-frequency Ca^{2+} oscillations.

Conclusion: Direct (XE-991) Kv7 channel inhibition resulted in Ca^{2+} oscillations in VSMCs through activation of L- and T-type VGCCs in norm and hypoxia. After hypoxic treatment XE-991-induced oscillations were enhanced. In both cases, the oscillations could be reversed with retigabine. According to our data, Kv7 channels play an essential role in the regulation of Ca^{2+} signalling in A7r5 cells which, in turn, is likely to affect cell contractility. By decreasing the expression of crucial Kv7 subunit (Kv7.5) and increasing Ca^{2+} signalling, hypoxia may potentiate Ca^{2+} overload, which most likely involving the vasospasm in the vascular smooth muscle.

[P09] STRUCTURAL PROPERTIES OF OMEGA-3 POLYUNSATURATED FATTY ACIDS (N-3 PUFAS) REQUIRED TO EVOKE VASODILATION MEDIATED BY KV7 CHANNELS IN RAT AORTA AND MESENTERIC ARTERIES

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Background: Omega-3 polyunsaturated fatty acids (n-3 PUFAs) evoke relaxation of isolated blood vessels, independent of activation of NO synthase or metabolism via COX. These relaxations involve activation of smooth muscle cell K^+ channels including K_v7. Here we characterise some of the major structural features of fatty acids required to K_v7 mediated relaxation in rat aorta and mesenteric arteries (MA)

Method: Male Wistar rats (200-300g) were humanely killed and the thoracic aorta and third order MAs excised. Rings (~2mm) were mounted on a wire myograph in Krebs solution bubbled with Carbogen (95% 0_2, 5% CO_2). Concentration-response curves (CRC 100nM-30μM) PUFAs including: n-3 PUFAs Docosahexaenoic acid (DHA, 22:6), Eicosapentaenoic acid (EPA, 20:5), α-linolenic acid (ALA, 18:3), and the n-6 PUFA arachidonic acid (AA, 20:4), were assessed following pre-constriction with U46619 (20-50 nM). CRC to PUFAs were repeated in the presence of the pan K_v7 blocker XE991 (1 μM) or the Kv7.1 selective blocker HMR 1556 (1 μM). Data are expressed as mean ± SEM from n animals. Comparisons were made using two-way ANOVA with Bonferroni's post-test. $P<0.05$ was considered statistically significant

Results: The n-3 PUFAs DHA and EPA evoked full relaxation (~90%) of the aorta and MA which was significantly inhibited by XE991 (Figure 1, $P<0.05$). Relaxation to DHA was insensitive to blockade by HMR1556 in either artery (figure 2). In aorta the n-3 PUFA ALA failed to evoke relaxation and in MA produced relaxation insensitive to XE991 (figure 3). In aorta the n-6 PUFA, AA evoked small relaxations (~20%) that were inhibited by XE991 ($P<0.05$) whereas in MA, AA evoked relaxation was insensitive to XE991 (figure 4).

Conclusion: We show that optimal PUFA-induced relaxation involving K_v7 channels in rat aorta and MA requires a minimum 20 carbon length chain and that the fatty acid is omega-3. In aorta shorter carbon chain lengths (ALA) or being an n-6 PUFA (AA) severely impaired relaxation. In MA whilst all PUFAs studied evoked relaxation only longer chain n-3 PUFAs (EPA and DHA) evoked Kv7 mediated relaxation. These properties of n-3 PUFA required to evoke relaxation are not consistent with those previously detailed for activation of $K_v7.1$ in cardiac myocytes. Furthermore, Kv7.1 are not involved in the relaxations observed. Other subtype(s) of Kv7, probably Kv7.4 or 5, mediate n-3 PUFA mediated relaxation of rat arteries. In summary despite heterogeneity between arteries optimal relaxation mediated via Kv7 channels requires an n-3 PUFA with a ≥20 carbon tail.

PHYSIOLOGICAL FUNCTIONS

[P10] HETEROZYGOUS LOSS OF EPILEPSY-ASSOCIATED KCNQ2 GENE ALTERS SOCIAL, REPETITIVE, AND EXPLORATORY BEHAVIORS IN MICE

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Background: KCNQ/K_v7 channels conduct voltage-dependent outward potassium currents that potently decrease neuronal excitability. Heterozygous inherited mutations in their principle subunits K_v7.2/KCNQ2 and K_v7.3/KCNQ3 cause benign familial neonatal epilepsy whereas patients with de novo heterozygous K_v7.2 mutations are associated with early-onset epileptic encephalopathy and neurodevelopmental disorders characterized by intellectual disability, developmental delay, and autism. However, the role of K_v7.2-containing K_v7 channels in behaviors especially autism-associated behaviors has not been described. Since pathogenic K_v7.2 mutations characterized to date are typically heterozygous loss-of-function mutations, we investigated the contributions of K_v7.2 to exploratory, social, repetitive, and compulsive-like behaviors by behavioral phenotyping of both male and female ***KCNQ2***$^{+/-}$ mice that were heterozygous null for the ***KCNQ2*** gene.

Method: A total of 23 male mice were used (***KCNQ2***$^{+/+}$ n= 12; ***KCNQ2***$^{+/-}$ n=11) and a total of 11 female mice were used (***KCNQ2***$^{+/+}$ n= 5; ***KCNQ2***$^{+/-}$ n=6). All behavioral tests were performed in a separate room from the colony, which were maintained on a reverse light:dark schedule. All mice were tested on all described tests in the following order: open field, habituated home cage activity, elevated plus maze, rotarod, self-grooming, marble burying, social interaction test, social dominance tube, and urine marking test.

Results: Compared to their wild-type littermates, male and female KCNQ2+/- mice displayed increased locomotor activity in their home cage during the light phase but not the dark phase and showed no difference in motor coordination, suggesting a possible circadian abnormality. In the dark phase, KCNQ2+/- group showed enhanced exploratory behaviors and repetitive grooming but decreased sociability with sex differences in the degree of these behaviors. While male KCNQ2+/- mice displayed enhanced compulsive-like behavior and social dominance, female KCNQ2+/- mice failed to display normal social approach. Lastly, the KCNQ2+/- mice exhibited enhanced hippocampal excitability and seizure propensity to chemoconvulsant kainic acid.

Conclusion: Our findings together indicate that heterozygous loss of K_v7.2 induces behavioral abnormalities including autism-associated behaviors such as reduced sociability and enhanced repetitive behaviors. Therefore, our study is the first to provide a tangible link between loss-of-function K_v7.2 mutations and the behavioral comorbidities of K_v7.2-associated epilepsy.

[P11] THE MOTOR PROTEIN DYNEIN COORDINATES NORMAL ADRENOCEPTOR-MEDIATED RELAXATIONS IN ARTERIAL SMOOTH MUSCLE

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Background: Dyneins are molecular motor proteins that move along the microtubule network to transport "cargo" away from the cell membrane. They are essential for the transport and distribution of certain membrane proteins. However, the physiological role of the dynein motor protein in vascular smooth muscle is poorly understood. Recently, it has been shown that microtubules can regulate β-adrenoceptor mediated relaxations in rat mesenteric and renal arteries by controlling membrane expression and function of the voltage-gated potassium channel, Kv7.4, in smooth muscle cells. The precise mechanism underlying this microtubule-dependent regulatory pathway, however, is yet unknown. The aim of this study was to investigate the physiological role of dynein in mesenteric arteries.

Method: Experiments were performed on 10- to 18- weeks-old male Wistar rats. For ***ex vivo*** experiments third-order mesenteric artery segments were isolated from rats and mounted in a wire myograph for isometric tension recording. For ***in vivo*** experiments, rats were anaesthetized by subcutaneous injection with ketamine (3 mg kg^{-1}) and xylazine (0.75 mg kg^{-1}) and changes in internal diameter of isolated small mesenteric arteries were studied using video imaging. Mesenteric arteries were incubated with the dynein inhibitor ciliobrevin ***ex vivo*** (10μM) and ***in vivo*** (30μM) for one hour, after which β-adrenoceptor agonist, isoprenaline, or Kv7.2-7.5 activators, S-1 or NS15370, were applied at increasing concentrations to precontracted artery segments.

Results: Ciliobrevin enhanced isoprenaline-mediated relaxations in rat mesenteric arteries ***ex vivo*** and ***in vivo***, compared to non-treated controls. The ciliobrevin-enhanced isoprenaline relaxations were attenuated by blocking the Kv7 channels with linopirdine, however, blockade of the BKCa channels with iberiotoxin had no effect on the ciliobrevin-enhanced relaxations. Ciliobrevin also enhanced relaxations when applying increasing concentration of Kv7 activators S-1 or NS15370. With morpholino-induced knockdown of Kv7.4 channels in mesenteric arteries we prevented ciliobrevin from enhancing S-1-mediated relaxations and partially attenuated the enhanced isoprenaline relaxations. We determined that ciliobrevin or colchicine enhanced Kv7.4 channel membrane expression in HEK cells. In addition, proximity ligation experiments showed that dynein co-localized with Kv7.4 and Kv7.5 in mesenteric artery myocytes. Finally, co-immunoprecipitation assays confirmed the interaction of Kv7.4 with dynein.

Conclusion: This study is the first to show regulatory effects of dynein on β-adrenoceptor-mediated relaxations of rat mesenteric arteries. We show that dynein inhibition enhanced Kv7.4 channel function, which contributed to increased β-adrenoceptor mediated relaxations.

[P12] EVIDENCE FOR KV7.4 CHANNELS IN NEURONAL MITOCHONDRIA

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Background: The potassium permeability represents a multifaceted aspect of mitochondrial function involved in apoptosis, energy metabolism, autophagy, and protection against ischemia-reperfusion (I/R) injury. In addition to the first two K^+ channels identified in mitochondria, mitoK_{ATP} and mitoBK, several other players were identified, and, recently, Kv7.4 channels were found in cardiac mitochondria where they could play a role in I/R cardioprotection (Testai et al., 2016). As in cardiomyocites, I/R is a primary injury mechanism for neurons, thus the putative occurrence of Kv7.4 in neuronal mitochondria was investigated.

Method: F11 cells (mouse neuroblastoma/embryonic rat dorsal-root ganglion neurons ibridoma) were used as neuronal cell model. Mitochondrial membrane potential (ΔΨ) of F11 isolated mitochondria was monitored by measuring safranine O fluorescence (λ_{ex} 520 nm; λ_{em} 570 nm). Kv7.4 subunits expression was investigated by Western blot (WB) experiments in total lysates or subcellular fractions of F11 cells, CHO cells and mouse brain. The subcellular distribution of Kv7.4 subunits was also studied by immunocitochemistry on intact F11 cells using anti-Kv7.4 antibodies and the mitochondrial marker Mitotraker. Heterologous expression of BK and Kv7.4 channels in CHO cells was achieved by transient transfections using Lipofectamine 2000.

Results: K^+ exposure to isolated mitochondria energized with succinate resulted in ΔΨ decrease whose rate showed a dependence on K^+ concentration showing hyperbolic nature; K-dependent ΔΨ decrease was not affected by iberiotoxin (mitoBK blocker) and only partially inhibited by either glybenclamide or ATP (mitoK_{ATP} blockers); furthermore, kinetic analysis of glybenclamide inhibition confirmed the occurrence of a glybenclamide-insentitive K^+ transport in these mitochondria. Interestingly, K-dependent ΔΨ decrease was reduced by XE-991 (a Kv7 blocker) and enhanced by retigabine (a Kv7 activator); the stimulatory effect of retigabine was abolished in the presence of XE-991, but not glybenclamide. Accordingly: ***i)*** RT-PCR experiments revealed that Kv7.4 mRNAs were expressed in F11 cells; ***ii)*** WB experiments detected Kv7.4 expression in mitochondrial fraction where no BK signal was found; ***iii)*** immunocytochemistry experiments showed a strong overlap between the distribution of anti-Kv7.4 antibodies and Mitotracker signals. Finally, Kv7.4 subunits were also detected in mitochondria isolated from mouse brain samples, revealing that mitoKv7.4 occurrence is not limited to F11 cell model.

Conclusion: Kv7.4 channels may regulate K^+ permeability of neuronal mitochondria; their possible contribution to neuroprotection during I/R injury is currently being investigated.

[P13] REGULATION OF HUMAN URINARY BLADDER SMOOTH MUSCLE FUNCTION BY KV7 CHANNELS

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Background: Detrusor smooth muscle (DSM) facilitates urinary bladder function. Voltage–gated potassium channels (Kv) determine DSM excitability and contractility. Among all Kv channels, Kv7 subtypes have emerged as key regulators of DSM excitation–contraction coupling in guinea pigs, pigs, and rats. Recent studies from our laboratory suggest a role of heteromeric Kv7.4/Kv7.5 channels in guinea pig DSM function, but they are yet to be explored in human DSM.

Method: DSM specimens were obtained from patients lacking symptoms of overactive bladder (OAB) undergoing open bladder surgeries. DSM whole tissue muscle strips (urothelium–free) and freshly–isolated single DSM cells were prepared and studied by RT–PCR, immunohistochemistry, immunocytochemistry, Western blot, Proximity Ligation Assay (PLA), DSM tissue contractility, and DSM whole–cell perforated patch–clamp electrophysiology.

Results: We found out that human DSM whole tissue and single smooth muscle cells expressed Kv7.4 and Kv7.5 mRNAs and proteins. In situ PLA demonstrated a high degree of spatial co–localization of Kv7.4 and Kv7.5 proteins in single DSM cells. Heteromeric Kv7.4/Kv7.5 complexes were highly expressed in the plasma membrane of DSM cells. In contrast, neither Kv7.4 nor Kv7.5 co–localized with inositol trisphosphate receptors (used as negative controls). Retigabine (Kv7.2–Kv7.5 channel activator) at 10 μM or ML213 (Kv7.4/Kv7.5 channel activator) at 10 μM reduced spontaneous phasic contractions of DSM tissue strips while XE991 (Kv7.1–Kv7.5 channel inhibitor) at 10 μM enhanced DSM contractility. Patch–clamp recordings in DSM cells revealed hyperpolarization induced by retigabine (10 μM) or ML213 (10 μM) and depolarization by XE991 (10 μM). Here for the first time, we recorded native human DSM Kv7 currents (using a ramp protocol) that were activated by retigabine (10 μM).

Conclusion: The close proximity of protein detections for both Kv7.4 and Kv7.5 subtypes in human DSM cells supports a role of native Kv7 channels composed of heteromeric Kv7.4/Kv7.5 channels whose activation leads to DSM hyperpolarization and attenuation of DSM contractility facilitating urine storage. Targeting heteromeric Kv7.4/Kv7.5 channels, displaying properties distinct from homomeric Kv7 channels, provides a potential novel therapeutic approach for the treatment of urinary bladder dysfunction including OAB and underactive bladder.

Funding: NIH R01DK106964

PHYSIOLOGICAL FUNCTIONS

[P14] ACTIVATION OF KV7 CHANNELS AS A NOVEL MECHANISM FOR NO/CGMP-INDUCED PULMONARY VASODILATION

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Background: The nitric oxide-NO/cGMP pathway represents a major physiological signalling controlling pulmonary arterial (PA) tone and drugs activating this pathway are used to treat pulmonary arterial hypertension. Kv channels expressed in PA smooth muscle cells (PASMC) are key determinants of vascular tone. We aimed to analyse the contribution of Kv1.5 and Kv7 channels in the electrophysiological and vasodilating effects evoked by NO donors and the guanylate cyclase (GC) stimulator riociguat in PA.

Method: Kv currents were recorded in isolated rat PASMC using the patch-clamp technique. Vascular reactivity was assessed in a wire myograph.

Results: The NO donors DEA-NO and SNP hyperpolarized the membrane potential and induced a bimodal effect on Kv currents (augmenting the current between -40 to -10 mV and decreasing it at more depolarized potentials). The hyperpolarization and the enhancement of the current were suppressed by Kv7 channel inhibitors and by the GC inhibitor ODQ but preserved when Kv1.5 channels were inhibited. Additionally, DEA-NO enhanced Kv7.5 currents in COS7 cells expressing KCNQ5 gene and this effect was prevented by ODQ. Riociguat increased Kv currents at all potentials ≥-40 mV and induced membrane hyperpolarization. Both effects were prevented by Kv7 inhibition. Likewise, PA relaxation induced by NO donors and riociguat was attenuated by Kv7 inhibitors.

Conclusion: In conclusion NO donors and riociguat enhance Kv7 currents, leading to PASMC hyperpolarisation. This mechanism contributes to NO/cGMP-induced PA vasodilation. Our study identifies Kv7 channels as a novel mechanism of action of vasodilator drugs used in the treatment of pulmonary arterial hypertension.

Supported by the Spanish Ministry of Economy and Competitiveness (SAF2016-77222-R), the Cardiovascular Medical Research and Education Fund and Fundación Contra la Hipertensión Pulmonar-EMPATHY.

PHYSIOLOGICAL FUNCTIONS

[P15] MAPPING THE FUNCTIONAL EXPRESSION PROFILE OF KV7 CHANNELS IN SOMATOSENSORY NEURONS OF DIFFERENT SENSORY MODALITIES

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Background: Voltage dependant potassium (K+) channels (Kv) are important in controlling excitability and repolarising neurons after depolarisation has occurred. One subset of these channels is the Kv7 family, which is composed of Kv7.1-7.5 subunits. Kv7 channels are promising targets for treating excitability disorders, including epilepsy and pain, as they are active at negative voltages, thus clamping the resting potential and reduce the excitability of a neuron. Kv7 channels are expressed in many types of excitable cells, including neurons and muscle cells, though subunit differences exist in different tissues. Therefore, identifying the expression pattern of Kv7 subunits in different cell types will aid development of more targeted therapeutic strategies.

Method: Here we used immunofluorescent techniques and electrophysiology to identify Kv7 expression profile within rat peripheral somatosensory system, which is responsible for detection and transmission of somatic and visceral stimuli, including these resulting in pain and itch sensations. To this end, rat dorsal root ganglia (DRGs) were co-labelled with antibodies against Kv7.1 - Kv7.5 and modality-specific markers (NF200 for A fibres, Peripherin for C fibres).

Results: Kv7.1 showed very limited expression in the DRG (4%±1.5 of total neurons), whereas 83% and 71% of Peripherin positive neurons were Kv7.2 and Kv7.3 positive respectively. Kv7.2 and Kv7.3 were also expressed in approximately 50% of NF200 positive neurons. Kv7.5 was expressed in approximately 70% of NF200 positive neurons and ≈50% of Peripherin positive neurons, suggesting that Kv7.5 is similarly distributed between large, medium and small diameter neurons. This data was supported by recording of Kv7 current (M-current) in capsaicin responsive (presumed nociceptive) DRG neurons in the presence of Kv7.2/7.3 heteromer selective agonist, ICA-27243. ICA-27243 increased outward current at -60mV by 62%±11 ($p<0.01$ n=7) compared to baseline; non-selective Kv7 channel agonist Retigabine, when applied after ICA-27243, produced no further current augmentation (while being as efficacious as ICA-27243, when applied alone) (n=12).

Conclusion: Together, these data suggest that Kv7.2, Kv7.3 and Kv7.5 are the major Kv7 subunits expressed in sensory neurons with Kv7.2 and Kv7.3 bei ng the predominant subunits in C fibers.

[P16] DEFINING THE ROLE OF KCNQ2 AND KCNQ3 POTASSIUM CHANNELS IN SETTING THE NEURONAL EXCITABILITY OF SUBICULUM NEURONS

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Background: The main distal output of the hippocampus is the subiculum, a region that projects to the entorhinal cortex topographically, the medial and lateral mammillary bodies, the nucleus accumbens, and amygdala. Consequently, due to its connectivity, it is not surprising that the subiculum has been implicated in epilepsy disorders. Despite its importance, the ion channels critical for its function are not fully known. Earlier studies have shown that KCNQ3 and to a lesser extent KCNQ2 potassium channels are highly expressed in subiculum, but the role of KCNQ2 and KCNQ3 channels in setting the excitability of subiculum neurons has yet to be addressed. This is particularly important as KCNQ3 and KCNQ2 pathogenic variants could lead to neonatal and infantile epilepsy disorders. To address this question, we are examining the effect of ***Kcnq2*** and ***Kcnq3*** deletions in proximal and distal subiculum neurons using our previously published knockout mice and our recently acquired ***Kcnq2*** knock-in mice; mice carrying a KCNQ2 loss-of-function variant.

Method: To determine the firing properties of subiculum neurons we use whole cell electrophysiology in acute slices prepared from P14-P20 mice. Subiculum pyramidal neurons are visually identified by differential interference contrast optics. All recordings are performed at a temperature of 31-32 degree celsius. We record from both proximal and distal subiculum neurons as they exhibit differing firing properties.

Results: Our current data indicate subiculum neurons from constitutive ***Kcnq3*** knockout mice and ***Kcnq2*** knock-in mice have an elevated neuronal excitability. The increased excitability is observed in both the proximaland distal subiculum neurons.

Conclusion: Our data raise the possibility that subiculum activity is controlled by KCNQ2/3 channels similar to our previous findinds in CA1 pyramidal neurons of the hippocampus.

[P17] THE IKS CHANNEL COMPLEX, NOT ONLY FLEXIBLE SUBUNIT COUPLING, BUT ALSO SINGLE CHANNEL KINETICS, CONDUCTANCE, AND MODULATION BY ML277, AN IKS OPENER

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Background: I_{Ks}, the slow delayed rectifier current important in cardiac repolarization during sympathetic activation, is known to occupy multiple subconductance levels, particularly on initial pore opening. Subconductance behaviour allows continuous modulation of current levels over time, after channel activation, subject to accessory subunit association and second messenger regulation. The prominent subconductance behavior of I_{Ks} makes it an obvious target for modulation, either physiologically or pharmacologically to stabilize different subconductance states and produce a range of conductance possibilities.

Method: Concatenated copies of KCNQ1 (Q) with and without KCNE1 (E) were used to titrate in the number of *E160R mutations within the heteromeric complex. Recordings of single channels with two restrained voltage-sensor domains (VSD; EQ*Q + KCNE1-GFP) or 3 restrained VSD (EQ*QQ*Q* + KCNE1-GFP) were collected and analyzed using the single channel function of Clampfit (Molecular Devices).

Results: As more VSD are restrained by the E160R mutation, the occupancy of closed states increases from 75.3 % in the wild-type channel, to 87.1% and 92.8% with 2 and 3 E160R subunits respectively, while occupancy of higher subconductance states is reduced but not eliminated. Initial experiments with the KCNQ1 activator ML277, which may bind the pore domain to enhance conductance similar to KCNE1, show that there is some rescue of function of E160R containing complexes through an increase in open probability, more active sweeps, shorter first latencies and slower deactivation.

Conclusion: We conclude that activation of all VSDs is not necessary for conductance through the I_{Ks} pore, but when all four VSD activate, conductance is larger and more stable. This regulation of conductance and kinetics at the level of individual VSDs allows for wide-ranging modulation of the channel by, for instance, co-assembly with multiple other β subunits (KCNE2-4) that have differing effects on channel properties.

REGULATORY MECHANISMS

[P18] A BFNE CAUSING MUTATION IN THE IQ SITE OF THE KV7.2 CHANNEL DISRUPTS CO-TRANSLATIONAL FOLDING OF THE CALICUM SENSOR

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Background: Mutations in helix A of the K_V7.2 channel calmodulin (CaM) binding domain (CaMBD) disrupt the interaction with CaM and channel´s function. There is a strong correlation between CaM binding and current density for several helix A mutants. However, the W344R is a remarkable exception, since the mutant channel is not functional, yet, the binding of CaM to the CaMBD is not affected in vitro. One possible explanation is that this residue somehow interferes with a critical movement within the channel that allows it to gate. Alternatively, the in vitro binding experiments could not represent the behavior of the protein within the cell. To address this possibility, we studied the interaction in cellulo by FRET.

Method: In cellulo FRET interaction studies have been carried out in HEK-293 cells co-expressing different fluorescence constructs. Interaction studies between CaM and channel, as well as assembly assays in the presence and the absence of CaM, have been performed.

Results: Whereas WT channels, either monomeric or tetrameric, displayed clear FRET signals consistent with the interaction, those properties were abolished for the W344R mutant.

Conclusion: We conclude that the relationship between CaM binding and function holds true also for the W344R mutant, and we hypothesize that there is a disruption during co-translational folding that prevents the mutant adopting a conformation recognized by CaM, leading to channel dysfunction. These results reveal a dramatic difference between in vitro and in cellulo behavior of a protein domain, stressing the care that should be exerted when drawing conclusions based on experiments performed in vitro.

[P19] THE HUMAN KCNQ2 EPILEPTIC ENCEPHALOPATHY MUTATION M546V INDUCES SUBUNIT ACCUMULATION, NEURODEGENERATION, AND SEIZURE IN MICE

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Background: Neuronal K_v7/KCNQ channels are voltage-gated potassium channels composed of $K_v7.2$/KCNQ2 and $K_v7.3$/KCNQ3 subunits. Enriched at the axonal membrane, they potently suppress neuronal excitability. ***De novo*** heterozygous mutations in $K_v7.2$ are associated with early-onset epileptic encephalopathy characterized by drug resistant seizures and profound psychomotor delay. However, their precise pathogenic mechanisms remain elusive.

Method: We investigated selected epileptic encephalopathy mutations in calmodulin (CaM)-binding helix B of $K_v7.2$ using interdisciplinary approach including electrophysiology, biochemistry, imaging, and knock-in mice. One of the mutations which we have focused on is M546V which is located at the CaM contact site in helix B. A patient with M546V mutation display drug-resistant seizures, mental retardation, and autism spectrum disorder.

Results: We discovered that the M546V mutation caused multiple severe defects in Kv7 channels including marked reductions in their CaM binding and their current and surface expression on hippocampal neuronal axon. This mutation also induced ubiquitination and accelerated proteasome-dependent degradation of Kv7.2, whereas the presence of Kv7.3 blocked this degradation, resulting in increased neuronal death in dissociated hippocampal culture. To test if such multiple Kv7 channel defects could exert more severe impacts on neuronal excitability and health in vivo, we generated conditional knock-in mice, in which a single copy of mouse Kv7.2-M547V (equivalent to human Kv7.2-M546V) and EGFP are expressed in forebrain excitatory neurons. We confirmed that Kv7.2-M546V knock-in mice display Kv7.2 aggregation and neurodegeneration in their hippocampus and cortex and display increased seizure propensity.

Conclusion: Taken together, these findings led to a surprising conclusion that epileptic encephalopathy M546V mutation in helix B of Kv7.2 involves a combination of distinct defects in Kv7 channels as well as neuronal injury. We are excited about our current studies on Kv7.2-M546V knock-in mice which should yield valuable insights into the pathogenic mechanisms underlying ***KCNQ2*** epileptic encephalopathy.

[P20] GENOTYPE-PHENOTYPE CORRELATIONS IN KV7.2-RELATED EPILEPSIES CAUSED BY DISTINCT MUTATIONS ON THE SAME RESIDUE

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Background: One of the major challenges in the channelopathy field is the understanding of the correlation between in vitro phenotypes and the clinical disease characteristics. In Kv7.2-related disorders, mutations occurring in benign/familial cases are mainly associated to mild loss-of-function (LoF) effects, whereas quantitatively stronger LoF deficits are caused by variants responsible for more severe sporadic cases of early-onset epileptic encephalopathy. To complicate this pattern, variants causing gain-of-function (GoF) effects have been more recently found in small series of patients with characteristic clinical phenotypes, often in the absence of neonatal seizures. These observations suggest that in vitro testing has a critical role not only in defining the pathogenic role of a specific variant, but also in predicting distinct clinical phenotypes. To support this view, we herein present genetic and clinical, as well as functional and pharmacological in vitro data on two patients carrying Kv7.2 mutations on the same residue (S195): the first patient, carrying the S195P variant, had no history of neonatal seizures but displayed infantile spasms at 5 months of age (Weckhuysen ***et al.***, 2013); the second patient, in which the S195F variant was found, showed the characteristic clinical pattern of neonatal-onset KCNQ2-epiletic encephalopathy.

Method: Genetic analysis was performed by next-generation sequencing. Mutations were engineered by QuickChange mutagenesis; channel subunits were expressed in CHO cells by transient transfection. Currents were recorded using the whole-cell configuration of the patch-clamp technique.

Results: Electrophysiological recordings revealed that, when compared to wild-type Kv7.2 channels, the voltage-dependence of Kv7.2 S195P or Kv7.2 S195F channels was voltage-shifted in opposite directions, namely toward the left or the right of the voltage axis, respectively. These results are consistent with GoF for the S195P or LoF for the S195F in vitro effects. Qualitatively similar, although quantitatively smaller, effects were also observed upon co-expression of mutant Kv7.2 subunits with Kv7.2/Kv7.3 subunits, to mimic the genetic balance of each proband. Selective Kv7 activators (retigabine) or blockers (ML252 and NH17) (Cheung ***et al.,*** 2012; Kornilov ***et al.,*** 2014) restored wild-type behaviour in Kv7.2 S195F or S195P channels, respectively.

Conclusion: These results suggest that clinical phenotypes of Kv7.2-related epilepsies associated to different variants affecting the same residue can lead to distinct and even opposite in vitro phenotypes, and point toward variant-specific personalized pharmacological approaches.

[P21] KV7 ACTIVATORS AS TARGETED THERAPIES FOR EPILEPTIC ENCEPHALOPHATY CAUSED BY KV7.2 MUTATION ASSOCIATED TO AN ALTERATION IN CURRENT REGULATION BY BOTH PIP2-AND CALMODULIN

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Background: Epileptic Encephalopathies (EEs) are severe forms of epilepsy in which epileptiform activity contributes to a progressive cerebral dysfunction. Recently, ***novel*** mutations in the ***KCNQ2*** (Kv7.2) gene have been identified in patients affected by early-onset EEs (EOEEs; www.rikee.org). Kv7.2, together with Kv7.3 subunits, underlie the M-current (I_{KM}), a K^+ current that regulates neuronal excitability. Mutations in Kv7.2 often affect the C-terminus where the binding site for calmodulin (CaM) and phosphatidylinositol-bisphosphate (PIP_2) have been identified. Most of Kv7.2 mutations induce loss-of-function (LoF) effects, leading to the suggestion that Kv7 activators may represent a precision medicine approach. Retigabine is the first-in-class Kv7 activator, showing high selectivity and efficacy; however, because of safety concerns, this drug has been recently withdrawn. Kv7 activation by gabapentin has been recently described (Manville and Abbott, 2018). In this study, we have characterized the functional and pharmacological properties of channels incorporating a ***novel*** Kv7.2 mutation (c.928G>A; p.Gly310Ser) found ***de novo*** in a patient affected by EE.

Method: CHO cells were transiently-transfected with plasmids containing the cDNA for wild-type or mutant Kv7.2 or Kv7.3 subunits and used 24 h later for biochemical, functional and pharmacological experiments.

Results: Patch-clamp recordings revealed that CHO cells expressing mutant Kv7.2 channels were not functional, despite being expressed at the plasmamembrane. Furthermore, Kv7.2 Gly310Ser subunits prompted a strong inhibition of the currents expressed by Kv7.2, Kv7.3, or Kv7.2/Kv7.3 subunits, suggesting a mutation-induced LoF effects. Co-expression with a mutant CaM isoform (CaM*****) unable to bind Ca^{2+} (but not with CaM), partially rescued Kv7.2 Gly310Ser currents; similar effects were observed in the presence of PIP5K, an enzyme increasing endogenous PIP_2 levels; additive effects were observed upon co-expression of CaM* and PIP5K. Exposure to retigabine (10 μM) restored Kv7.2/Kv7.2 Gly310Ser/Kv7.3 currents to wild-type levels; preliminary ***in vitro*** experiments also revealed that gabapentin (10 μM) activated both Kv7.2/Kv7.3 and Kv7.2/Kv7.2 Gly310Ser/Kv7.3 currents. On these basis, treatment of the proband with gabapentin (600 mg/die) was safe, and resulted in a mild improvement in terms of seizures and postural control, allowing the withdrawal of the anti-epiletic drug levetiracetam.

Conclusion: Overall, the results obtained suggest that the p.Gly310Ser variant causes a complex alteration in CaM/PIP_2-dependent regulation of Kv7.2 currents. Moreover, Kv7 activators could be considered as valuable therapeutic tools for EE-affected patients carrying LoF Kv7.2 mutations.

[P22] IN-VITRO AND EX-VIVO CHARACTERIZATION OF A NOVEL HOMOZYGOUS KCNQ3 P.PHE534ILE VARIANT IN A PATIENT DIAGNOSED WITH A NON-SYNDROMIC INTELLECTUAL DISABILITY AND NEONATAL-ONSET PHARMACODEPENDENT EPILEPSY

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Background: Variants in KCNQ3 have been associated with neonatal-onset epilepsies showing broad clinical heterogeneity and diverse genetic transmission mechanisms. Notably, in both familial and sporadic cases, KCNQ3 pathogenic variants mostly carry single missense heterozygous variants, either with autosomal-dominant inheritance or arising de novo, respectively (www.rikee.org). Two exceptions are a recently-described patient who carries two missense variants in compound heterozygosity (Ambrosino et al., 2018), and a family with three siblings affected with neonatal-onset seizures and intellectual disability due to a homozygous frameshift variant in KCNQ3 (Kothur et al., 2018). The aim of this work was to report the clinical, molecular and functional properties of a new KCNQ3 variant found in homozygous configuration in a 9-year-old girl born to consanguineous healthy parents with pharmacodependent neonatal-onset epilepsy and non-syndromic intellectual disability.

Method: Exome sequencing was used for genetic investigation. Fibroblasts for ***ex-vivo*** experiments were obtained from punch skin biopsies and used for mRNA abundance assessment by RT-PCR and iPSC (induced Pluripotent Stem Cells) generation, by a non-integrated, episomal reprogramming system. Neural precursor cells were obtained from iPSC upon culturing in neural induction medium and then differentiated into neurons according to Verpelli et al. (2013). ***In vitro*** studies were performed by patch-clamp electrophysiology in CHO cells heterologously-expressing KCNQ3 subunits.

Results: A novel single-base duplication in exon 12 of KCNQ3 (NM_004519.3:c.1599dup) was found in homozygous configuration in the proband; this frameshift variant introduced a premature termination codon (PTC), thus deleting a large part of the C-terminal region. Mutant KCNQ3 transcript and protein abundance was markedly reduced in primary fibroblasts from the proband when compared to those from the wild-type brother. The mutation fully abolished the ability of KCNQ3 subunits to assemble into functional homomeric or heteromeric channels with KCNQ2 subunits. To investigate the effects of the KCNQ3 Phe534Ile fs*15 variant in neurons, iPSC from proband and isogenic control fibroblasts were derived and used in neural induction experiments. Functional, morphological and molecular experiments are ongoing.

[P23] SPECIALIZED SIGNAL PROCESSING OF THE DENTATE GYRUS RELIES ON KCNQ2/3 CHANNEL INHIBITION

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Background: The dentate gyrus is the first pathway in the trisynaptic circuit of the hippocampus. The dentate gyrus (DG) filters afferent inputs into the hippocampal circuit as a "gatekeeper" of excitability, which is crucial to the processing of physiological and pathophysiological network functions. DG granule cell (DGGC) neurons receive many excitatory inputs into the hippocampus coming from the entorhinal cortex and basal forebrain for downstream neurotransmission. Despite the clearly defined role for KCNQ2/3 channels in inhibitory control of neurons, their function in acquired epileptogenesis is not very well understood.

Method: We investigated the role of KCNQ2/3 channels in the dentate gyrus in response to neuromodulation and hyperexcitability using patch-clamp electrophysiology in brain slice and ***in vivo*** seizure models in mice.

Results: Upon stimulation of muscarinic acetylcholine receptors and other classes of Gq-coupled receptors, DGGCs were observed to have enhanced M current amplitudes, rather than suppression of M current. This was determined to be dependent on net-synthesis of PIP_2 after initial Gq-coupled phospholipase C activity in these cells. In hippocampus, this was a specialized property of DGGCs, as CA1 pyramidal neurons, in contrast, demonstrated suppression of M current after muscarinic receptor stimulation. Therefore, the signal processing demands of these classes of neurons appear to be different in response to cholinergic activity. After a chemoconvulsant challenge to the brain, DGGCs exhibit significantly greater firing, and the hippocampal network is hyperexcitable. When exposed to a secondary challenge with the $GABA_A$ receptor antagonist pentylenetetrazole, mice demonstrated greater seizure susceptibility. However, this seizure susceptibility could be attenuated by pre-treatment with the M-channel opener retigabine. In mice with KCNQ2-deficiency exclusive to the DG (using Cre-POMC mice crossed with floxed KCNQ2 mice), we observed greater seizure susceptibility to challenge with pentylenetetrazole. Furthermore, mice that were haploinsufficient in both KCNQ2 and KCNQ3 demonstrated greater seizure susceptibility than wild-type counterparts.

Conclusion: Therefore, we report a pivotal role of the DG as a filter for the hippocampus in controlling excitability. The regulatory cascades involving KCNQ2/3 channels may be instrumental to disorders of hyperexcitability or hypoexcitability, such as epilepsy or cognitive dysfunction.

[P24] A NOVEL VARIANT IN THE S4 VOLTAGE-SENSOR OF KCNQ3 IMPLICATED IN BENIGN FAMILIAL NEONATAL EPILEPSY

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Background: ***KCNQ2*** variants account for the majority of pedigrees with Benign Familial Neonatal Epilepsy (BFNE) and ***KCNQ3*** for a much smaller subgroup, but the reasons for this imbalance remain unclear. While heterozygous missense ***de novo*** variants in ***KCNQ2,*** usually clustered in S4, the pore domain, and C-terminal regulatory segments, are a common cause of neonatal-onset epileptic encephalopathy, this has not emerged for ***KCNQ3.*** Analysis of additional pedigrees is needed to further clarify genotype-phenotype relationships and to improve prediction of pathogenicity and severity for novel variants.

Method: As part of ongoing investigations of genetic epilepsy, we identified a family where a BFNE history was present in two siblings and their parent. We performed tetrad exome sequencing and bioinformatic analysis on samples from both parents and siblings and collected related family and clinical histories. Patch clamp studies of the variant are anticipated but not completed to date.

Results: Sequencing revealed the novel variant, ***KCNQ3*** c.719T>G (p.Met240Arg) in the three affected individuals, absent from the unaffected parent. This Met is fully conserved among human ***KCNQ1-5*** and lies between the R5 (Arg239) and R6 (Arg242) positions of the voltage-sensing S4 transmembrane segment. The variant is absent from gnomAD and predicted to be deleterious (polyphen-2, 0.989; SIFT, 0.9122; CADD score 27.3). We found no previous reports of neonatal-onset epilepsy associated with KCNQ3 S4 variants, nor of patients with variants at the paralogous KCNQ2 Met211 residue. However, the variant introduces a positive charge and this portion of S4 is a hotspot for charge-changing KCNQ2 variants causing BFNE (KCNQ2 Asp212Gly[1]) variable phenotypes with BFNE, myokymia, and epileptic encephalopathy (e.g., KCNQ2 Arg207Gln;[2,3]), or epileptic encephalopathy (Arg210His;[4,5] RIKEE.org n=8; Arg210Cys[6]). Where studied, these have reduced current via slowing of activation and/or depolarizing shifts of $v_{1/2}$.

Conclusion: We speculate that KCNQ3 Met240Arg will reduce current, but this awaits experimental proof. Quantitative comparison of the effects of this ***KCNQ3*** BFNE variant with nearby ***KCNQ2*** variants causing epilepsy with a spectrum of phenotypes may shed additional light on the distinct genotype-phenotype relationships of the two subunits and aid future efforts towards early prognosis prediction.

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[P25] CYSTEINE-MODIFICATION OF KV7 CHANNELS AS ANALGESIC MECHANISM OF ACTION OF PARACETAMOL

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Background: Application of NAPQI (N-acetyl-p-benzoquinone imine), an active metabolite of paracetamol, reduces neuronal excitability in first- and second-order neurons of the pain pathway. The reduction in action potential frequency and hyperpolarization is mediated by an activation of Kv7 channels. NAPQI is known to modify cysteine residues and both cysteine-alkylation as well as oxidative modification of cysteine residues in Kv7 channels were shown to increase M-currents. The aim of the study is to determine, if modification of cysteine residues in Kv7 channels by NAPQI is responsible for the reported increase of Kv7 currents.

Method: Heterologously expressed Kv7.2 to Kv7.5 subtypes were recorded in the perforated voltage clamp mode. For time course experiments, cells were clamped to -30 mV. Once every 15 s, the cells were hyperpolarized to -80 mV to elicit a deactivation current. A baseline was recorded for two minutes, followed by application of 3 µM NAPQI for ten minues and a subsequent 5-minute wash-out phase. Activation curves were recorded between -150 mV and +50 mV in 10 mV increments. Current levels at +60 mV were evaluated. Activation curves were recorded before, after three and ten minutes of NAPQI application. The effects of NAPQI were tested on wt and mutated versions of the respective Kv7 channel subtypes.

Results: While currents through Kv7.3 channels remained unchanged, deactivation currents through heterologously expressed Kv7.2, Kv7.4 and Kv7.5 channels increased about two-fold after ten minutes of NAPQI perfusion. The currents remained unaltered during a subsequent five-minute wash out phase. Half-maximal activation of these subtypes was left shifted and their maximal current levels were increased. Mutation of a triple-cysteine stretch in the S2-S3 linker region prevented the NAPQI-mediated increase.

Conclusion: This module of three subsequent cysteine residues was previously shown to mediate the current increase induced by oxidative modification. We conclude that covalent modification of this stretch of three cysteine residues is responsible for the increase of Kv7 currents in response to NAPQI application.

[P26] GOSLO-SR-5-6 ACTIVATES KV7 CHANNELS AND IT'S EFFECTS ARE REDUCED BY A F322A MUTANT IN KV7.4

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Background: K_v7.4/7.5 channels help regulate airway diameter and activators of these channels induce relaxation[1]. We previously demonstrated that GoSlo-SR-5-6 (SR-5-6) activates BK channels, but its proposed mechanism of action[2] suggests that it may activate other K^+ channels, including K_v7.

Method: HEK cells were transiently transfected with K_v7.1-K_v7.5 cDNA and whole cell currents recorded under voltage clamp in cells perfused with a K^+ rich pipette solution. Cells were held at -80 mV, stepped for 1s from -100 to +40 mV in 10 mV steps and repolarised to -120 mV. G-V curves were plotted from tail currents and fitted with the Boltzmann equation.

Results: SR-5-6 (10 mM) constitutively activated K_v7.1 and K_v7.5 currents and significantly increased G_{MAX} (at +40 mV) to 3 ± 0.4 (mean±SEM, n=5) to 2.9 ± 0.2 (n=7), respectively. However, SR-5-6 failed to alter G_{MAX} of either K_v7.2 or K_v7.3, but modestly shifted the voltage of half maximal activation ($V_{1/2}$) by -20 ± 3 mV (n=6) and -16 ± 1 mV (n=5). In contrast, SR-5-6 increased G_{max} to 1.7 ± 0.1 and significantly shifted $V_{1/2}$ from -19 ± 2 mV to -62 ± 2 mV ($\Delta V_{1/2}$ =-43 mV, n=11) in K_v7.4. These effects were not altered by a mutation[3] (W242L) which abolished the effects of the retigabine analogue, ML213 (10 mM). An S6 mutant (F322A) of K_v7.4 reduced the effects of SR-5-6 ($\Delta V_{1/2}$ -15 ± 3 mV, G_{MAX} 1.12 ± 0.1, n=6), but not ML213 (n=6).

Using a homology model based on the cryo-EM structure[4] of K_v7.1, we identified a potential binding pocket near the distal end of S6. However, mutation of residues in this pocket failed to reduce the effects of SR-5-6. We next constructed a K_v7.3/7.4 chimera in which the S6 transmembrane helix of K_v7.3 was swapped into the K_v7.4 background. These chimeric channels activated more positively than WT K_v7.4 ($V_{1/2}$=0 ± 2 mV, n=9) and SR-5-6 shifted $V_{1/2}$ by -33 ± 5 mV and increased the G_{MAX} to 2.3 ± 0.1 (n=9).

Conclusion: Taken together, these data suggest that although the F322A mutant can reduce the effects of SR-5-6 on K_v7.4, S6 is unlikely to be involved in the binding of this compound.

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[P27] EXPRESSION PROFILE OF KV7 CHANNELS AND THEIR FUNCTIONAL INTERPLAY WITH CANNABINOID RECEPTORS AS A POTENTIAL STRATEGY FOR RESTORING COMPROMISED AUTOPHAGY IN DUCHENNE'S MUSCULAR DYSTROPHY

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Introduction: Duchenne's muscular dystrophy (DMD) represents the most frequent form of hereditary myopathy affecting about 1:3,500 newborn boys. Mutations in the gene encoding for dystrophin, a key protein connecting the cytoskeleton of a muscle fiber to the surrounding extracellular matrix through the cell membrane, are the cause of the disease. Recent studies demonstrated that a progressive decline in the autophagy process is one of the main causes leading to inefficient muscle regeneration. The involvement of K_v7 K^+ channels in skeletal muscle dystrophies as well as autophagy remains unknown. Only recently, we have demonstrated that: i) K_v7 stimulation by Retigabine promotes myotubes formation; ii) the endocannabinoid 2-Arachidonoylglycerol (2-AG) inhibits myoblast differentiation via CB1 receptor-mediated inhibition of $K_v7.4$ channels; iii) in gastrocnemius and quadriceps of dystrophic mice, compared to control tissues, CB1 transcripts showed a highest degree of expression at disease onset (5 weeks) and then declining over time (8 weeks); vi) Rimonabant, a selective CB1 receptor antagonist, increased the number of healthy/regenerating myofibers by reducing inflammation and concomitantly promoting autophagy. In the light of this evidence, we characterized the expression profile of K_v7 K^+ channels and subsequently explored their potential involvement in DMD onset and progression.

Materials and methods: Transcript levels of Kv7 and autophagy genes were measured in dissected skeletal muscles (gastrocnemius and quadriceps femoralis) isolated from control and dystrophic mice at both 7 and 34 weeks by quantitative qPCR analysis.

Results: We found that, among the K_v7 genes, only $K_v7.4$ and $K_v7.5$ are abundantly expressed in control as well as DMD skeletal muscle tissues. Of note, the expression of $K_v7.4$ was found to be higher in DMD than in control muscles immediately after (8 weeks) the onset of pathology, and not at the onset as for CB1, and then to decline over time (34 weeks). Intriguingly, the transcript levels of ATG4 and ULK-1, two known master genes regulating autophagy, showed an expression profile very similar to $K_v7.4$. In contrast, the expression of $K_v7.5$ was unchanged between control and DMD mice at both 7 and 34 weeks.

Conclusions: In summary, we demonstrated that among all K_v7 genes, only $K_v7.4$ undergoes changes during DMD progression. Furthermore, we present here evidence in favor of a plausible functional interplay between $K_v7.4$ channels and endocannabinoid CB1 receptors that may provide the bases for an unpredicted strategy to ameliorate certain pathological aspects of DMD, including muscle differentiation and regeneration possibly through the restoration of autophagy.

[P28] PHARMACOLOGICAL CHARACTERIZATION OF IKS USING AUTOMATED PATCH CLAMP

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Background: The cardiac I_{Ks} channel, the slowly activating delayed rectifier potassium channel, consists of the pore-forming α subunit (KCNQ1) and a modulatory β subunit (KCNE1) and is an important ion channel for ventricular repolarization. Decreases in I_{Ks} due to genetic mutations cause Long QT syndrome, a potentially fatal cardiac disorder. Equally, compounds which inhibit I_{Ks} can also induce potentially fatal arrhythmia. I_{Ks} is one of the ion channels involved in the comprehensive in vitro proarrhythmia assay (CiPA) study for more accurate cardiac safety testing of new and existing drugs on the market. Patch clamp electrophysiology remains the gold standard for studying ion channels, but manual patch clamp requires skilled personnel and is unsuitable for high throughput screening efforts. Automated patch clamp is becoming widely accepted as a medium and high throughput alternative to manual electrophysiology.

Method: We have used CHO cells expressing KCNQ1/KCNE1 on automated patch clamp devices recording from 8 or 384 cells simultaneously. Voltage step protocols were run to establish the current-voltage relationship. Furthermore, pharmacological agents chromanol 293B and XE991 were applied to the cells and IC_{50} values were calculated. The presence of I_{Ks} in human stem cell-derived cardiomyocytes (hiPSCs) was also investigated.

Results: I_{Ks} started to activate at approximately -20 mV and current amplitude increased with each voltage step up to 60 mV. Pharmacological agents were applied via the external solution. I_{Ks} was elicited using a single voltage step protocol to 60 mV for 5 s or 40 mV for 2 s and either single point or cumulative concentration response curves were applied to each cell. Chromanol 293B inhibited I_{Ks} mediated responses with an IC_{50} between 2 and 14 µM. XE991 inhibited IK_s with an IC_{50} between 73 and 112 nM.

Conclusion: Using voltage protocols in accordance with CiPA guidelines, I_{Ks} could be recorded on two different automated patch clamp devices and pharmacology of known blockers was confirmed. The use of automated patch clamp to record IK_s at higher throughput for cardiac safety screening is feasible and can be performed routinely with good success rates for completed experiments.

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[P29] CHARACTERIZATION OF KCNQ2 G256W IN VITRO AND IN VIVO

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Background: Variants in ***KCNQ2*** are a common cause of neonatal onset epileptic encephalopathy[1]but a definite relationship has not been established. In this study we investigated whether KCNQ2/3 mutations are a frequent cause of epileptic encephalopathies with an early onset and whether a recognizable phenotype exists. METHODS We analyzed 80 patients with unexplained neonatal or early-infantile seizures and associated psychomotor retardation for KCNQ2 and KCNQ3 mutations. Clinical and imaging data were reviewed in detail. RESULTS We found 7 different heterozygous KCNQ2 mutations in 8 patients (8/80; 10%. In their neonatal presentation and subsequent outcome, KCNQ2 encephalopathy (Q2E) patients include a severity spectrum. Although the majority of Q2E patients have severe to profound global developmental delay after a neonatal presentation with refractory seizures, burst-suppression interictal EEG, and clinical encephalopathy, a smaller subgroup have milder neonatal presentations and subsequently have less severe developmental trajectories, with relative preservation of gross motor development but markedly impaired language, social, and cognitive abilities as well as autistic like features and breakthrough seizures[1]but a definite relationship has not been established. In this study we investigated whether KCNQ2/3 mutations are a frequent cause of epileptic encephalopathies with an early onset and whether a recognizable phenotype exists. METHODS We analyzed 80 patients with unexplained neonatal or early-infantile seizures and associated psychomotor retardation for KCNQ2 and KCNQ3 mutations. Clinical and imaging data were reviewed in detail. RESULTS We found 7 different heterozygous KCNQ2 mutations in 8 patients (8/80; 10%, [2]. Expressed in heterologous cells, most Q2E mutations act in dominant negative fashion, resulting in a >50% reduction of maximal current density, or strongly shifting voltage-dependence of activation. Construct and face valid murine models of Q2E have been difficult to develop, but are needed both to better understand the mechanisms of the complex associated phenotypes and as platforms for therapy development.

Method: We have studied the mechanisms of KCNQ2 p.Gly256Trp, a variant identified in a patient at the less severe end of the Q2E spectrum[2], in vitro and in vivo. We introduced the variant by site-directed mutagenesis and performed patch clamping in heterologous cells. We generated transgenic knock-in mice in the C57BL/6J background via CRISPR/Cas9. We performed current clamp recordings in hippocampal pyramidal cells and studied the impact of the variant on development, behavior and seizures.

Results: In patch clamp recordings from CHO cells, the Gly256Trp variant showed dominant negative effects. Our preliminary data indicate 100% P0 mortality in homozygous Gly256Trp mice, and 23% mortality by P3 in heterozygous mice. Heterozygous mice that survive the early neonatal period show no differences in the attainment of several developmental milestones including: weight gain, surface righting, negative geotaxis, cliff aversion, and forelimb grasp. We observed a spontaneous generalized, primarily tonic seizure in a P10 G256W heterozygote. Additional studies of behavior, spontaneous seizures, and seizure susceptibility are underway. CA1 pyramidal cells in slices from 21 day old G256W/+ mice demonstrate reduced action potential thresholds, increased firing rates, and increased input resistance.

Conclusion: We have created a construct valid mouse model of “less severe” Q2E. We have begun to elucidate links between the molecular effects of the variant on channel function and the cellular, circuit, developmental and behavioral consequences.

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OTHER

[P30] DISTINGUISHING BETWEEN BIOLOGICAL AND TECHNICAL REPLICATES: IMPLICATIONS FOR VASCULAR KV7 CHANNEL RESEARCH

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Background: Perivascular adipose tissue (PVAT) is a regulator of vascular tone. Voltage-gated K_v channels (K_v7) in vascular smooth muscle cells (VSMCs) are proposed to play a key role in this vasoregulation because the anti-contractile effects of PVAT are inhibited by the pan KCNQ channel blockers, such as XE991 and 4-AP. However, there is no direct evidence that K_v7 channel opening, for example by PVAT or synthetic Kv7 channel openers, can cause hyperpolarization of VSMCs to cause relaxation. Moreover, there is a major concern on how to distinguish between biological vs. technical replicates in this area of research.

Method: We tested that hypotheses that K_v7 channel opening can cause VSMC hyperpolarization in the vessel wall to cause relaxation in isolated arteries and determined the outcomes of different experimental study designs on PVAT control of arterial tone.

Results: We found that the Kv7 opener flupirtine (10 µM) caused VSMC hyperpolarization from -33 +/- 3 to -45 +/- 3 mV and from -32 +/- 2 to -39 +/- 2 mV in isolated mouse mesenteric arteries precontracted with 1 µM phenylephrine and 10 µM serotonin, respectively. This membrane potential hyperpolarization caused relaxation of the vessels to 59 +/-10 % and 56 +/- 8 % of pre-contracted tension levels, respectively. Furthermore, we examined possible outcomes of different experimental study designs on PVAT control of arterial tone using isolated arteries. Based on experimental data, we determined the sample size and power of statistical analyses for such experiments. We evaluated whether n-values should correspond to the number of arterial rings and analyzed the resulting effects if those numbers are averaged to provide a single N-value per animal, or whether the hierarchical statistical method represents an alternative for analyzing such kind of data. Our analyses showed that that the data ($logEC_{50}$) from (+) PVAT and (–) PVAT arteries are clustered. Intra-class correlation was 31.4%. Moreover, hierarchical approach was better than regular statistical tests as the analyses revealed by a better goodness of fit (v^2-2LL test).

Conclusion: We propose to use at least three independent arterial rings from each from three animals for each group, i.e. (+) PVAT vs. (–) PVAT. Overall, our data support the concept that K_v7 channel opening in VSMCs in the vessel wall is a powerful mechanism to cause arterial relaxation. Moreover, our sample size calculations could be used as for the design of future studies on isolated arteries using the wire-myography technology.