TRPA1 receptor as a potential drug target in the treatment of neurodegenerative and neuroinflammatory diseases

PhD-thesis

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Pécs, 2022

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1. List of abbreviations

Bcl-2 - B-cell Lymphoma 2

BNO – International Classification of Diseases

BSA – Bovine Serum Albumine

CIS – Clinically Isolated Syndrome

Cr+pCr – Creatine + phosphocreatine

CV – Cresyl Violet

DICOM – Digital Image Communication

DPA – Dynamic Plantar Aesthesiometer

DRG - Dorsal Root Ganglion

EAE - Experimental Autoimmune Encephalomyelitis

ERK - Extracellular Signal-Regulated Kinases

FGF-2 – Fibroblastic Growth Factor 2

FOV – Field of View

GABA – Gamma Amino Butter Acid

GFAP - Glial Fibrillary Acidic Protein

HLSVD – Hankel Lánczos Single Value Decompositon

IBA-1 - Ionized Calcium-binding Adaptor Molecule

ICH – Intracerebral Hemorrhage

ICV - Intracerebroventricular

IGF-1 – Inzuline-like Growth Factor

LFB – Luxol Fast Blue

LILFU – Low Intensity Low Frequency Ultrasound

MAO – Monoamine Oxidase

MAPK - Mitogen-Activated Protein Kinase

MBP - Myelin basic protein

MR – Magnetic Resonance

MRI – Magnetic Resonance Imaging

NAA – N-acethyl aspartate

NFKB – Nuclear Factor Kappa-B

OPC - Oligodendroglia Prekurzor Cell

OVS - Outer Volume Suppression

PBS – Phospahte Buffered Saline

PDGFα - Platelet Derived Growth Factor Subunit A

PPMS – Primary Progressive Multiple Sclerosis

QPCR – Qunatitative Polymerase Chain Reaction

RARE - Rapid Aquisition with Relaxation Enhancement

ROI – Region Of Interest

SM – Sclerosis Multiplex

RRMS – Relapsing Remitting Multiple Sclerosis

SPMS – Secondary Progressive Multiple Sclerosis

TRPA – Transient Receptor Potential Ankyrin

WT – Wild Type

2. Introduction

As health care improves, diseases affecting the nervous system, particularly in old age, are becoming an increasingly prominent problem in the world. These conditions tend to have a severe impact on quality of life, requiring prolonged medical care, which places a heavy load on the health care system and the patient's family and environment.

The biggest problem with these injuries is that, to our current knowledge, there is no definitive and effective therapy. In our research we are not looking for the "philosopher's stone", but only to contribute some new experimental results to the early detection of these diseases, alleviate their symptoms, slow down the progression, control or reverse the pathological processes that have developed. We have studied the pathomechanism and the symptoms of multiple sclerosis in an animal model, and have developed testing protocols that facilitate the monitoring of progression and allow the identification of potential intervention points.

When I started my Ph.D., I chose multiple sclerosis as the subject of my research. The interest in this topic at our institute is not without precedent, and I started my research structure on previous work. The relationship between neurodegenerative processes and TRPA (transient receptor potential ankyrin) ion channels has been previously reported by colleagues (Sághy, Sipos, et al. 2016). I was in the fortunate position to have the help of Professor János Szolcsányi, a world-renowned expert in sensory neuropharmacology, at the beginning of my work. So I had the privilege to join a major scientific workshop.

There was a strong need to develop a complex test method to study different neurodegenerative models. I had already started to work with magnetic resonance methods during my undergraduate years, so I had some experience in applying the methodology. Magnetic resonance is also extensively used in the diagnosis of human MS for both functional and morphological studies.

2.1. Multiple sclerosis

Multiple sclerosis (MS), a chronic inflammatory autoimmune disease affecting the central nervous system, which damages the myelin sheath covering the nerve fibres of the brain and spinal cord. Inflammation occurs in diverse areas of the nervous system, where nerve cells are ultimately destroyed during immune activation. The progressive course of the disease is subsequently characterized by multifocal symptoms, which may involve nerves in the brainstem, optic nerve, cerebellum and spinal cord (Sand 2015). Its pathomechanism is characterized by alternating relapses and remissions and a continuous slow progression. Its treatment currently consists of symptomatic management of relapses and control of chronic autoimmune processes. MS is not a classic neurological disease of old age, as alarming symptoms most often appear in young adulthood. The course of the disease is lifelong and eventually leads to disability. The clinical picture identifies several types (NMSS 2022) (National Multiple Sclerosis Society, USA).

2.1.1. Clinically Isolated Syndrome (CIS)

CIS is the first episode of neurological symptoms caused by inflammation and demyelination of the central nervous system. This episode should last for at least 24 hours, typical of multiple sclerosis, but is not enough to establish the diagnosis. If CIS is accompanied by lesions detectable by brain MRI, the person is likely to have a second episode of neurological symptoms and relapsing-remitting MS.

Individuals with CIS who are at high risk of developing MS can be treated with disease-modifying therapies that delay the onset of manifest MS.

2.1.2. Relapsing-Remitting Form (RRMS)

This form is characterized by typical seizures followed by periods of partial or complete recovery (remission). During remissions, all symptoms may disappear. However, the disease progresses even during periods of remission. RRMS can be further characterized as active or inactive (based on MRI findings), and worsening (if the quality of life deteriorates) or non-worsening.

2.1.3. Secondary progressive form (SPMS)

SPMS initially has a relapsing-remitting course. A proportion of patients diagnosed with RRMS progress over time to a secondary progressive course with a relatively slow decline in neurological function.

2.1.4. Primary progressive form (PPMS)

PPMS is characterized by rapid deterioration of neurological function from the onset of symptoms, without early remissions. It is the most aggressive form of the disease and has the worst prognosis.

2.1.5. Pathology of MS

In MS, plaques formed in the central nervous system. These plaques form in the brain and spinal cord, mainly in the white matter around the ventricles, in the optic nerves and tracts, in the corpus callosum, in the cerebellar peduncles, in the long tracts, and in the spinal cord and brainstem, but they can also be found in the grey matter. They are present in all forms of the disease, but their quantity and composition vary between the different forms of the disease and also vary during progression. These plaques, although heterogeneous, usually contain demyelinated axons and their remnants, oligodendrocytes, astrocytes, and perivenular or parenchymal infiltrates (including lymphocytes and macrophages). The progressive phase shows atrophy of grey and white matter, with inflammation and microglial activation at the plaque borders, together with diffuse damage to the normal-appearing white matter outside the plaques. Demyelination in all forms is also the result of an immunological reaction. The myelin sheath is extremely vulnerable to non-specific immune products, such as cytotoxic cytokines, excitotoxins, reactive oxygen species, or nitric oxide species. Antibody- and complement-associated lesions and hypoxia-like tissue injury are frequently observed, in which the initiation of demyelination is attributed to apoptosis of oligodendrocytes. Altered astrocyte signaling also leads to compromised myelination (Huang, Chen, et al. 2017).

Approximately 85% of cases can be classified as RRMS. In Hungary, the prevalence is 3-5/100 000 (based on BNO, G35H0), thus affecting about 8000 patients and resulting in 300-500 new diagnoses per year, approximately equal to the number of patients lost per year (OEP 2010).

The scientific community worldwide has devoted considerable resources to the treatment of this disease. To find a solution, it is of course essential to know the exact course of the disease, and my Ph.D. thesis is part of this task.

2.2. Characteristics of the cuprizone-induced demyelination model

The experimental cuprizone-induced demyelination is a suitable rodent model (in mice) to study the processes leading to myelin loss. No significant lymphocyte response is induced in the model (Remington, Babcock et al. 2007). The main histological features are in good conformity with those

observed in the course of human type III MS (Torkildsen, Brunborg et al. 2008, Kipp, Clarner et al. 2009, Acs and Kalman 2012). The first of the cuprizone-induced effects in time is the apoptosis of mature oligodendrocytes, which is manifested in the primary demyelination of affected areas, this phenomenon being most spectacular in the corpus callosum (Blakemore 1972, Komoly, Hudson et al. 1992, Mason, Langaman et al. 2001). Subsequently, diffuse astrocyte activation and microglia and macrophage invasion can be observed (Praet, Orije et al. 2015). A dual role can be suspected behind these reactions, with a role in the onset of demyelination and remyelination processes observed later in the experiments (Pasquini, Calatayud et al. 2007, Kang, Liu et al. 2012, Voß, Škuljec et al. 2012). In all possibility, the two processes start in parallel and a kind of imbalanced equilibrium may develop between the two phenomena.

In previous years, the role of some growth factors in pathogenesis has also been highlighted (Gudi, Gingele et al. 2014). Activated astrocytes and macrophages may produce multiple growth factors during the pathogenesis, which may exert different effects on different cell types and may also interfere with each other. An essential element of the system is the stimulation of oligodendrocyte progenitor cell proliferation and the regulation of differentiation of mature oligodendrocytes. Growth factors also play a fundamental role in determining the chances of apoptosis/survival of surrounding cells. Fibroblast-derived growth factor 2 (FGF-2) and platelet-derived growth factor (PDGFα) push cells towards differentiation and restrain proliferation. In contrast, insulin-like growth factor (IGF-1) slows oligodendrocyte differentiation (Gudi, Gingele et al. 2014).

A further complicating factor is that members of the Bcl-2 (B-cell lymphoma 2) gene family may interfere with this process, both on the pro- and anti-apoptotic side (Itoh, Itoh et al. 2003). Lindsten and colleagues described already in 2000 that they may be able to release, for example, cytochrome C (Lindsten, Ross et al. 2000). Li et al. have shown in vitro in oligodendrocyte cultures that p53 has very strong proapoptotic effects, resulting in extensive cell death and consistent demyelination, accompanied by microglial activation (Li, Zhang et al. 2008).

Together with this, it becomes clear how complex is the system, what we are trying to map, and how many different regulated processes are involved. On the other hand, it also shows that there are many opportunities for intervention. We have considered all this and decided to use the cuprizone model.

2.3. Role and importance of the TRPA-1 receptor (Transient Receptor Potential Ankyrin 1) in the demyelination process

The TRPA-1 receptor is a member of the TRP receptor superfamily, which includes a total of 28 similar receptors, non-selective cation channels (Clapham, Runnels et al. 2001). It can be activated by a variety of exogenous stimuli including allicin, mustard oil, allyl isothiocyanate, cinnamaldehyde, and other electrophilic agents (Bandell, Macpherson et al. 2007, Bautista 2015). In addition, endogenous substances can activate, for example, but not limited to; 8-iso-prostaglandin, 15-deoxy-Δ12,14-prostaglandin (Cruz-Orengo, Dhaka et al. 2008, Andersson, Gentry et al. 2012, Andersson, Gentry et al. 2013), long-chain polyunsaturated fatty acids (Motter and Ahern 2012), formaldehyde, hydrogen sulfide and hydrogen peroxide (Andersson, Gentry et al. 2012, Andersson, Gentry et al. 2013).

These suggest that its physiological role in signal transduction of inflammatory processes may be significant, as the metabolites listed, which are otherwise largely toxic, typically accumulate in inflamed, damaged tissue rather than in healthy tissue. The literature suggests that TRPA-1 may act as a kind of "oxidative stress sensor". Mechanical or cold damage can also open the channel (Story, Peier et al. 2003, Andersson, Gentry et al. 2012, Andersson, Gentry et al. 2013) and it also plays a crucial role in pain perception. It can be involved in a wide variety of physiological and pathological signaling processes and has been associated with colitis (Engel, Leffler et al. 2011), asthma (Grace, Baxter et al.

2014), migraine (Dussor, Yan et al. 2014), cystitis (DeBerry, Schwartz et al. 2014) and even the sensation of itching (Wilson and Bautista 2010).

It is expressed in higher amounts in the primary afferent somatosensory cortex. Its expression has been described in the trigeminal, ganglion, DRG (Nagata 2007). It is also present in several sites outside the nervous system, in keratinocytes, lung fibroblasts, and vascular endothelial cells (Nilius, Voets et al. 2005).

In electrophysiological terms, its most important role is in Ca^{2+} flux generation (non-selective cation channel). Shigetomi et al. described that inhibition of TRPA-1, either by antagonist or silencing RNA, can inhibit Ca^{2+} influx in primary astrocyte cultures (Shigetomi, Jackson-Weaver et al. 2013). It has also been observed that TRPA-1 inhibition is associated with GABA-ergic tone enhancement in hippocampal interneurons (Shigetomi, Tong et al. 2012). It has also been demonstrated in GFAP positive astrocytes in rats (Lee, Cho et al. 2012).

Its appearance on oligodendrocytes was first described in 2016 using an in situ hybridization technique (Hamilton, Kolodziejczyk et al. 2016). Also, Hamilton's group described that inhibition of TRPA-1 could reduce ischemia-induced Ca2+ current-mediated myelination in in-vitro studies, and therefore could potentially be a target for the development of a drug to counteract demyelination. A new paper on the topic has recently been published, describing how glial TRPA1 may regulate neuronal excitability in white matter under both physiological and pathological conditions (Lajoso, Flower et al. 2021).

Our institute has previously published a paper demonstrating that the TRPA-1 receptor is widely expressed in the mouse CNS and that knockout animals exhibit milder demyelination symptoms in response to cuprizone (Sághy, Sipos et al. 2016). Based on the above, it is important to note that the effect of the lesion is likely to be accompanied or closely followed by spontaneous remyelination (Mason, Langaman et al. 2001, Acs and Kalman 2012, Gudi, Gingele et al. 2014). However, it is not yet clear whether the protective effect generated by the inhibition or absence of TRPA-1 is through the attenuation of demyelination or possibly through the enhancement or promotion of spontaneous remyelination. Based on our previous results, we conclude that cuprizone administration does not increase the differentiation of OPC (oligodendroglial precursor cell) cells, even though myelination and oligodendrocyte destruction alone can activate both astrocytes and microglia/macrophage cells. It has also been shown that in the absence of the TRPA-1 receptor, the macrophage invasion pattern is altered, and less activation is observed along the astrocyte lineage (Sághy, Sipos et al. 2016). The milder course of these inflammatory responses correlates with a smaller degree of myelination. These results led us to conclude that the TRPA-1 receptor may play a role in oligodendroglia-astrocyte signaling and the resulting immune-mediated loss of myelin. This theory is further supported by the fact that a role for TRPA-1 in modulating astrocyte function has been described under physiological conditions (Shigetomi, Tong et al. 2012, Shigetomi, Jackson-Weaver et al. 2013) and that inflammatory factors released by astrocytes also directly and profoundly influence the apoptotic tendency of oligodendroglia (Linares, Taconis et al. 2006; Zeger, Popken et al. 2007; Kang, Liu et al. 2012; Gudi, Gingele et al. 2014). Cuprizone-induced apoptosis is most likely mediated by the ERK1/2 - p38-MAPK signaling pathway, which requires Ca^{2+} influx, which is partly mediated by TRP channels. The absence, reduced number, or dysfunction of these channels may confuse the apoptotic signaling cascade (Veto, Acs et al. 2010, Sághy, Sipos et al. 2016).

It should also be noted that TRPA-1 expression has not been detected on oligodendrocytes for a long time (Hamilton, Kolodziejczyk et al. 2016), and the effect has thus until recently been attributed exclusively to an astrocyte-oligodendrocyte-directed juxtacrine and/or paracrine communication, in which the astrocyte sends proapoptotic signals to surrounding oligodendrocytes, thereby inducing

their death. As the damage is much less severe and a bit modulated but occurs in the absence of the TRPA-1 receptor, it is very probably that several ultimately redundant proapoptotic signaling pathways are initiated, one of which is inhibited to produce the protective effect.

3. Objectives

As can be seen from the above, this is a complex and diverse topic with considerable potential for drug development. As a pharmacist, this is exceptionally exciting.

After an analysis of the literature, it suggests that this model could be suitable for a true preclinical drug development system if coupled with appropriate investigational methods. As the first step in my work, I needed to develop a complex testing protocol that was both sufficiently robust and precise to allow us to better investigate disease processes. Since there was no known testing protocol in the literature that could allow the examination of this neurodegeneration model in a time-resolved, self-controlled setup with the instrumental capabilities available to us, I aimed to set up such a testing framework.

We aimed to map the morphological, microstructural changes during the course of the cuprizone model by electron microscopy and MRI, as well as the behavioral differences. Since we are practically in the dark about metabolic changes, we also attempted to monitor metabolic changes in vivo, which we planned to do using MR spectroscopy, for which there are currently very few examples in the literature (Orije, Kara et al. 2015), also with a different focus. We hope that this method may also be suitable for early diagnosis, which to our current knowledge is not possible in the clinical practice of human MS. We plan to observe the role of the TRPA-1 receptor in the development of these lesions in a genetically modified TRPA-1 total knock-out and GFAP-Cre conditioned TRPA-1-KO mouse model, which will require the creation of the TRPA-1 GFAP-Cre conditioned KO mouse strain itself, as no such strain is currently available.

The series of experiments can be divided into 2 distinct phases based on the genetic conditioning of the mice used in the experiments, and therefore I have divided my thesis into two parts.

4. Materials and methods

4.1. TRPA-1 total knockout mouse model

First, we investigated the role of TRPA-1 in the cuprizone model in a TRPA-1 total-KO mouse model.

Both TRPA-1 $^{+/+}$ and TRPA-1 $^{-/-}$ animals were bred at our institute and maintained during the experiments. Up to 8 animals were kept in a standard polycarbonate box. The heterozygous pairs of the original strain were a gift from Professor Pierangelo Geppetti (University of Florence, Italy). The strain is based on C57/Bl6 mice and no evidence of any harmful phenotype has been described in the literature, nor did we observe any differences between TRPA-1 $^{+/+}$ and TRPA-1 $^{-/-}$ animals by any of the evaluated methods. Nor did the animals show any differences in behavior compared to either the control TRPA-1 $^{+/+}$ or the "standard, Wild-type" C57/Bl6 strain.

4.2. Testing protocol

A widely accepted experimental procedure was used (Hiremath, Saito et al. 1998, Matsushima and Morell 2001, Kipp, Clarner et al. 2009, Praet, Orije et al. 2015). Cuprizone (Bis(cyclohexanone)oxaldihydrazone) was administered to animals at a concentration of 0.2 m% ad libitum for 6 weeks in powdered rodent food. At the end of the experiments, the animals were sacrificed, perfused, and extensive histopathological examinations were performed on the dissected brains. Male mice 8-12 weeks old were used for the model. The physical/behavioral parameters of the animals were monitored daily in all experiments and body weight was also measured. During our work, the animals did not generally show signs of pain or other neurological problems, but such phenomena are known in the literature (Liebetanz and Merkler 2006, Franco-Pons, Torrente et al. 2007), so special attention was paid to this. If any signs were detected that affected the animals' welfare more than necessary, they were removed from the experiment, but this was extremely rare.

However, behavioral and social changes have been described in some studies. In particular, lower social interaction skills and anxiety, as well as increased exploratory behavior, have been observed previously (Xiao, Xu et al. 2008, Makinodan, Yamauchi et al. 2009, Xu, Yang et al. 2009, Xu and Li 2011). These factors were investigated in my work too.

4.3. Behavioral test procedures used

4.3.1. Open field test

One method of determining general anxiety involved placing animals in a relatively large arena for 10 minutes. The animals' movements were recorded with a digital camera and then valued using behavioral analysis software.

4.3.2. Y-maze test

The test used to determine the working memory function is the "Y-maze"

In this experiment, animals spend 5 minutes in a Y-shaped arena, during which they alternate between the three arms of the Y. The number and quality of alternations can be used to deduce the functional capacity of working memory (Kraeuter, Guest et al. 2019)

4.4. Mechanociceptive threshold measurement

Two methods were used to measure this parameter, the von Frey filament method and the dynamic plantar aesthesiometer (DPA). Mice were placed on a raised, gridded test platform and allowed to rest for at least 15 min. The two measurements were performed "in one run", without removing the animals from the test module.

First, the von Frey filament test was performed, in which a filament of a given thickness and stiffness was pressed 5 times on the sole of the mouse foot, the value corresponding to the given filament thickness was recorded as the pain threshold.

A few minutes after the first test, a DPA measurement was also performed. In this test, a blunt-tipped needle was used to apply increasing pressure to the animal's plantar fascia until it pulled away. The compressive force applied to the foot is fixed as a threshold.

4.5. Motor performance test

Motor power was also determined using two methods. Firstly, we measured a self-weighted grip test. The animals were placed on a grid on which they could comfortably cling, and then the grid was inverted, on which the mice clung while hanging down. We measured the time it took the animal to hold its own weight above the box and recorded the time it jumped down into the box.

For the second test, we used a device called a RotaRod. This is a cylinder that rotates at an accelerating speed and on which the mouse has to balance. The speed increased from 4 to 40 rpm in 5 minutes. The cut-off value was given by the fall-off time.

4.6. MRI scan (Magnetic Resonance Imaging)

The experiments were headed by a development period of several weeks, during which a large number of pilot measurements were carried out on phantoms and later on live animals. Due to the nature of the MRI measurements, the animals had to be anesthetized during the measurements, for which we used isoflurane (1mg/ml) vaporization, with respiratory monitoring and body temperature control. After the pilot measurements, the T2 RARE - (Rapid Acquisition with Relaxation Enhancement) sequence was found to be appropriate. Due to the nature of the T2-weighted ^{1}H measurement, the higher water content tissue appears as a brighter (higher pixel intensity) area in the image. The myelin-rich healthy corpus callosum appears darker in the image, as myelin is a rather apolar, lipid-like protein with very low water content. Cuprizone treatment causes this myelin to loosen and then to break and be replaced by cerebrospinal fluid in increasing proportions. This increase in water content can be observed in the T2 image, where the increase in pixel intensity is proportional to the increase in water content, and hence to the decrease in myelin. Based on the histological image, the extent of damage correlates well with the values determined by MRI.

4.7. Histopathological tests

4.7.1. Luxol Fast Blue (LFB)

LFB staining is used to mark myelin in the tissue (Acs and Kalman 2012). The stained sections are evaluated by two independent operators on a scale of 0-3 in a semi-quantitative way. 0 indicates intact myelin and 3 indicates the complete absence of myelin. Three sections from each animal were evaluated from the corpus callosum.

4.7.2. Immunohistochemistry, Glial Fibrillary Acidic Protein (GFAP) and Ionized Calcium-binding Adaptor Molecule – 1 (Iba-1)

Specific markers of these cells were used to label the accumulated and activated astrocyte (GFAP) and microglia/macrophage (Iba-1) populations. Sections were photographed with a microscope equipped with a digital camera (Olympus BX51 microscope frame and Olympus DP50 camera, 200x magnification). The evaluation was performed blindly based on the optical density of ROIs of equal size placed in the medial corpus callosum area using ImageJ software (Schneider, Rasband et al. 2012).

4.8. Electron microscopy

The properly prepared sections were examined by electron microscopy using a Jeol 1200EX-II electron microscope. The quantification was based on the ratio of myelinated/demyelinated axons in the visual field and the size of the axon cross-section. The group assignment was not known to the operator and evaluator.

5. Statistics

Statistical analysis of the results was performed in all cases using GraphPad Prism software (GraphPad Prism version 8.0.1 for Windows, GraphPad Software, San Diego, California USA), qPCR results, and MRI results were compared using one-way ANOVA and Tukey's comparison test to determine significance values. For the comparison of histological results, paired T-test was used and individual p-values were indicated in all cases. If the result did not reach the desired level of significance ($p<0,05$), the p-values were not reported.

6. Results 1 (total TRPA-1 gene-deficient mice)

6.1. Mechanociceptive threshold measurement

No significant differences were found between the groups in the pain threshold tests, either with von Frey filaments or with DPA, which had much lower variability.

6.2. Motor performance test

The weekly tests, for both scraping and RotaRod, proved to be highly robust and reproducible, but no significant differences were found in these tests (two-way ANOVA with Bonferroni post-hoc test).

6.3. Y-maze test

We did not find any significant difference, but we did find several phenomena that could explain this. In the weekly repeated experiments, the animals were habituated to the test conditions by the third or fourth time, and thus lost interest in the experimental environment, and in both cuprizone groups, the alternation numbers were much higher initially due to the increased exploratory behavior we observed and described earlier.

6.4. Open-field test

Cuprizone-treated WT mice spent more time moving and had a higher average speed than untreated WT mice in weeks 2 and 3. ($p<0.01$ and $p<0.001$, two-way ANOVA and Bonferroni post hoc test).

There was a significant difference between the behavior of treated and control TRPA1 KO animals only in week 3 in the time spent moving ($p<0.05$) and in week 2 in the average speed ($p<0.1$) and distance traveled ($p<0.01$). No significant differences were found between the treated TRPA-1 WT and KO groups in these parameters. On the other hand, significantly higher "branching" numbers were produced by cuprizone treatment in the WT group from week 2 onwards, which was maintained until week 5, compared to the KO group. No difference in time spent in the open field was found in either group.

6.5. T2-weighted ^{1}H MRI measurements

Cuprizone treatment caused a significant increase in pixel intensity in the WT group from week 2 to the end of the experiment compared to the control group ($p<0.001$, all time points). In contrast, cuprizone was only able to produce a significantly smaller change in the TRPA-1 KO group and the difference was observed throughout the experiment. The largest protective effect was observed at week 3 at 149% in the WT group vs. 126% in the KO group. The percentage is defined as the increase in intensity relative to the baseline measurement of the individual animals, with 100% taken as the initial condition.

6.6. Luxol-Fast-Blue staining to determine the degree of demyelination at the end of week 3

Examination of the LFB of the corpus callosum at week 3 in the WT group indicated practically complete loss of myelin in response to cuprizone. Demyelination was also observed in KO animals, but the degree of that was much more moderate. The histological study, therefore, confirmed the MRI results obtained during the experiment.

6.7. Astrocyte and microglia/macrophage accumulation and activation

GFAP and Iba-1 labeling showed a significant increase in microglia/macrophage and astrocyte activation at the end of week 3. This indicator also recorded significantly lower values in the TRPA-1 KO group. The immunohistochemical studies thus clearly support a significant protective role of TRPA-1 receptor deficiency against the effects of cuprizone.

6.8. Electron microscopy

Electron microscopy was used to measure the number of myelin-loss axons and the diameter of the axons. Both values were dramatically reduced by treatment compared to the control groups, but the lesions were much more severe in the TRPA-1 WT group.

7. Effect of inhibition of TRPA-1 with a selective antagonist

It was obvious to attempt to inhibit the receptor with a drug candidate, so a pilot preclinical study was conducted with AMG0902. Compound AMG0902 was administered to the experimental animals by twice-daily oral probing. The data did not show any effect of the compound, either in the control animals alone or in the cuprizone-treated group. By the end of the experiment, we concluded that we were unable to create sufficient concentration in the brain of mice due to the limited amount of drug administered. We wanted to eliminate this problem from our experimental system by using a more sophisticated drug delivery method. In the next period, we tried an intracerebroventricular (ICV) route of administration using a single-use, sterile, subcutaneous osmotic pump (ALZET Osmotic Pumps, 10260 Bubb Road Cupertino, CA 95014-4166). In this case, however, the insufficient solubility of the agent was a barrier to establishing an effective concentration. It was not possible to dissolve and present sufficient amounts of the agent into the matrix of the osmotic pump, so the same results were obtained with this delivery method.

8. Summary

The fact that the absence of the TRPA-1 receptor alone can exert such a marked protective effect has confirmed our opinion that it warrants further investigation as a potential drug target. Since these are in vivo experiments, any further investigation must be based on a properly selected model animal. Based on the considerations described so far, we have decided to switch to GFAP-Cre conditioned TRPA-1 KO animals for the further phase of the studies.

9. GFAP-Cre conditioned TRPA-1 KO mouse model

In the second phase of our work, we investigated the lack of TRPA-1 receptor in mGFAP-Cre conditioned TRPA-1 deleted mice, which we generated ourselves in the institute's animal facility.

Animals homozygous for floxed TRPA-1 were crossed with GFAP promoter-directed Cre recombinase gene hemizygous mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Only the female mice carried the GFAP-Cre transgene to avoid non-specific Cre activation during spermatogenesis, and only one copy of the GFAP-Cre transgene was carried to avoid aspecific recombination. In a first step, GFAP-$Cre^{+/-}$ females were coupled with floxed TRPA-1 homozygous TRPA-1 $^{Fl/Fl}$ males. The resulting females heterozygous for both genes were backcrossed with TRPA-1 $^{Fl/Fl}$ males. The $Cre^{+/-}$ TRPA1 $^{Fl/Fl}$ males born from this constellation were used as the focus of our studies, and their littermates ($Cre^{+/-}$ TRPA-$1^{Fl/-}$ and Cre $^{-/-}$ TRPA-1 $^{Fl/Fl}$) served as hetero and floxed controls. The genotype of each animal was determined using tissue samples from the tail.

10. Applied tests

In the second phase of the experiment, we also performed the previously described assays for Luxol-Fast-Blue, GFAP, and Iba-1, and an additional immunohistochemical assay for myelin basic protein (MBP). The previously described T2-weighted MRI assays were also performed.

11. TRPA-1 qRT-PCR

Total RNA isolation was also performed from homogenized cortex samples. Purified RNA was quantified by spectrophotometer and the sample was treated with DNAse I enzyme to eliminate genomic DNA contamination. The first cDNA strand was synthesized using Maxima First Strand cDNA Kit. The PCR reaction was performed using SensiFastProbe Lo-ROX Kit. The forward primer is bound on exon 23, the reverse primer is bound on exon 24 and the candidate probe (56FAM/tgggcagct/ZEN/tattgccttcacaat/3IABkFQ) is also bound on exon 23. The reaction was performed on an Applied Biosystems Quantstudio5 quantitative PCR instrument. As the reaction was designed such that the complement of the primer and probe would be knocked out during recombination, only intact TRPA-1 mRNA would be able to give a detectable signal. Mouse β-actin was used as reference and expression was determined. All qPCR reactions were performed in duplicate to determine Ct values. To determine dCt values, the Ct value of the reference β-actin was subtracted from the Ct value of TRPA-1. And the ddCt value was calculated for each sample by subtracting the Cre-/- dCt value (100%) from the sample. Finally, the relative expression level was represented by the following formula; 100/(2ddCt).

12. Development of MR spectroscopy

Since we have still very limited information about the metabolomic background of cuprizone-induced neurotoxicity, we also aimed to develop an in vivo MR spectroscopy technique that can detect changes in energy metabolism. The literature does not currently describe an in vivo spectroscopic procedure performed in mice on similar models, given the hardware setup available to us. There have also been very few studies at higher field strengths and with different profiles (Praet, Orije et al. 2015). A previous publication, although with a different instrumental setup, attempted to map a broad metabolic profile (Orije, Kara et al. 2015) of the long-term effects of cuprizone, with just a few changes in metabolites detected (on a dedicated instrument with higher field strength). Based on this, we decided to focus on those few metabolites (NAA, creatine and phosphocreatine, taurine), and thus attempted to optimize our own setup to detect these metabolic changes.

13. Results 2 (GFAP-Cre TRPA-1 conditioned KO mouse model)

13.1. TRPA-1 quantitative RT-PCR results

A significant reduction in the amount of mRNA produced was observed in both the Cre $^{+/-}$ TRPA-1 $^{Fl/-}$ group (68%) and the Cre $^{+/-}$ TRPA-1 $^{Fl/Fl}$ group (77%). This result suggests that Cre-lox recombination results in a significant reduction in the expression of the gene of interest.

13.2. T2-weighted MRI Results

In the untreated control group, the intensity was practically unchanged and no other changes were detected during the whole experiment. In contrast, the intensities were significantly increased in the cuprizone-treated GFAP-Cre$^{-/-}$TRPA-1$^{Fl/-}$ group compared to the GFAP-Cre$^{+/-}$TRPA-1$^{Fl/-}$ and GFAP-Cre$^{+/-}$TRPA-1$^{Fl/Fl}$ groups in both weeks 3 and 4. The largest difference was observed in week 4 between the GFAP-Cre$^{-/-}$TRPA-1$^{Fl/-}$ group (179.75%) and the GFAP-Cre$^{+/-}$TRPA-1$^{Fl/-}$ group (134.75%). This result shows that both homozygous and heterozygous GFAP-Cre conditioned KO animals developed significantly milder demyelination in response to treatment. At later time points, the difference in this case is reduced, as in our previous experiments, only in the total KO genotype. At the end of the experiment, no significant difference was detected between cuprizone-treated GFAP-Cre$^{-/-}$TRPA-1$^{Fl/Fl}$, GFAP-Cre$^{+/-}$TRPA-1$^{Fl/-,}$ and GFAP-Cre$^{+/-}$TRPA-1$^{Fl/Fl}$ animals. It is important to note, however, that all cuprizone treated groups show significant differences compared to their untreated control group from week 3 to the end of the experiment.

13.3. Assessment of the degree of demyelination by LFB staining and MBP immunohistochemistry

At the end of the experiment, in agreement with the MRI results, all three genotypes showed significant demyelination by cuprizone compared to the untreated group. In contrast, no significant difference was detected in the three genotypes compared to each other. This suggests that the effect of the GFAP conditioned KO genotype, which was shown to be protective against demyelination, is reduced or below the significance level by the end of the experiment. Both the LFB staining score and the software quantification of MBP immunohistochemistry show this picture. The results were evaluated using blinded operators, of course, independently, following the parallel work of two operators.

13.4. Analysis of microglia/macrophage activation and accumulation by immunohistochemistry

At the end of week 6, we obtained results with Iba-1 and GFAP immunohistochemistry that closely correlated with the degree of myelination. Significant increases were observed for both markers in all cuprizone-treated groups. However, no significant difference was detected in the untreated groups for the three different genotypes. The GFAP conditioned KO genotype was also unable to produce a significant difference at week 6 in the cuprizone-treated groups compared to the control.

13.5. MR-Spectroscopy

The results of our spectroscopy measurements are twofold. After performing in vivo ^{1}H RARE measurements, we found that a significant decrease in NAA (N-acetyl aspartate) was observed early in the treatment, even in the first week, and persisted afterward (week 1, 75%, week 2, 83%, week 3, 88%). No change in the control group, the amount of NAA is normalized to the peak of Cr+pCr (creatine, phospho-creatine), as we know from previous studies (Orije et al., 2015) that it remains constant throughout the experimental series. If we relate the result to an internal standard, the quantification will be less sensitive to external confounders and to biological diversity between animals. It also

compensates for possible metabolic variation due to the natural aging of the animals. Unfortunately, it was also found that changes in the levels of other metabolites could not be reliably detected due to the instrumental limitations mentioned earlier.

This discovery means that metabolic changes can be detected even before morphological changes are visible on T2-weighted MR imaging. This fact certainly warrants further research, with an instrument specific to this task, as further development could potentially provide us with an early diagnostic tool, not only of preclinical but also of clinical relevance. However, in my view, it is also a valuable achievement - especially for the MR community - that it is possible to perform such a study with the lower field strength instrumental setup, like what is available to us. This will provide a point of reference for research groups with similar capabilities in terms of applicable methodologies. For this consideration, I would like to publish this part of our work in an MR-focused methodology journal, which is currently in progress.

14. Discussion

We have investigated in detail the role of the TRPA-1 receptor in demyelination processes associated with neurogenic inflammation in both complete KO and GFAP-Cre TRPA1-conditioned KO mice. GFAP is widely accepted in the literature as an astrocyte marker, and various neurodegenerative pathologies routinely cause activated astrocyte accumulation with consequent GFAP overproduction (Siracusa et al., 2019).

By performing a series of experiments, it has been demonstrated that the TRPA-1 receptor plays an important role in the development of neurogenic inflammation, demyelination, and consequent oligodendroglial destruction. A genetically determined absence of the receptor clearly results in a protective effect in the cuprizone-induced demyelination mouse model between weeks 3 and 5. The effect is confirmed by MR and electron microscopic imaging as well as histological and immunohistological tests. However, the significant protective effect disappears on week 6 of the experiments.

In the first period of our work, we demonstrated that the TRPA-1 total KO genotype is able to reduce the apoptotic propensity of mature oligodendrocytes. As TRPA-1 expression on oligodendroglia has recently been described, this suggests a direct effect (Hamilton et al., 2016). Since the TRPA-1 receptor is expressed at significantly higher levels in astrocytes, an indirect effect may also play an important role in these processes by regulating the release of astrocyte inflammatory cytokines and growth factors. Astrocyte-oligodendroglial communication also plays an important role under physiological conditions. Since the TRPA-1 receptor can be activated by various stimuli - inflammation, tissue damage, oxidative stress - and the astrocyte is capable of mediating and amplifying toxin-induced proapoptotic processes, the receptor may be involved in the pathomechanism through at least two pathways.

Several previous studies have described that the TRPA-1 receptor is involved in the regulation of normal Ca^{2+} levels in the physiological condition (Lee et al., 2012; Shigetomi et al., 2012; Shigetomi et al., 2013; Lee et al., 2016; Bosson et al., 2017).

In a recent study, Oh et al. described that TRPA-1 receptors expressed on astrocytes are targets of low-intensity low-frequency ultrasound (LILFU)-induced neuromodulation (Oh et al., 2019). In this case, neuromodulation occurs as a consequence of the Ca^{2+} flux generated in the TRPA-1 channel in

response to ultrasound. The opening of the channel leads to glutamate release from the astrocyte, which excites NMDA receptors in the surrounding neurons.

In another recent publication, TRPA1 has been described to be involved in the expansion of consequent demyelination in a mouse intracerebral hemorrhage (ICH) model. The oxidative stress generated by ICH activates the channel, resulting in Ca^{2+} influx and consequent NOX1 and Calpain1 release, which exacerbates myelination (Xia et al., 2019).

The fact that a stronger protective effect was induced in total KO animals than in the GFAP conditioned KO genotype suggests that not only GFAP positive cells (most of them astrocytes) are involved in the regulation of this process, but that several other GFAP negative cell types (oligodendrocytes, microglia, other neurons) expressing TRPA1 receptors may also play an important role. The disappearance of the significant effect in the late phase of the experiment also supports the theory that a multi-step, multi-cellular, and complexly modulated process is involved. These cell types are certainly worthy of further attention.

In our work, we have developed a highly reproducible low-field MRI technique that is well suited for monitoring and semi-quantitative assessment of demyelination. This procedure has been validated by both electron microscopy-derived microstructural analysis and by robust and redundant histological and immunohistochemical data performed on a large scale. It is now possible to follow not only the cuprizone model but also any other pathomechanism involving demyelination in a time-resolved way since the agent or process that induces demyelination is practically irrelevant for imaging purposes.

It is true that a change in the amount of NAA has been observed, but we do not yet have sufficient information on the cause of this change, as we see contradictory results in the literature and no convincing explanation for this phenomenon (Praet et al., 2015) (Orije et al., 2015). Our research group has not yet conducted any experiments toward the elucidation of NAA metabolism, these are currently in the planning stage.

15. Summary of novelties

-Development of a new, robust and reproducible experimental procedure that can be used as a standard for pre-clinical testing of drug candidates for demyelinating diseases (Such projects have been carried out since then, commissioned by external pharmaceutical R&D companies, but are subject to confidentiality obligations)

- Creation and breeding of an induced GFAP-Cre TRPA1-KO mouse model with a novel genetic constellation in the Institute

- Mapping the time course of the cuprizone model, quantification of its severity by in vivo MRI using a non-destructive, self-controlled procedure on a low field instrument

- Development of an in vivo MR spectroscopy technique at low field strength to monitor and semi-quantitatively evaluate metabolic changes in cuprizone-induced demyelination processes.

16. List of publications on which the thesis is based

Kriszta, G., Nemes, B., Sándor, Z., Ács, P., Komoly, S., Berente, Z., Bölcskei, K., and Pintér, E. (2020). Investigation of cuprizone-induced demyelination in mGFAP-driven conditional transient receptor potential ankyrin 1 (TRPA1) receptor knockout mice. Cells 9, 81.

Bölcskei, K., Kriszta, G., Sághy, É., Payrits, M., Sipos, É., Vranesics, A., Berente, Z., Ábrahám, H., Ács, P., and Komoly, S. (2018). Behavioral alterations and morphological changes are attenuated by the lack of TRPA1 receptors in the cuprizone-induced demyelination model in mice. Journal of neuroimmunology 320, 1-10.

Kriszta G., Pintér E., Berente Z. In vivo magnetic resonance spectroscopy in the cuprizone mouse model, an early diagnostic tool for demyelination. (Under submission); Cells Special Issue "Mechanisms of Neurodevelopment and Neurodegeneration"

17. List of further publications

Kriszta, G., Kriszta, Z., Váncsa, S., Hegyi, P. J., Frim, L., Erőss, B., ... & Pintér, E. (2021). Effects of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers on angiotensin-converting enzyme 2 levels: A comprehensive analysis based on animal studies. Frontiers in pharmacology, 12, 254.

Botz, B., Kriszta, G., Bölcskei, K., Horváth, Á. I., Mócsai, A., & Helyes, Z. (2021). Capsaicin-Sensitive peptidergic sensory nerves are anti-inflammatory gatekeepers in the hyperacute phase of a mouse rheumatoid arthritis model. International Journal of Molecular Sciences, 22(4), 1682.

Bencze, N., Schvarcz, C., Kriszta, G., Danics, L., Szőke, É., Balogh, P., ... & Botz, B. (2021). Desensitization of capsaicin-sensitive afferents accelerates early tumor growth via increased vascular leakage in a murine model of triple-negative breast cancer. Frontiers in Oncology, 2597.

18. Acknowledgments

This work could not have been done without the following people:

prof. Dr. Erika Pintér, my supervisor, who always spared no time and energy to help me both professionally and personally,

Dr. Zoltán Berente, who taught me everything I know about MR,

Dr. Kata Bölcskei, who has provided invaluable professional assistance

Prof. Dr. Zsuzsanna Helyes, who, as Scientific Director of the Szentágothai János Research Centre, helped me by providing me with instrumental facilitators, and countless colleagues inside and outside the Institute who contributed to the preparation of this thesis with their work and expertise.

I would also like to thank Zsolt Nyisztor and László Jánosi for guiding me towards a career in science and for showing me a perspective through their example during my student years.

I would also like to thank the Richter Gedeon Centenary Foundation for the financial support for my work.

Special thanks go to my wife Dr. Zsófia Kriszta, who endured the often long weekends and nights and supported me all the way and all the time.

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