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## Original Article

Genetic deletion of TRPA1 receptor attenuates amyloid beta- 1-42 (A $\beta$ <sub>1-42</sub>)-induced neurotoxicity in the mouse basal forebrain in vivoM. Payrits<sup>a,b,1</sup>, E. Borbely<sup>a,b,1</sup>, S. Godo<sup>b,c</sup>, D. Ernszt<sup>b,c</sup>, A. Kemeny<sup>a,b,d</sup>, J. Kardos<sup>e</sup>, E. Szoke<sup>a,b</sup>, E. Pinter<sup>a,b,\*</sup><sup>a</sup> Department of Pharmacology and Pharmacotherapy, Medical School, University of Pécs, Hungary<sup>b</sup> Centre for Neuroscience, Szentágotthai Research Center, University of Pécs, Pécs, Hungary<sup>c</sup> Institute of Physiology, University of Pécs, Pécs, Hungary<sup>d</sup> Department of Medical Biology and Central Electron Microscope Laboratory, University of Pécs, Hungary<sup>e</sup> ELTE NAP Neuroimmunology Research Group, Department of Biochemistry, Eötvös Loránd University, Budapest, Hungary

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## ABSTRACT

Amyloid  $\beta$  1–42 peptide (A $\beta$ <sub>1–42</sub>) accumulates in Alzheimer's disease (AD) that is toxic to the basal forebrain cholinergic (BFC) neurons in substantia innominata-nucleus basalis magnocellularis complex (SI-NBM). Transient Receptor Potential Ankyrin1 (TRPA1) receptor is present in murine brain, however its role in neurotoxic processes is unclear. We investigated the A $\beta$ <sub>1–42</sub>-induced neurotoxicity in TRPA1 wild-type (TRPA1<sup>+/+</sup>) and knockout (TRPA1<sup>-/-</sup>) mice.

Expression and neuroanatomical localization of TRPA1 receptor were examined using RT qPCR. Cholinergic fibre loss was determined on acetylcholinesterase (AChE) stained brain slices, and choline acetyltransferase (ChAT) immunohistochemistry was used to assess the cholinergic cell loss. Novel object recognition (NOR), radial arm maze (RAM) and Y-maze tests were used to investigate memory loss.

A $\beta$ <sub>1–42</sub>-injected WT mice showed marked loss of cholinergic fibres and cell bodies, which was significantly attenuated in TRPA1<sup>-/-</sup> animals. According to the NOR and RAM tests, pronounced memory loss was detected in A $\beta$ <sub>1–42</sub>-injected TRPA1<sup>+/+</sup> mice, but not in TRPA1<sup>-/-</sup> group.

Our findings demonstrate that TRPA1 KO animals show substantially reduced morphological damage and memory loss after A $\beta$ <sub>1–42</sub> injection in the SI-NBM. We conclude that TRPA1 receptors may play an important deteriorating role in the A $\beta$ <sub>1–42</sub>-induced cholinergic neurotoxicity and the consequent memory loss in the murine brain.

## 1. Introduction

One of the major pathomorphological hallmarks of Alzheimer's disease (AD) is the significant neuronal death in the cortex, hippocampus and basal forebrain. The cholinergic system of the basal forebrain (BFC) is the pivotal source of cholinergic connections to the cortex and hippocampus, which plays a major role in attention and memory (Zaborszky et al., 1999; Beninger et al., 2001). Accumulation of neurotoxic  $\beta$ -amyloid peptides, such as A $\beta$ <sub>1–42</sub> contributes to neuronal loss in BFC in AD (Maccioni et al., 2001). The A $\beta$  activates astrocytes and microglial cells, also it has direct neurotoxic effect by ligand-like interaction with the N-methyl-D-aspartate (NMDA) glutamate receptors (Harkany et al., 2000). A $\beta$ <sub>1–42</sub> microinjection into the nucleus

basalis magnocellularis (NBM) results in severe damage of neurons in BFC and memory deficits in rodents (Harkany et al., 2000, 1998; Giovannelli et al., 1995; Kwakowsky et al., 2016).

There is an increasing evidence that neuro-immune interactions may play a critical role in the pathomechanism of several neurodegenerative diseases (Candore et al., 2007; Masters et al., 2015, Sághy et al., 2016) and regulatory role of the transient receptor potential (TRP) ion channels is strongly suggested in these processes. TRP receptors form non-selective cation channels, with preferentially high calcium ion permeability. Calcium influx triggers several intracellular pathways. TRP channels are sensitive for various stimuli including mechanical, thermal triggers or chemical ligands. Considering these interactions, they are likely to be sensors for several physiological or

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pathophysiological stimuli (Khalil et al., 2018). One of the TRP receptors, the transient receptor potential ankyrin 1 (TRPA1) is best known as a sensor for environmental irritants participating in pain, cold and itch sensation. TRPA1 has a wide range of ligands; specifically it is sensitive to low and high temperature, osmotic changes, natural and synthetic irritants. Several TRPA1 agonists are reactive electrophilic ligands (Zimova et al., 2018). Channel gating effect of these compounds is based on covalent modification of cysteine and lysine residues within the N-terminus and transmembrane domains of the receptor (Zygmunt and Högesätt, 2014). Since TRPA1 can be triggered by endogenous compounds generated by tissue injury and inflammation, it can be a potential target for anti-nociceptive and anti-inflammatory drug candidates (Nishida et al., 2015; Horváth et al., 2016). Recent data showed that TRPA1 receptors are also expressed in the brain and play modulatory role in neurodegenerative disorders and neuroinflammation, such as multiple sclerosis and AD (Sághy et al., 2016; Lee et al., 2016; Bölskei et al., 2018; Dalenogare et al., 2020; Ritter et al., 2020). TRPA1 channels have also been found on astrocytes of corpus callosum (Sághy et al., 2016), also in oligodendrocytes of the cerebellum (Hamilton et al., 2016) and in the endothelium of cerebral arteries (Sullivan et al., 2015; Pires and Earley, 2018). Nowadays, Lee and colleagues have provided immunohistochemical evidence that hippocampal astrocytes express TRPA1 in wild-type mice; and immunoreactivity of the receptor protein also occurs in the cortical neurons of amyloid precursor protein/presenilin1 (APP/PS1) transgenic mice (Lee et al., 2016). However, only few *in vivo* data are available to corroborate the function of TRPA1 in the brain. The principal aims of the present study were to investigate the morphological and the functional aspects of TRPA1-mediated events in BFC using  $A\beta_{1-42}$ -induced neurotoxicity model in TRPA1 knockout mice.

## 2. Materials and methods

### 2.1. Animals

Experiments were carried out on 3–4 month-old male C57BL/6, TRPA1 knockout mice (TRPA1<sup>-/-</sup>) and their wild-type siblings (TRPA1<sup>+/+</sup>) (obtained from Prof. P. Geppetti, University of Florence, Italy). The animals were bred and kept in the Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs at 24 °C and provided with standard rodent food and water *ad libitum*. Mice were housed under conditions of 12-h light/dark cycle in groups of 3–5 in polycarbonate cages (530 cm<sup>2</sup> floor space, 14 cm height) on wood shavings bedding. The animals had a 60-min acclimatization period prior to each behavioural experiment. Six animal groups were created, stated as untreated wild-type (TRPA1<sup>+/+</sup> naïve) and TRPA1 receptor gene-deficient mice (TRPA1<sup>-/-</sup> naïve), vehicle-injected wild-type (TRPA1<sup>+/+</sup> Veh) and TRPA1 knockout (TRPA1<sup>-/-</sup> Veh),  $\beta$ -amyloid-treated wild-type (TRPA1<sup>+/+</sup>  $A\beta_{1-42}$ ), and TRPA1 knockout (TRPA1<sup>-/-</sup>  $A\beta_{1-42}$ ). Eight TRPA1<sup>+/+</sup> and seven TRPA1<sup>-/-</sup> animals were used for AChE histochemistry. Eight TRPA1<sup>+/+</sup> and six TRPA1<sup>-/-</sup> mice prepared for ChAT immunohistochemistry. In behavioural measurements the number of animals was 12–12 in TRPA1<sup>+/+</sup> naïve and TRPA1<sup>-/-</sup> naïve groups, 8–8 in TRPA1<sup>+/+</sup> Veh and TRPA1<sup>-/-</sup> Veh groups, 10 in TRPA1<sup>+/+</sup>  $A\beta_{1-42}$  group and 12 in TRPA1<sup>-/-</sup>  $A\beta_{1-42}$  group. All experimental procedures were approved by the Animal Welfare Committee of University of Pécs, licence no.: BA 02/2000–24/2016, BA 02/2000–38/2016.

### 2.2. Preparation of $A\beta_{1-42}$ peptide samples

$A\beta_{1-42}$  was expressed recombinantly in *E. coli* and purified according to Ikenoue and colleagues (Ikenoue et al., 2014). Briefly, the DNA sequence coding the human  $A\beta_{1-42}$  peptide was artificially constructed using codons preferred by *E. coli* with an extra Met residue at the N-terminal of the peptide. The DNA construct was inserted into

pAED4 vector (Doering and Matsudaira, 1996) and the protein expression was carried out in *E. coli* BL21 (DE3) pLysS strain (Novagen, Inc., Madison, WI), using 1 mM IPTG for induction. The peptide accumulated in the inclusion bodies of the bacterial cells. The purified inclusion bodies were solubilized in 20 mM NaOH and the peptide was further purified by repeated cycles of amyloid growth at low pH and monomerization in hexafluoroisopropanol combined with centrifugation steps. The monomeric lyophilized peptide was first dissolved in 10 mM NaOH on ice, then it was diluted into appropriate amount of TBS buffer resulting in a final concentration of 300  $\mu$ M peptide and a pH of 7.4. The solution was aged for 5 days at room temperature. Before administration, the solution was spun down in a tabletop centrifuge at 15,000 g for 2 min to remove larger aggregates.

### 2.3. $A\beta_{1-42}$ injection

Mice were anesthetized with isoflurane, mounted in a stereotaxic apparatus, and slowly injected (0.1  $\mu$ l/min) with 1  $\mu$ l of aged  $A\beta_{1-42}$  diluted in Tris-buffered saline (TBS) into the SI-NBM of the right hemisphere for the morphological studies or bilaterally for the behavioural studies.  $A\beta_{1-42}$  was injected at the stereotaxic coordinates relative to Bregma at anteroposterior (–0.7 mm) and mediolateral (–2 mm), and dorsoventral from dura mater (–3.75 and –4.75 mm, 0.5  $\mu$ l at both coordinates). Based on previously published results the 300  $\mu$ M  $A\beta_{1-42}$  dose and the 12-day survival time were chosen (Kwakowsky et al., 2016) for morphological and behavioural experiments (Fig. 1).

### 2.4. Measurement of *Trpa1* expression

Brains and trigeminal ganglia of TRPA1<sup>+/+</sup> (n = 6) and TRPA1<sup>-/-</sup> mice (n = 6) were quickly dissected after decapitation. The samples were immediately frozen in liquid nitrogen and stored at –80 °C. Then, brains were sliced by razor blades using a coronal brain matrix (cat.nr. 15050, Ted Pella, Redding, CA) to obtain 1 mm coronal sections (Palkovits et al., 1978). A microdissection needle (cat.nr. 15091, Ted Pella) of 1 mm diameter was used to collect micropunches containing the following brain areas in the marked coronal planes (the distances of coronal sections from the Bregma were indicated in brackets: SI-NBM (0–(–1 mm)), hippocampus and somatosensory cortex (–1 mm–(–2 mm)) (Paxinos and Franklin, 2001). Total RNA content was isolated using Direct-zol RNA MiniPrep kit (Zymo Research, cat.nr: R2060), according to the manufacturer's instruction. Samples were diluted to equal amount of total RNA and cDNA pools were generated by reverse transcription using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, cat.nr: K1971). The expression level of *Trpa1* mRNA was determined with Stratagene Mx3000 qPCR instrument using custom-designed *Trpa1* TaqMan assay (forward primer: atgccttcag-caccctcattg, reverse primer: gacctcagaatgcccacaa, probe: 56FAMtggg-cagctZENattgctcacaat3IABkFQ) and normalised with the predesigned hypoxanthine phosphoribosyltransferase (*Hprt*) TaqMan assay (Mm.PT.39a.22214828). The thermal cycling was initiated at 95 °C for 5 min followed by 45 cycles of 35 s at 95 °C and 30 s at 62 °C and 1 min synthesis at 72 °C. Primers and probes were obtained from IDT, Leuven, Belgium.

### 2.5. Immunohistochemistry

To examine the cholinergic cell loss in the SI-NBM, ChAT immunohistochemistry was accomplished with free-floating technique (Kwakowsky et al., 2016). To determine the rate of cholinergic fibre loss in the somatosensory cortex, AChE histochemistry with silver nitrate intensification was performed (Koszegi et al., 2011). On day 13,  $A\beta_{1-42}$ -injected and non-injected control animals were deeply anesthetized and perfused as described above. Dissected brains were postfixed for 4 h at room temperature, then incubated overnight in 30 % sucrose diluted in phosphate buffer. Four sets of 30  $\mu$ m thick coronal

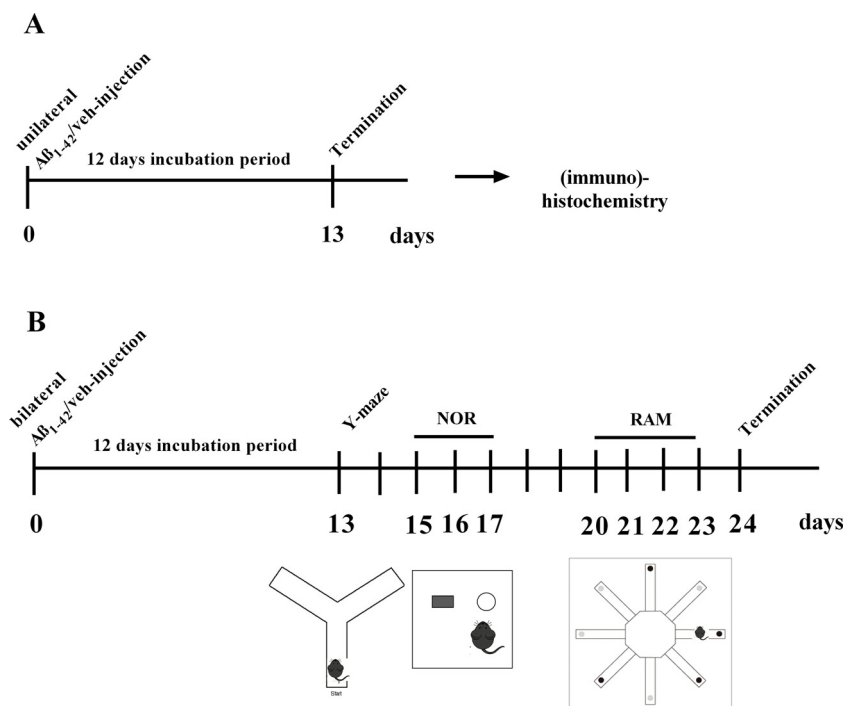


Fig. 1. Chronological flowchart for immunohistochemistry (A) and behavioural studies (B).

sections were cut on a freezing microtome (Leica, Germany).

## 2.6. Behavioural experiments and analysis

### 2.6.1. Novel object recognition test (NOR)

NOR tests the recognition memory (Li et al., 2011; Bevins and Besheer, 2006; D'Souza et al., 2015). We applied a 3-day-long protocol. On day 1, mice were allowed to move around in the  $45 \times 45 \times 30$  cm wooden box for 5 min. On day 2, animals were let freely explore two identical objects for 5 min. On day 3 (24 h later), the animals were presented to one familiar and one novel object, which was similar in size but different in color and shape compared to the familiar one (Fig. 4A). Mice could explore the objects freely for 5 min. Behaviour of animals was video-recorded and analysed with Ethovision XT 11 software (Noldus Information Technology, Netherlands). The obtained data are calculated and represented as discrimination index (DI) = difference in time exploring the novel and familiar object/total exploration time.

### 2.6.2. Radial arm maze test (RAM)

RAM is a suitable tool for the assessment of both short and long-term memory. Mice on normal diet were trained for three days (habituation and learning period) to find food pellets (Dustless Precision Pellets 45 mg, Sucrose; BioServ, US) in four selected arms of the eight-arm radial maze ( $5 \times 35$  cm for each arm and 5 cm in diameter for the central platform). The sessions lasted until the animals collected all the four food pellets or 5 min, whichever came first. The learning ability of the animals was assessed on the fourth day of the experiment. Behaviour was video-recorded and analysed with Ethovision XT 11 Software (Noldus Information Technology, Netherlands). The obtained data are calculated and represented as: reference memory error = entries into unbaited arms (Zhang et al., 2012; Li et al., 2011).

### 2.6.3. Y-maze test

Spatial working memory was measured after placing the animals in one of the arms of a Y-shaped maze (arms 5 cm wide  $\times$  35 cm long  $\times$  10 cm high) for 5-mins (D'Souza et al., 2015). The track of the animals was obtained with Ethovision XT 11 software (Noldus Information

Technology, Netherlands). The sequence and number of arm entries were used to determine the percentage of spontaneous alternation by the following formula: the sequence of visiting the 3 arms (A, B and C) consecutively in any order was considered as correct alternating behaviour (ABC, ACB, BAC, BCA, CAB, CBA) and was divided by the number of arm entries minus two (representing all possible alternation sequences). Total number of arm entries was also measured for the assessment of locomotor activity.

### 2.6.4. Statistical analysis

Data in all experiments were expressed as mean  $\pm$  SEM. Data were analysed by factorial ANOVA followed by Bonferroni's post hoc test with a value of  $p < 0.05$  considered significant. In case of cell/fibre loss, Mann-Whitney test was used. Learning curve was analysed by repeated measures two-way ANOVA followed by Bonferroni's post hoc test. All statistical analysis was performed using GraphPad Prism Software 6.0, except for the factorial ANOVA analysis that was performed using STATISTICA Software (TIBCO Software Inc., CA).

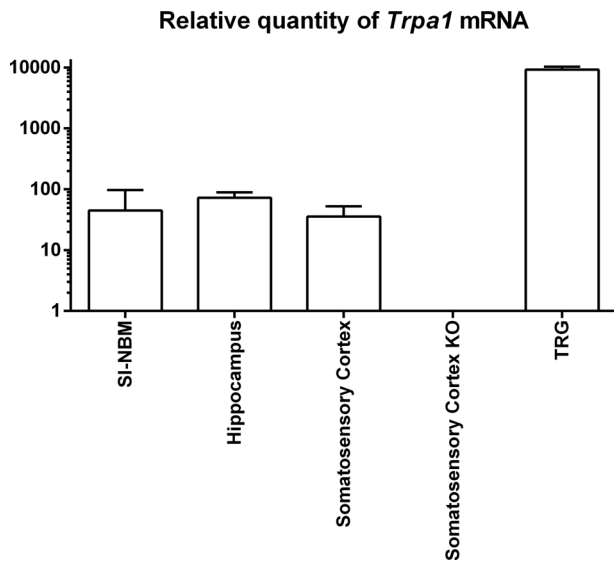
## 3. Results

### 3.1. *Trpa1* mRNA expression in the brain

To elucidate whether *Trpa1* is expressed in brain regions relevant to this work, we performed RT-qPCR in micropunched areas obtained from TRPA1<sup>+/+</sup> or TRPA1<sup>-/-</sup> animals. *Trpa1* expression became significant at 35–36 cycles. *Trpa1* mRNA was detectable in SI-NBM, hippocampus, somatosensory cortex and trigeminal ganglion isolated from TRPA1<sup>+/+</sup> mice. The *Trpa1* expression level of the TRPA1<sup>+/+</sup> samples was compared to their TRPA1<sup>-/-</sup> counterparts (Fig. 2).

### 3.2. $A\beta_{1-42}$ -induced cholinergic cell body and fibre loss is significantly attenuated in TRPA1<sup>-/-</sup> animals

Cholinergic fibre loss and cholinergic cell loss were detected in the ipsilateral somatosensory cortex (layer IV-V) and SI-NBM, in WT mice after  $A\beta_{1-42}$  injection into SI-NBM. The cholinergic fibre loss labelled with AChE immunohistochemistry was  $30.56 \pm 1.39$  % in TRPA1<sup>+/+</sup>



**Fig. 2. Relative quantity of *Trpa1* mRNA in the brain and the trigeminal ganglion.** The expression level of *Trpa1* mRNA was determined in the following brain regions: SI-NBM, hippocampus, somatosensory cortex of TRPA1<sup>+/+</sup> and TRPA1<sup>-/-</sup> mice along with trigeminal ganglion by RT qPCR. Samples collected from the somatosensory cortex of TRPA1<sup>-/-</sup> animals served as negative tissue control.

mice, while it was only 1.55 ± 0.91 % in TRPA1<sup>-/-</sup> animals. The ChAT-immuno-reactive cell loss in the SI-NBM was 28.68 ± 2.85 % in WT mice, and it was 2.19 ± 1.70 % in the TRPA1<sup>-/-</sup> mice (Figs. 3A and B).

**3.3. The effect of Aβ<sub>1-42</sub> injection on cognitive functions is attenuated in TRPA1<sup>-/-</sup> mice**

In the NOR test Aβ<sub>1-42</sub> injection had no effect on the locomotion of mice during habituation (Fig. 4B). There was no side preference during the exploration phase of the test in all studied groups (Fig. 4C). However, during the test phase the discrimination ability was significantly attenuated in Aβ<sub>1-42</sub>-injected TRPA1<sup>+/+</sup> mice (-0.02 ± 0.11) compared to the naïve controls (0.43 ± 0.08, p = 0.015703). This difference was not observed between the Aβ<sub>1-42</sub>-treated and control

TRPA1<sup>-/-</sup> groups (DI: 0.36 ± 0.07 and 0.47 ± 0.14; respectively) showing intact discrimination abilities. This resulted in a significant difference between Aβ<sub>1-42</sub> treated TRPA1<sup>+/+</sup> and TRPA1<sup>-/-</sup> mice (p = 0.037785). Vehicle injection had no significant effect (TRPA1<sup>+/+</sup>: 0.20 ± 0.11 and TRPA1<sup>-/-</sup>: 0.42 ± 0.09) (Fig. 4D).

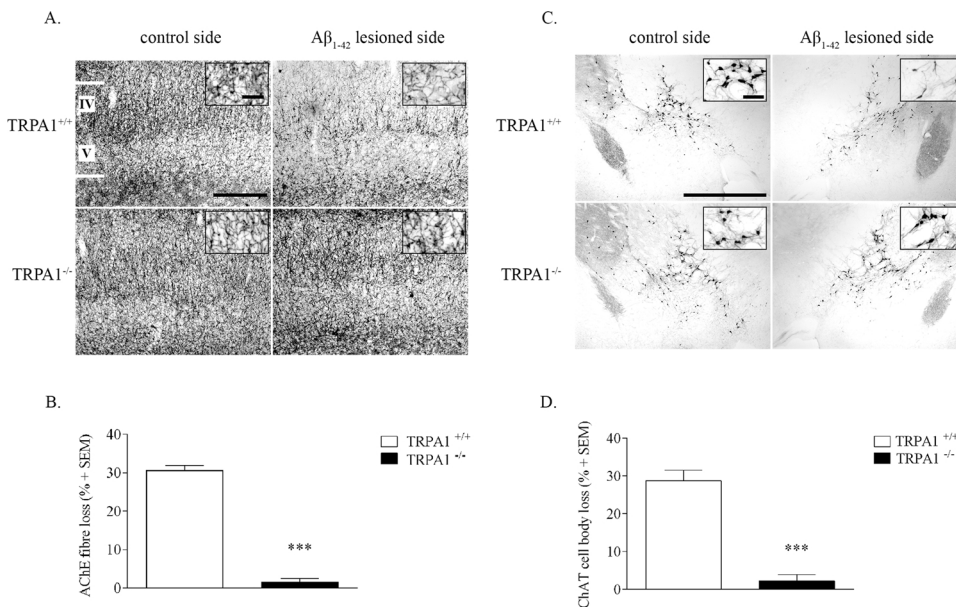
In the RAM test animals showed learning ability during the experimental days except for Aβ<sub>1-42</sub> injected TRPA1<sup>+/+</sup> mice (Fig. 5A). There was no change in reference memory error of Aβ<sub>1-42</sub> injected TRPA1<sup>+/+</sup> mice over the time (4.0 ± 0.63 on day 2 and 4.0 ± 0.26 on day 4), while in the vehicle-treated group there was a decrease (4.33 ± 1.76 on day 2 and 2.50 ± 0.86 on day 4). The same tendency could be detected in the TRPA1<sup>-/-</sup> groups (Aβ<sub>1-42</sub>-injected group: 4.0 ± 2.0 on day 2 and 2.6 ± 0.49 on day 4; vehicle-treated group: 3.75 ± 0.48 on day 2 and 2.4 ± 0.51 on day 4). This means, that Aβ<sub>1-42</sub>-injected TRPA1<sup>-/-</sup> animals made significantly less errors (p = 0.019726) on day 4 compared to Aβ<sub>1-42</sub>-treated TRPA1<sup>+/+</sup> mice (Fig. 5B). There was no significant difference between the two naïve groups and there was no detectable change in learning ability over time in these groups (TRPA1<sup>+/+</sup>: 2.67 ± 0.33 and TRPA1<sup>-/-</sup>: 1.4 ± 0.24 on the day 4).

In the Y-maze test there was no difference in locomotion between groups reflected by the total number of arm entries. Examining the spontaneous alterations, each group showed intact working memory (naïve TRPA1<sup>+/+</sup>: 15.71 ± 1.91 and 68.42 % ± 2.32 and TRPA1<sup>-/-</sup>: 17 ± 1.19 and 66.31 % ± 3.61, Aβ<sub>1-42</sub>-treated TRPA1<sup>+/+</sup>: 16.4 ± 1.69 and 67.8 % ± 2.79; TRPA1<sup>-/-</sup>: 15.6 ± 2.4 and 58.92 % ± 4.73) (Figs. 5C and D).

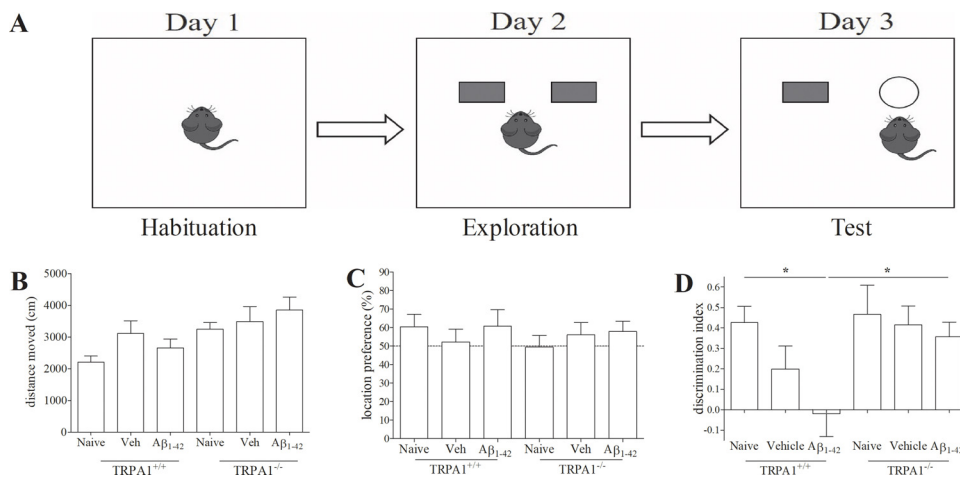
**4. Discussion**

Our study provides the first evidence for the putative role of TRPA1 receptor in neuronal loss and cognitive impairment in Aβ<sub>1-42</sub>-induced murine neurotoxicity model. With the help of neuroanatomical evaluation and *in vivo* functional studies we have demonstrated that genetic deletion of TRPA1 receptor attenuates Aβ<sub>1-42</sub> toxicity in cholinergic neurons in the SI-NBM.

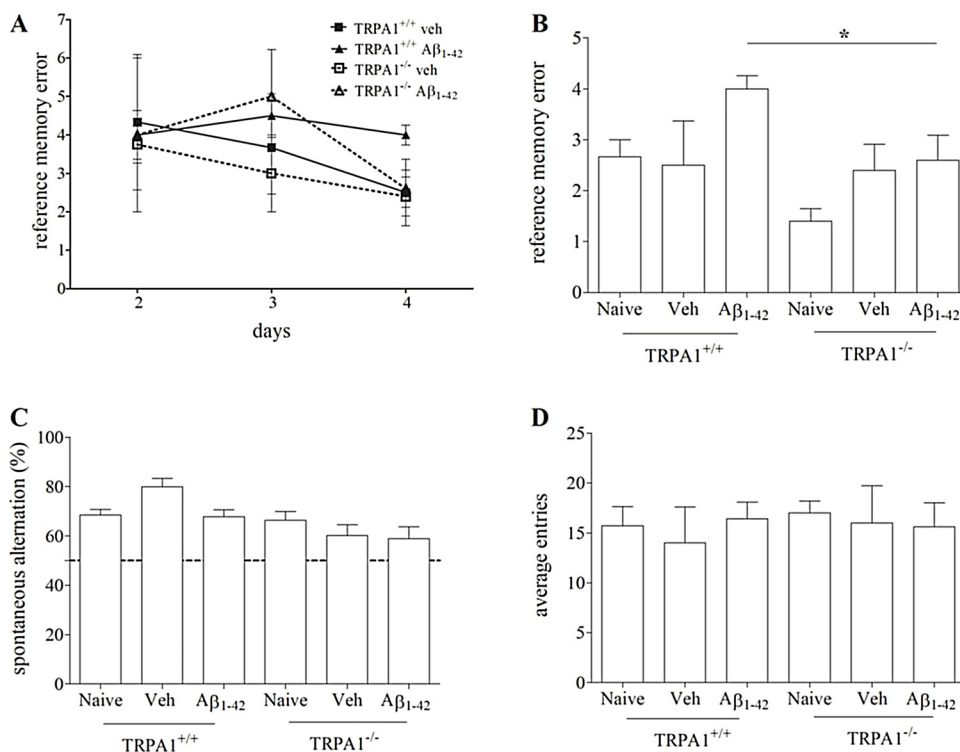
There are several dementia models available: spontaneous, chemically induced (e.g. Aβ, scopolamine, okadaic acid) and transgenic (e.g. APP/PS1 knockout mice, tau-related) models (Tayebati, 2006; Neha Sodhi et al., 2014). None of them is able to show all pathophysiological features of the human AD. Transgenic models are widely used, because non-invasive procedures are needed and intracellular Aβ-toxicity can



**Fig. 3. Morphological changes in the brain of TRPA1<sup>+/+</sup> and TRPA1<sup>-/-</sup> mice after Aβ<sub>1-42</sub> injection.** Representative pictures (A) and numerical values (B) show the cholinergic fibre loss in the ipsilateral somatosensory cortex (layer IV-V) detected with acetylcholinesterase histochemistry. Scale bar: 200 µm, insert scale bar: 20 µm. Representative pictures (C) and numerical values (D) show the cholinergic cell body loss in the SI-NBM detected with choline acetyltransferase immunohistochemistry. Scale bar: 1000 µm, insert scale bar: 250 µm. Data were presented as the mean ± SEM (n = 6-10) and were analysed with Mann-Whitney test (\*\*\* p < 0.001).



**Fig. 4.** Effects of vehicle or  $A\beta_{1-42}$  injection on the performance in the novel object recognition (NOR) task in  $TRPA1^{+/+}$  and  $TRPA1^{-/-}$  mice. On day 1 of the 3-day-long NOR test (A) distance moved (B), on day 2 location preference (C) was measured. On day 3 memory function was determined as discrimination index (D). Data are presented as the mean  $\pm$  SEM ( $n = 6-10$ ) and were analysed by factorial ANOVA followed by Bonferroni's post hoc test and location preference with one sample  $t$ -test in comparison to 50.



**Fig. 5.** Effects of vehicle and  $A\beta_{1-42}$  injection on the performance in the radial arm maze (RAM) and Y-maze task in  $TRPA1^{+/+}$  and  $TRPA1^{-/-}$  mice. Learning curve on the basis of reference memory error (A) and the reference memory error on day 4 (B) was determined in RAM. Spontaneous alternation (C) and average entries (D) was measured in Y-maze test. Data are presented as the mean  $\pm$  SEM ( $n = 6-10$ ). Learning curve was analysed by repeated measures two-way ANOVA followed by Bonferroni's post hoc test, and reference memory error and average entries were analysed by factorial ANOVA followed by Bonferroni's post hoc test (\* $p < 0.05$ ).

also be triggered (LaFerla and Green, 2012). However, in gene-manipulated animals, the massive loss of neurons and fibres, as one of the most typical changes of human disease is not characteristic. In contrast to the genetic models of AD (e.g. APP/PS1 knockout mice) (Lee et al., 2016), our experimental approach with intracerebral injection of  $A\beta$  acutely generate AD-like neurotoxicity and cognitive deficits. Early recognition of the AD is almost impossible because of the atypical symptoms of the slow progression. The current treatment focuses on the maintenance of the remaining cholinergic functions applying cholinesterase inhibitors and NMDA receptor antagonists (Talesa, 2001; Eleti, 2016). Despite of some moderately effective drugs, the pharmacotherapy of the AD is still an unsolved problem. Therefore, useful animal models that properly mimic the pathophysiological characteristics of AD are still essential for the discovery of novel therapeutic targets.  $A\beta$  microinjection elicits significant loss of neuronal elements with remarkable memory impairment that can be detected with classical behavioural tests (Morris water maze, Y/T-maze, NOR). This model represents the later phase of the disease when patients' relatives realize the memory problems.

$TRPA1$  is a widely studied non-selective cation channel, expressed by the primary afferent neurons regulating pain sensation (Nishida et al., 2015; Jardín et al., 2017). In addition, increasing evidence has been published about its function in the CNS:  $TRPA1$  can be found in neurons of the rodent hippocampus, magnocellular neurosecretory cells, the visceral afferent pathway and cortex (Nilius et al., 2012; Kheradpezhough et al., 2017). Also its expression has been investigated in glial cells and its putative role in the pathophysiology of AD (Lee et al., 2016, Shigetomi et al., 2013). Since it is well-known, that  $TRPA1$  is a key regulating receptor of calcium homeostasis, it may influence cell death or survival (Bosson et al., 2017, Stueber et al., 2017).

$A\beta_{1-42}$  stereotaxic microinjection caused robust damage of cholinergic neurons and fibres in the WT animals, which indicates that this method is suitable model of the typical pathomorphological changes of cholinergic neurotoxicity. Interestingly, in  $TRPA1^{-/-}$  animals the  $A\beta_{1-42}$ -induced cell and fibre loss of cholinergic neurons were nearly absent. These results suggest pivotal role of  $TRPA1$  in the neurodegenerative processes of the brain, especially in the basal forebrain cholinergic neurons. Morphological changes evoked by  $A\beta$  injection

have also been supported by functional investigations. Bilateral administration of A $\beta$ <sub>1-42</sub> induced significantly impaired memory function in TRPA1<sup>+/+</sup> mice. These changes could be detected in both NOR and RAM, but Y-maze was not suitable for the demonstration this type of cognitive impairment. NOR test is one of the most appropriate methods assessing memory loss in rodents. It has been used in numerous different (transgenic and non-transgenic) AD models. It has several advantages, such as short testing period, no food restriction is needed, and it is based on the natural exploratory behaviour of the mice (Zhang et al., 2012; Grayson et al., 2015). RAM is also frequently used in AD mouse models (Webster et al., 2014), and it is a suitable for evaluation of both short- and long-term memory (Puzzo et al., 2014). Despite of the A $\beta$  treatment, the memory performance of the TRPA1<sup>-/-</sup> animals was comparable with the naïve mice in these tests. This means, that the measured morphological changes are manifested at the behavioural level. These results verify the observations of the former transgenic model, that genetic lack of TRPA1 can significantly attenuate the memory loss (Lee et al., 2016), nevertheless not all of the tests showed the same results. Our Y-maze methodology was slightly different from the data of the above mentioned research group. Lee et al. divided Y-maze into start, familiar and novel arms, and they measured the visiting activity of mice in the novel arm, which is very similar to the aspect of NOR. On the contrary, we considered the arms as equal, and the alternation activity was assessed during the experiments. In this case we gain information about the spatial memory.

Our findings showed that genetic deletion of TRPA1 receptors substantially inhibited the A $\beta$ <sub>1-42</sub>-induced cholinergic cell body loss and fibre loss. We demonstrated that A $\beta$ <sub>1-42</sub> injection into the SI-NBM caused significantly reduced neuronal damage and memory loss in the TRPA1 KO animals. The exact molecular mechanism, how TRPA1 receptors contribute to the A $\beta$ <sub>1-42</sub>-induced cholinergic cell loss is still not fully elucidated. Previously, Harkany and colleagues published that A $\beta$ <sub>1-42</sub> induces cholinergic cell death via NMDA receptor mediated intracellular Ca<sup>2+</sup> increase in SI-NBM (Harkány et al., 2000). We speculate that TRPA1 receptor-mediated calcium influx may enhance the neurotoxic effect of the A $\beta$ <sub>1-42</sub> due to the cytotoxic Ca<sup>2+</sup> overload in cholinergic neurons in SI-NBM. This theory warrants further investigation.

## 5. Conclusion

Our study has presented the first evidence that the genetic lack of TRPA1 receptor significantly attenuates the A $\beta$ <sub>1-42</sub>-induced cholinergic neurotoxicity in the SI-NBM. We also demonstrated that TRPA1 KO animals show substantially reduced memory loss after A $\beta$ <sub>1-42</sub> lesion compared to the wild-type counterparts. Based on our findings, inhibition of the TRPA1 receptors might be a novel promising prospect in the pharmacotherapy of AD. The limitation of our study is, that its main conclusions are based exclusively on morphological and functional data provided by experiments on TRPA1 wildtype and KO mice.

## Ethical approval and consent to participate

All experimental procedures were performed according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection, Consideration Decree of Scientific Procedures of Animal Experiments (243/1988), Hungarian regulations (40/2013, II.14.) and Directive 2010/63/EU of the European Parliament. The studies were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments and licence was given (licence no.: BA 02/2000 – 24/2016; BA 02/2000 – 38/2016).

## Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Author's contribution

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors read, revised and approved the final version of the submitted manuscript. EP had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: MP, ÉB, EP. Acquisition of data: MP, ÉB, SG, DE, ÁK, EK, ÉSZ, JK. Analysis and interpretation of data: MP, ÉB, SG, ÁK, EK, JK, ÉSZ, EP.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

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## References

- Beninger, R.J., Wirsching, B.A., Jhamandas, K.B.R., 2001. Animal studies of brain acetylcholine and memory. *Arch. Gerontol. Geriatr.* 1, 71–89.
- Bevins, R.A., Besheer, J., 2006. Object recognition in rats and mice: A one-trial non-matching-to-sample learning task to study 'recognition memory'. *Nat. Protoc.* 1, 1306–1311.
- Böleskei, K., Kriszta, G., Sághy, É., Payrits, M., Sipos, É., Vranesics, A., Berente, Z., Ábrahám, H., Ács, P., Komoly, S., Pintér, E., 2018. Behavioural alterations and morphological changes are attenuated by the lack of TRPA1 receptors in the cuprizone-induced demyelination model in mice. *J. Neuroimmunol.* 320, 1–10.
- Bosson, A., Paumier, A., Boisseau, S., Jacquier-Sarlin, M., Buisson, A., Alsbriex, M., 2017. TRPA1 channels promote astrocytic Ca<sup>2+</sup> hyperactivity and synaptic dysfunction mediated by oligomeric forms of amyloid- $\beta$  peptide. *Mol. Neurodegener.* 12, 53.
- Colonna-Romano, G., Lio, D., Caruso, C., 2007. Polymorphisms of pro-inflammatory genes and Alzheimer's disease risk: a pharmacogenomic approach. *Mech. Ageing Dev.* 128, 67–75.
- D'Souza, Y., Elharram, A., Soon-Shiong, R., Andrew, R.D., Bennett, B.M., 2015. Characterization of Aldh2<sup>-/-</sup> mice as an age-related model of cognitive impairment and Alzheimer's disease. *Mol. Brain* 8, 27.
- Dalenogare, D.P., Theisen, M.C., Peres, D.S., Fialho, M.F.P., Lückemeyer, D.D., Antoniazzi, C.T.D., Kudsi, S.Q., Ferreira, M.A., Ritter, C.D.S., Ferreira, J., Oliveira, S.M., Trevisan, G., 2020. TRPA1 activation mediates nociception behaviors in a mouse model of relapsing-remitting experimental autoimmune encephalomyelitis. *Exp. Neurol.* 328, 113241.
- Doering, D.S., Matsudaira, P., 1996. Cysteine scanning mutagenesis at 40 of 76 positions in villin headpiece maps the F-actin binding site and structural features of the domain. *Biochemistry* 35, 12677–12685.
- Eleti, S., 2016. Drugs in Alzheimer's disease Dementia: an overview of current pharmacological management and future directions. *Psychiatr. Danub.* 28, 136–140.
- Giovannelli, L., Casamenti, F., Scali, C., Bartolini, L., Pepeu, G., 1995. Differential effects of amyloid peptides  $\beta$ (1-40) and  $\beta$ (25-35) injections into the rat nucleus basalis. *Neuroscience* 66, 781–792.

- Grayson, B., Leger, M., Piercy, C., Adamson, L., Harte, M., Neill, J.C., 2015. Assessment of disease-related cognitive impairments using the novel object recognition (NOR) task in rodents. *Behav. Brain Res.* 285, 176–193.
- Hamilton, N.B., Kolodziejczyk, K., Kougioumtzidou, E., Attwell, D., 2016. Proton-gated Ca<sup>2+</sup>-permeable TRP channels damage myelin in conditions mimicking ischaemia. *Nature* 529, 523.
- Harkany, T., O'Mahony, S., Kelly, J.P., Soós, K., Törő, I., Penke, B., Luiten, P.G., Nyakas, C., Gulya, K., Leonard, B.E., 1998.  $\beta$ -amyloid(Phe(SO<sub>3</sub>H)<sub>24</sub>)<sub>25-35</sub> in rat nucleus basalis induces behavioral dysfunctions, impairs learning and memory and disrupts cortical cholinergic innervation. *Behav. Brain Res.* 90, 133–145.
- Harkany, T., Abrahám, I., Timmerman, W., Laskay, G., Tóth, B., Sasvári, M., Kónya, C., Sebens, J.B., Korf, J., Nyakas, C., Zarándi, M., Soós, K., Penke, B., Luiten, P.G., 2000.  $\beta$ -Amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. *Eur. J. Neurosci.* 12, 2735–2745.
- Horváth, Á., Tékus, V., Boros, M., Pozsgai, G., Botz, B., Borbély, É., Szolcsányi, J., Pintér, E., Helyes, Z., 2016. Transient receptor potential ankyrin 1 (TRPA1) receptor is involved in chronic arthritis: in vivo study using TRPA1-deficient mice. *Arthritis Res. Ther.* 18, 6.
- Ikenoue, T., Lee, Y.H., Kardos, J., Saiki, M., Yagi, H., Kawata, Y., 2014. Goto Y.COLD denaturation of  $\alpha$ -synuclein amyloid fibrils. *Angew. Chemie - Int. Ed.* 53, 4502–4512.
- Jardín, I., López, J.J., Díez, R., Sánchez-Collado, J., Cantonero, C., Albarrán, L., Woodard, G.E., Redondo, P.C., Salido, G.M., Smani, T., Rosado, J.A., 2017. TRPs in pain sensation. *Front. Physiol.* 8, 382.
- Khalil, M., Alliger, K., Weidinger, C., Yerinde, C., Wirtz, S., Becker, C., Engel, M.A., 2018. Functional role of transient receptor potential channels in immune cells and epithelia. *Front. Immunol.* 9, 174.
- Kheradpezhoh, E., Choy, J.M.C., Daria, V.R., Arabzadeh, E., 2017. TRPA1 expression and its functional activation in rodent cortex. *Open Biol.* 7, 160314.
- Kozegi, Z., Szego, É.M., Cheong, R.Y., Tolod-Kemp, E., Ábrahám, I.M., 2011. Postlesion estradiol treatment increases cortical cholinergic innervations via estrogen receptor- $\alpha$  dependent nonclassical estrogen signaling in vivo. *Endocrinology* 152, 3471–3482.
- Kwakowsky, A., Potapov, K., Kim, S., Peppercorn, K., Tate, W.P., Ábrahám, I.M., 2016. Treatment of beta amyloid 1–42 (A $\beta$ <sub>1–42</sub>)-induced basal forebrain cholinergic damage by a non-classical estrogen signaling activator in vivo. *Sci. Rep.* 6, 21101.
- LaFerla, F.M., Green, K.N., 2012. Animal models of alzheimer's. *Cold Spring Harb. Perspect. Med.* 2, a006320.
- Lee, K.I., Lee, H.T., Lin, H.C., Tsay, H.J., Tsai, F.C., Shyue, S.K., Lee, T.S., 2016. Role of transient receptor potential ankyrin 1 channels in Alzheimer's disease. *J. Neuroinflammation* 12, 92.
- Li, Y.F., Cheng, Y.F., Huang, Y., Conti, M., Wilson, S.P., O'Donnell, J.M., Zhang, H.T., 2011. Phosphodiesterase-4D knock-out and RNA interference-mediated knock-down enhance memory and increase hippocampal neurogenesis via increased cAMP signaling. *J. Neurosci.* 31, 172–183.
- Maccioni, R.B., Muñoz, J.P., Barbeito, L., 2001. The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch. Med. Res.* 32, 367–381.
- Masters, C.L., Bateman, R., Blennow, K., Rowe, C.C., Sperling, R.A., 2015. Cummings J.L. Alzheimer's disease. *Nat. Rev. Dis. Prim.* 1, 15056.
- Neha Sodhi, R.K., Jaggi, A.S., Singh, N., 2014. Animal models of dementia and cognitive dysfunction. *Life Sci.* 109, 733–786.
- Nilius, B., Appendino, G., Owsianik, G., 2012. The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflügers Arch. Eur. J. Physiol.* 464, 425–458.
- Nishida, M., Kuwahara, K., Kozai, D., Sakaguchi, R., Mori, Y., 2015. TRP Channels: Their Function and Potentiality as Drug Targets. *Innov. Med. Basic Res. Dev.* 195–218.
- Palkovits, M., Graf, L., Hermann, I., Borvendeg, J., Acs, Z.S., Lang, T., 1978. Regional Distribution of Enkephalins, Endorphins and ACTH in the Central Nervous System of Rats Determined by Radioimmunoassay. *Endorphins' 78*, Acad. Press, Budapest, pp. 187–195.
- Paxinos, G., Franklin, K.B.J., 2001. *Mouse Brain in Stereotaxic Coordinates*. Academic Press.
- Pires, P.W., Earley, S., 2018. Neuroprotective effects of TRPA1 channels in the cerebral endothelium following ischemic stroke. *Elife.* 7, e35316.
- Puzzo, D., Lee, L., Palmeri, A., Calabrese, G., Arancio, O., 2014. Behavioral assays with mouse models of Alzheimer's disease: practical considerations and guidelines. *Biochem. Pharmacol.* 88, 450–467.
- Ritter, C., Dalenogare, D.P., de Almeida, A.S., Pereira, V.L., Pereira, G.C., Fialho, M.F.P., Lückemeyer, D.D., Antoniazzi, C.T., Kudsí, S.Q., Ferreira, J., Oliveira, S.M., Trevisan, G., 2020. Nociception in a progressive multiple sclerosis model in mice is dependent on spinal TRPA1 channel activation. *Mol. Neurobiol.* 57, 2420–2435.
- Sullivan, M.N., Gonzales, A.L., Pires, P.W., Bruhl, A., Leo, M.D., Li, W., Oulidi, A., Boop, F.A., Feng, Y., Jaggar, J.H., Welsh, D.G., Earley, S., 2015. Localized TRPA1 channel Ca<sup>2+</sup> signals stimulated by reactive oxygen species promote cerebral artery dilation. *Sci. Signal.* 8 ra2.
- Talesa, V.N., 2001. Acetylcholinesterase in alzheimer's disease. *Mech. Ageing Dev.* 122, 1961–1969.
- Tayebati, S.K., 2006. Animal models of cognitive dysfunction. *Mech. Ageing Dev.* 127, 100–108.
- Webster, S.J., Bachstetter, A.D., Nelson, P.T., Schmitt, F.A., Van Eldik, L.J., 2014. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet.* 5, 88.
- Zaborszky, L., Pang, K., Somogyi, J., Nadasdy, Z., Kallo, I., 1999. The basal forebrain corticopetal system revisited. *Ann. N. Y. Acad. Sci.* 877, 339–367.
- Zhang, R., Xue, G., Wang, S., Zhang, L., Shi, C., Xie, X., 2012. Novel object recognition as a facile behavior test for evaluating drug effects in A $\beta$ PP/PS1 Alzheimer's disease mouse model. *J. Alzheimers Dis.* 31, 801–812.
- Zimova, L., Sinica, V., Kadkova, A., Vyklicka, L., Zima, V., Barvik, I., Vlachova, V., 2018. Intracellular cavity of sensor domain controls allosteric gating of TRPA1 channel. *Sci. Signal.* 11 eaan8621.
- Zygmunt, P.M., Högesätt, E.D., 2014. TRPA1. *Handb. Exp. Pharmacol.* 222, 583–630.