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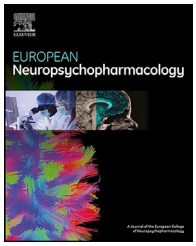

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Expression of the Transient Receptor Potential Vanilloid 1 ion channel in the supramammillary nucleus and the antidepressant effects of its antagonist AMG9810 in mice

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Abstract
The Transient Receptor Potential Vanilloid 1 (TRPV1) non-selective cation channel predominantly expressed in primary sensory neurons of the dorsal root and trigeminal ganglia mediates pain and neurogenic inflammation. TRPV1 mRNA and immunoreactivity were described in the central nervous system (CNS), but its precise expression pattern and function have not been clarified. Here we investigated *Trpv1* mRNA expression in the mouse brain using ultrasensitive RNAScope in situ hybridization. The role of TRPV1 in anxiety, depression-like behaviors and memory functions was investigated by TRPV1-deficient mice and pharmacological antagonism by AMG9810. *Trpv1* mRNA is selectively expressed in the supramammillary nucleus (SuM) co-

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localized with *Vglut2* mRNA, but not with tyrosine hydroxylase immunopositivity demonstrating its presence in glutamatergic, but not dopaminergic neurons. TRPV1-deleted mice exhibited significantly reduced anxiety in the Light-Dark box and depression-like behaviors in the Forced Swim Test, but their performance in the Elevated Plus Maze as well as their spontaneous locomotor activity, memory and learning function in the Radial Arm Maze, Y-maze and Novel Object Recognition test were not different from WTs. AMG9810 (intraperitoneal injection 50 mg/kg) induced anti-depressant, but not anxiolytic effects. It is concluded that TRPV1 in the SuM might have functional relevance in mood regulation and TRPV1 antagonism could be a novel perspective for anti-depressant drugs.

1. Introduction

The Transient Receptor Potential Vanilloid 1 (TRPV1) capsaicin receptor is a non-selective cation channel activated and sensitized by a broad range of physical stimuli including noxious heat, as well as several exogenous and endogenous chemicals like protons, bradykinin, prostaglandins, etc. produced by inflammation and tissue injury (Vennekens et al., 2012). David Julius and his team cloned TRPV1 and the discovered its molecular structure, gating mechanisms, and role in heat and pain sensation, which was awarded by Nobel Prize in Physiology or Medicine in 2021 (Caterina et al., 1997a; Tominaga et al., 1998a).

While TRPV1 was predominantly described in the peripheral nervous system (Caterina et al., 1997b; Helliwell et al., 1998; Szallasi and Blumberg, 1999; Tominaga et al., 1998b), it has been long accepted that TRPV1 is also present in the central nervous system (CNS). *In situ* hybridization (ISH) suggested *Trpv1* mRNA expression in the rat olfactory cortex, septal nucleus, hippocampus, locus coeruleus, substantia nigra and the inferior olive (Mezey et al., 2000). TRPV1-like immunoreactivity was also shown in several rodent brain structures, including the hippocampus, basal ganglia, hypothalamus, thalamus, and cerebellum (Cristino et al., 2006; Mezey et al., 2000; Tóth et al., 2005). Reverse transcription (RT)-PCR and Western blot demonstrated of *Trpv1* mRNA and TRPV1 immunopositivity in rodent hippocampus, cerebellum, olfactory bulb (Mezey et al., 2000; Tóth et al., 2005). Besides, capsaicin injection targeting selective rodent brain regions including the cortex, hippocampus, hypothalamus, thalamus, cerebellum, substantia nigra, periaqueductal gray was showed to elicit electrical and physiological responses, such as changed in the firing activity of neurons or enhanced synaptic transmission (Hori et al., 1988; Mezey et al., 2000; Steenland et al., 2006; Szabo et al., 2002).

However, in contrast to the widespread and robust distribution of TRPV1 in the CNS suggested by these studies, *Trpv1*-specific bacterial *lacZ* reporter gene expression results confirmed *Trpv1* expression only in few mouse brain areas, including the intrafascicular nucleus, the periaqueductal gray matter, the supramammillary nucleus, hypothalamus and hippocampus (Cavanaugh et al., 2011). Therefore, TRPV1 expression in the CNS remains controversial presumably due to selectivity and sensitivity problems of the detection techniques, most importantly several non-specific antibodies (Supplementary Table 1).

Besides the well-established sensory and nociceptive functions of TRPV1 (Bölcskei et al., 2005; Caterina et al., 1997b; Szolcsányi, 1977; Szolcsanyi et al., 2011; Tominaga et al., 1998b; Walker et al., 2003), it was proposed to contribute to a wide range of physiological and pathophysiological processes in the CNS. TRPV1-deficient mice exhibited less anxiety-like behaviors in the Light-Dark Box and the Elevated Plus Maze test (Marsch et al., 2007a) and impaired memory in the Novel Object Recognition and Passive Avoidance test (You et al., 2012), but TRPV1 deficiency also rescued memory deficit in a mouse model with Alzheimer's disease (Kim et al., 2020). Capsaicin decreased anxiety in the Elevated Plus Maze and the Vogel conflict test (Terzian et al., 2009), reduced depression-like behaviors in the Forced Swim and the Tail Suspension Test (Hayase, 2011), and enhanced memory functions (Bashiri et al., 2018). On the other hand, TRPV1 antagonist capsazepine reduced immobility time in the Forced Swim Test (Manna and Umathe, 2012), and dual blockade of fatty acid amide hydrolase and TRPV1 receptor decreased anxiety levels in the Elevated Plus Maze test (Micale et al., 2008). TRPV1, therefore, can be a promising target for the treatment of various CNS disorders, including anxiety, stress, or movement disorders (Lee et al., 2006; Li et al., 2008; Marzo et al., 2008; Pegorini et al., 2006; Terzian et al., 2009). The CNS expression and role of TRPV1 need to be elucidated to determine further drug developmental perspectives.

Here we aimed to elucidate *Trpv1* mRNA expression pattern in the mouse brain using the ultrasensitive RNAScope ISH technique, as well as the role of TRPV1 in anxiety, depression-like behaviors, learning and memory functions by genetic deletion and pharmacological inhibition of the receptor.

2. Materials and methods

2.1. Animals

3- 4-month-old male C57Bl/6 mice were used for RNAscope ISH and immunohistochemical staining. Similarly, 3-5-month-old male C57Bl/6 mice (wild type – WT) and TRPV1 knock-out (TRPV1 KO,

purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred as homozygotes) mice were used for behavioral experiments. Mice were randomized in both the TRPV1-deleted and WT groups containing subjects from all age categories.

All mice were bred and raised in temperature and humidity controlled 12 h light-dark cycle environment in standard polycarbonate cages in the Animal House of the Department of Pharmacology and Pharmacotherapy of the University of Pécs following the regulations of the Animal Welfare Committee at the University of Pécs, the National Scientific Ethical Committee on Animal Experimentation in Hungary (permission no: BA02/2000-53/2022.), and in agreement with the directive of the 1986 European Communities Council, as well as with the 1998 Law of XXCIII on Animal Care and Use in Hungary. Mice were provided ad libitum with standard rodent chaw and drinking water.

2.2. Brain sectioning

Mice ($N = 3$) were deeply anesthetized with an overdose of urethan (2.4 g/kg) and transcardially perfused with ice-cold 0.1 M PBS (pH: 7.4), followed by 4% paraformaldehyde (PFA) solution in Millonig buffer (pH 7.4). Brains were dissected, postfixed for 24 h at room temperature, rinsed in 0.1 M PBS, and stored at 4 °C. Brains were embedded in 4% freshly prepared agarose blocks and coronally sectioned (by 30 μm) using a Leica VT1000S vibratome (Leisca Biosystems, Wetzlar, Germany). Sections were collected into RNase-free tubes and stored in 1x PBS-0.1% Na-azide at 4 °C.

Several brain regions such as the olfactory bulb, prelimbic cortex, septum, hypothalamus, hippocampus, piriform cortex, amygdala, primary somatosensory cortex, cerebellum, supramammillary nucleus were sectioned and selected for singleplex RNAScope, where *TRPV1* mRNA expression was expected based on literature data expression (Paxinos et al., 2001) (Supplementary Table 1). Two representative sections from the mouse supramammillary nucleus (SuM, from Bregma −2.50 to −3.50 mm according to (Paxinos et al., 2001)) per animal were selected for RNAScope in situ hybridization combined with immunohistochemistry studies.

2.3. RNAScope in situ hybridization (ISH)

Singleplex RNAScope ISH was performed using the ultrasensitive RNAscope Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics (ACD), Newark, CA, USA) according to the manufacturer's protocol ($N = 3$) with minor modification (Kecskés et al., 2020). After tissue pretreatment, sections were hybridized with probes specific to mouse *Trpv1* (ACD, Cat. No.: 313,331-C2). Signal amplification and channel development were conducted sequentially. Cyanine 3 (Cy3) (1:750 diluted in TSA buffer) were used as fluorophores for *Trpv1* mRNA detection. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, ACD) and mounted with Prolong Glass antifade mountant (Thermo Fisher Scientific) for confocal imaging. Slides were stored at −20 °C.

Multiplex RNAScope ISH on mouse brain sections was performed using the same procedure and reagent kit for singleplex RNAScope, with some minor differences (Kecskés et al., 2020). Pre-treated sections were instead hybridized with probes designed to mouse *Trpv1* (ACD, Cat. No.: 313,331-C2) and *Vglut2* (ACD, Cat. No.: 319,171-C3) mRNA. Cy3 (1:750 diluted in TSA buffer) and Cyanine 5 (Cy5) (1:1500 diluted in TSA buffer) were selected as fluorophores for *Trpv1* and *Vglut2* mRNA signals, respectively. RNAscope 3-plex mouse positive control probes (ACD, Cat. Nr.: 320,881, probes specific to RNA polymerase II subunit A (*Polr2a*), peptidyl-prolyl cis-trans isomerase B (*Ppib*), ubiquitin C (*Ubc*)) and negative controls (ACD, Cat. Nr.: 320,871, probe designed to bacterial, 4-hydroxy-tetrahydrodipicolinate reductase, *dapB*) were used in parallel to ensure interpretable and standardized results ($N = 1$).

2.4. Immunohistochemistry (IHC)

Tyrosine hydroxylase-specific IHC was performed in coupled with multiplex RNAScope ISH according to the manufacturer's protocol ($N = 3$) (TM323100, Tech Note: Dual ISH IHC manual Multiplex Fl v2, Advanced Cell Diagnostics). Sections were washed in 1% 0.1 M PBS and incubated with rabbit anti-tyrosine hydroxylase primary antibody (1:2000 diluted in 2% normal goat serum (NGS), Cat. Nr.: Ab112, Abcam, Cambridge, UK) overnight at room temperature. Sections were washed 3×15 min then incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:1000 prediluted in 2% NGS, A-11,008, Thermo Fisher Scientific) for 3 h at room temperature. For IHC, sections were counterstained with DAPI (ACD) and mounted with Prolong Glass antifade mountant (P36980, Thermo Fisher Scientific). Slides were stored at −20 °C for confocal imaging.

2.5. Confocal imaging and image analysis

Fluorescent images (z-stacks with 1 μm intervals) of mouse brain sections according to (Paxinos et al., 2001) were acquired using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany). Virtual colors were selected to depict fluorescent signals: green for Alexa 488 (tyrosine hydroxylase IHC and *Polr2a* mRNA), red for Cy3 (*Trpv1* and *Ppib* mRNA), white for Cy5 (*Vlgut2* and *Ubc* mRNA), and blue for DAPI.

Brightness/contrast adjustment and z-projection (15 stacks/image, 1 μm-intervals) with maximum intensity of separate channels were processed using (Fiji, 1.53c, NIH, USA).

2.6. Pharmacological treatment

Mice were separated into four groups receiving intraperitoneal injection (i.p) of either saline (control group), citalopram hydrobromide, a well-known anti-depressant drug targeting the serotonergic system as a positive control (10 mg/kg i.p., Sigma-Aldrich Gmbh) (Bezchlibnyk-Butler et al., 2000), AMG9810, a potent TRPV1 antagonist (50 mg/kg) (Alawi et al., 2015), or vehicle for AMG9810 (composed of: 20 μl DMSO+50 μl Tween80+9.30 μl saline), which served as a control group for AMG9810 treatment. All measurements were performed 30 mins after the treatments.

2.7. Open field test (OFT)

OFT represents a conflict between the willingness to explore new environments and the innate aversion of rodents to light. Mice (WT $N = 19$, TRPV1 KO $N = 18$) were placed in a brightly lit wooden box (60 cm x 40 cm) with a floor divided into 16 equal squares (4 x 4), where the animals could move freely. The movement of each mouse was recorded for 5 min with the EthoVision XT11 software (Noldus Information Technology, Netherlands). At the beginning of the measurement, mice were placed in the same corner of the box. The time spent moving and the behavior of the mouse was analyzed using the video track (Holland and Weldon, 1968).

2.8. Light-Dark box test (LDB)

LDB test evaluates the anxiety level based on the innate light-aversive behavior and exploratory behavior of rodents (Bourin and

Hascoët, 2003). Mice (WT $N = 9$, TRPV1 KO $N = 10$) were placed in a 60 cm x 60 cm x 45 cm wooden box, consisting of two equally sized compartments, one closed dark, and one open lit compartment. The illumination for the lit compartment (thermal neutral fiber optic source, Fiber-lite) was provided by an intense light (800 lx) source that did not produce heat. The two compartments were separated by a wall and connected via a 7 x 7 cm opening at the floor level. Mice were individually investigated for 20 min, and the amount of time spent in the light compartment was measured (Scheich et al., 2017). The same experiment was performed for mice receiving saline ($N = 6$), vehicle ($N = 6$) or AMG9810 ($N = 4$).

2.9. Elevated plus maze test (EPM)

EPM test is another test for the assessment of the anxiety level of rodents (Komada et al., 2008). Two opposite open arms (50 cm x 10 cm) and two opposite closed arms (50 cm x 10 cm), connected via a central platform and located 1 meter above the floor were used in the experiment. Each mouse (WT $N = 7$, TRPV1 KO $N = 7$) was individually placed on the central platform and allowed to explore the arms. The time spent in the open arms during the 5-minute experimental period was registered using the EthoVision XT11 software (Noldus Information Technology, Netherlands) (László et al., 2010). The same experiment was performed for mice receiving saline ($N = 4$), vehicle ($N = 4$), AMG9810 ($N = 7$), or citalopram ($N = 7$).

2.10. Forced swim test (FST)

FST is a widely used test to assess depression-like behaviors and the antidepressant effect of drugs or genetic manipulations in rodents (Can et al., 2012). Mice (WT $N = 12$, TRPV1 KO $= 12$) react to an inescapable acute stress situation by alternating mobility (escaping) and immobility (floating). Animals were individually placed in a transparent cylinder (height 25 cm, diameter 20 cm) filled with 19 cm depth of water (24 - 25 °C). The total duration of stress exposure was 6 min, and the time of immobility, referring to depression-like behaviors, was registered during the final 4 min of stress exposure (Borbély et al., 2017). The same experiment was performed for mice receiving saline ($N = 9$), vehicle ($N = 7$), AMG9810 ($N = 7$), or citalopram ($N = 4$).

2.11. Radial arm maze test (RAM)

RAM test is a suitable method for assessment of short- and long-term memory alterations (Penley et al., 2013). Three-day-long habituation and learning period are used before the test trial. During this time, mice (WT $N = 13$, TRPV1 KO $N = 12$) have to learn where they can find the food pellets (Dustless Precision Pellets® 45 mg, Sucrose; BioServ, USA) placed in 4 previously chosen arms of the eight-arm radial maze (arms 5-cm-wide x 35-cm-long, central platform diameter 5 cm). The trials last for 5 min or until the animals have found all the four food pellets. On the fourth day, the animals also have to find the four pellets placed in the same arms as it was during the learning period. The data for the fourth day was used to assess the learning capabilities, as follows: working memory errors = entries into the baited arms that had already been visited during the same trial, referring to the short-term memory, and reference memory error = entries into empty arms, showing the status of long-term memory (Payrits et al., 2020; Szentes et al., 2019).

2.12. Y-Maze test (YMZ)

YMZ test was used for the assessment of spatial memory (Kraeuter et al., 2019). One 5-minute trial was performed during the experiment (three 35-cm-long x 5-cm-wide, stated as A, B and C arms constituting the Y-maze) in which mice (WT $N = 13$, TRPV1 KO $= 12$) were allowed to freely explore the maze. The spontaneous alternation, correct alternating behavior (ABC, ACB, BAC, BCA, CAB, CBA)/ the total number of arm entries minus two, was calculated on the basis of video track by EthoVision XT11 software (Noldus Information Technology) (Borbély et al., 2019).

2.13. Novel object recognition test (NOR)

NOR test is a widely used and validated test for the assessment of recognition memory (Morellini, 2013; Zhang et al., 2012). On the first habituation day, animals were (WT $N = 13$, TRPV1 KO $N = 12$) allowed to freely explore the 45 × 45 × 30 cm wooden box (open field box) for 5 min, which can be considered a simple open field test. On the second day, mice could examine the two identical objects for 5 min. On the third day, one novel object replaced one of the two identical objects, and mice could choose from the novel object and the other familiar object (from the second day) for 5 min. The time spent for each object was registered using the EthoVision XT11 software (Noldus Information Technology). Data can be assessed as follows: recognition index = (time exploring the novel object/total exploration time) × 100 and discrimination index = difference in time exploring the novel and familiar objects/total exploration time, exploration time of the familiar and novel objects.

2.14. Data analysis

Data was analyzed using Prism 8 software (GraphPad Software, San Diego, CA, USA). The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to determine the normality of data distribution. For normally distributed data unpaired Student's test was used for comparing data of two groups, while one-way ANOVA followed by Dunnett's test as post-hoc test was used for dataset consisting of more than two groups. For non-normal distribution, the Mann-Whitney test was used for the dataset with two groups, and Kruskal-Wallis Test followed by Dunn's test as post-hoc test for comparing more than two groups. A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. *Trpv1* mRNA is selectively expressed in *vglut2*-positive neurons of the mouse sum

Trpv1 mRNA expression is specifically detected in mouse SuM via singleplex fluorescent RNAscope (Fig. 1A). In contrast, no *Trpv1* mRNA signals were detected in the olfactory bulb, prelimbic cortex, septum, hypothalamus, hippocampus, piriform cortex, amygdala, primary somatosensory cortex and cerebellum (Supplementary Fig. 1).

Strong colocalization of *Trpv1* with *Vglut2* mRNA, but not with tyrosine hydroxylase immunopositivity was demonstrated in the mouse SuM (Fig. 1B).

RNAScope 3-plex mouse positive control probes visualized moderate to strong signals of mouse housekeeping genes RNA polymerase II subunit A (*Polr2a)*, Peptidylprolyl cis-trans isomerase B (*Ppib)*, and ubiquitin C *(Ubc)* (Fig. 1D). Negative controls designed for the bacterial 4-hydroxy-tetrahydrodipicolinate reductase *(dapB)* gene gave no detectable fluorescent signal (Fig. 1E).

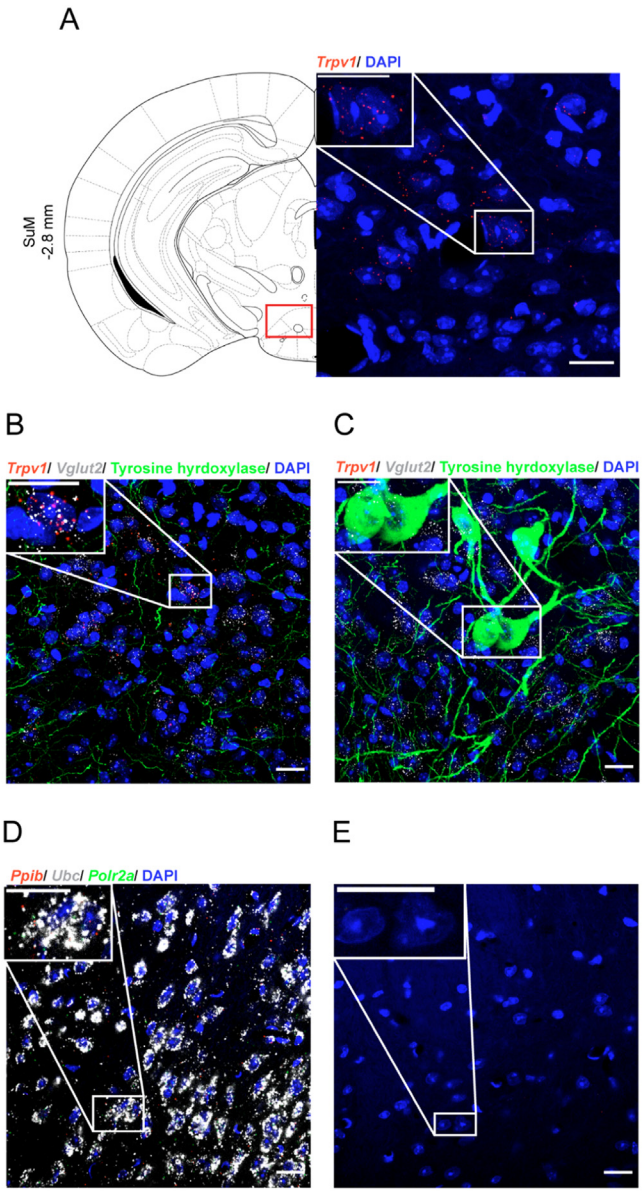

Fig. 1 Representative RNAScope and IHC multiplex fluorescent staining in the mouse supramammillary nucleus (SuM). Selective Trpv1 mRNA expression in the mouse SuM, Bregma −2.8 mm (A). Trpv1 mRNA co-localized with Vglut2 mRNA (B). There was no overlap between Trpv1 mRNA and tyrosine hydroxylase immunoreactivity (C). RNAScope 3-plex mouse positive control probes visualized moderate to strong signals of mouse housekeeping genes (D). Negative controls for RNAScope gave no detectable fluorescent signal (E). Nuclei were stained with DAPI. 63x objective, scale bar 20 μm, inset scale bar 20 μm.

In order to select the most behavioral tests to investigate TRPV1's function, we summarized the available literature data linking the potential function of TRPV1 and the SuM (Supplementary Table 2). We found a remarkable overlap between the roles of TRPV1 and SuM in terms of anxiety and memory functions highlighted in bold. Based on these results we decided to investigate TRPV1-deleted mice in the aspect of anxiety and memory, as well as depression-like behavior, since anxiety and depression are related to several common mechanisms.

3.2. Deletion of TRPV1 does not influence spontaneous locomotor activity and anxiety level in the OFT

WT ($N = 19$) and TRPV1 KO mice ($N = 18$) spent 45.23 ± 4.127 s and 38.60 ± 4.091 s in the center zone of the OFT, respectively (Fig. 2A), showing no significant difference. The number of entries into the center zone of the two groups also did not remarkably differ (WT 36.32 ± 2.007, TRPV1$^{-/-}$ 33.78 ± 2.552, Fig. 2B). WT mice moved 2864 ± 113.5 cm with a velocity of 9.555 ± 0.380 cm/*sec*, respectively while the values of TRPV1$^{-/-}$ animals were 3037 ± 151.6 cm and 10.15 ± 0.5076 cm/*sec*, respectively (Fig. 2C, D).

3.3. TRPV1-deficient mice showed lower anxiety and depression levels than WTs

In the LDB test, TRPV1$^{-/-}$ mice ($N = 10$) spent more time in the light compartment (544.2 ± 32.45 s) than the WTs ($N = 9$, 381.9 ± 37.43 s), implying lower anxiety levels in the gene-deficient group (Fig. 3A). The time spent in the open arms of the EPM was 61.28 ± 3.273 and 54.90 ± 11.62 s in the case of the WT ($N = 7$) and TRPV1$^{-/-}$ ($N = 7$) groups, respectively (Fig. 3B).

TRPV1$^{-/-}$ mice exhibited significantly shorter immobility time in the FST ($N = 12$, 72.25 ± 5.706 s) compared to WTs ($N = 12$, 101.1 ± 11.81 s), demonstrating decreased depression-like behaviors (Fig. 3C).

3.4. Systemic TRPV1 antagonist treatment induces anti-depressant, but not anxiolytic effects in mice

Saline-treated control mice ($N = 6$) spent 283.9 ± 66.87 s, while the vehicle-treated ($N = 6$) and the AMG9810-treated ones ($N = 4$) spent 345.2 ± 35.70 and 471.3 ± 48.93 s in the light compartment of the LDB, respectively with no significant differences suggesting similar anxiety levels of all groups (Fig. 4A).

In the EPM test, saline-treated mice ($N = 4$) spent 44.76 ± 3.811 s, vehicle-treated animals ($N = 4$) 59.35 ± 16.64 s, while the AMG9810-treated ones ($N = 7$) 63.85 ± 10.70 s in the open arms, which did not differ significantly from each other. The antidepressant compound citalopram induced significant anxiolytic effects ($N = 7$; 85.24 ± 4.283 s in the open arm) (Fig. 4B).

The immobility times in the FST were 126.6 ± 11.10 s and 85.57 ± 26,78 s in the saline-($N = 9$) and vehicle-treated ($N = 7$) control groups, respectively. AMG9810-treated mice ($N = 7$) were significantly less immobile (11.43 ± 5.018 s), suggesting anti-depressant effect of TRPV1 antagonism. Citalopram treatment ($N = 4$) also significantly reduced the immobility time to 50.5 ± 18.67 s (Fig. 4C).

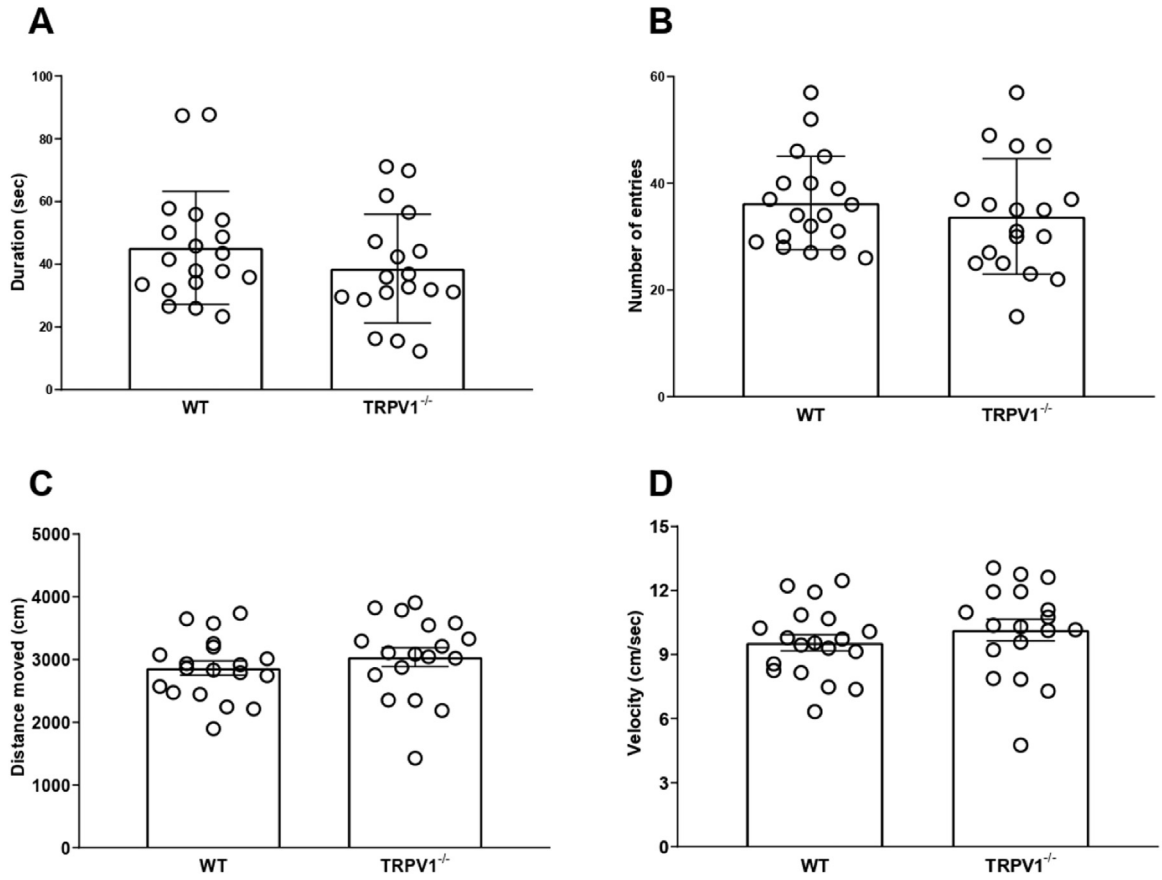

Fig. 2 Similar performance in the Open Field Test of the wild-type (WT) and TRPV1$^{-/-}$ mice. (A) Time spent in the center zone, t(35) = 1.14, $p = 0.26$; (B) entries into the center zone, t(35) = 0.79, $p = 0.44$; (C) distance moved, t(35) = 0.92, $p = 0.36$; and (D) velocity, t(35) = 0.94, $p = 0.35$. Data are demonstrated as individual dots, error bars represent the means ± SEM.

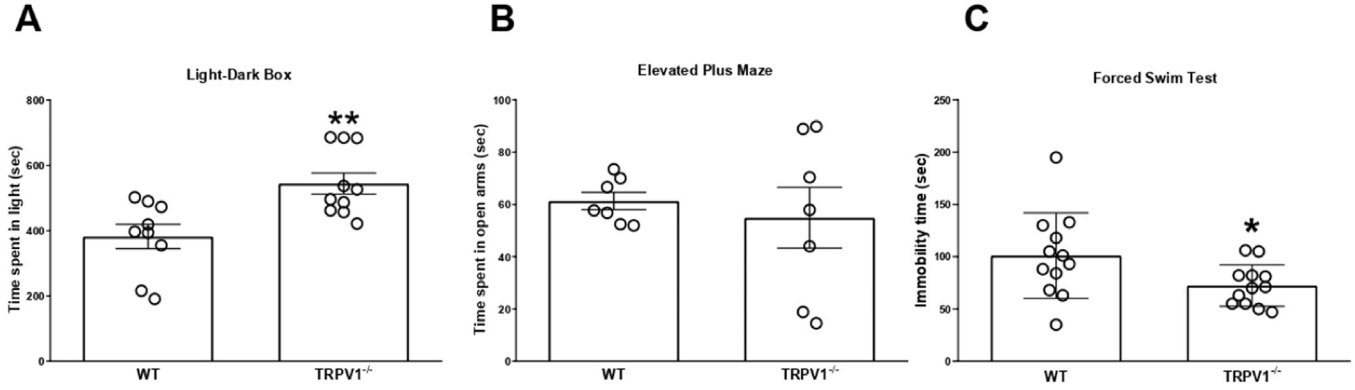

Fig. 3 Anxiety and depression tests comparing wild-type (WT) and TRPV1$^{-/-}$ mice. (A) Time spent in the light compartment of the Light-Dark Box test; unpaired Student's test, t(17) = 3.29, $p = 0.004$, (B) time spent in open arms in the Elevated Plus Maze test, unpaired Student's test, t(12) = 0.52, $p = 0.61$, and (C) immobility time in the Forced Swim Test; unpaired Student's test, t(22) = 2.2, $p = 0.04$. Data are demonstrated as individual dots; error bars represent the means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$.

3.5. TRPV1 deficiency does not affect learning and memory functions

In the RAM test, WT mice showed reference memory error of 5.23±0.79 and working memory error of 2.54 ± 0.5. The corresponding values of TRPV1$^{-/-}$ mice were 5.83± 0.941 and 2.58± 0.83 ($N = 13, 12$), respectively, demonstrating no significant differences (Fig. 5A, B).

In the YMZ test, the spontaneous alternation index of the WT and TRPV1$^{-/-}$ groups were also similar, 0.72±0.04 and 0.62± 0.3, respectively (Fig. 5C).

In the NOR test no significant differences were observed in either the recognition index (50.95 ± 3.644% vs. 60.6 ± 2.843%) or the discrimination index (0.019± 0.07 vs. 0.22± 0.06) of the WT and TRPV1$^{-/-}$ mice (Fig. 5D, E).

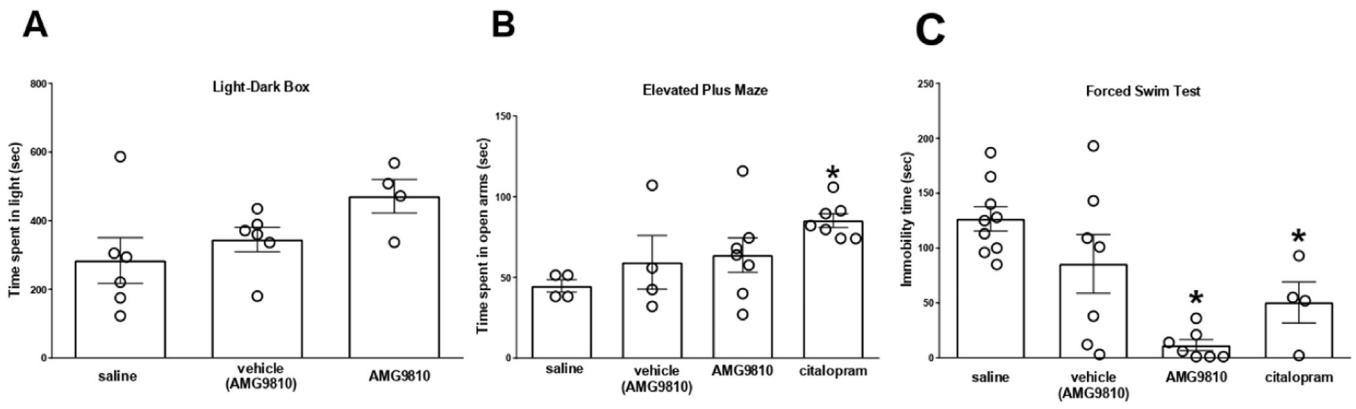

Fig. 4 Systemic TRPV1 antagonist AMG9810 treatment decreases depression-like behavior in mice. Effect of i.p. AMG9810 (50 mg/kg) (**A**) in the Light-Dark Box, one-way ANOVA test, F (2, 13) = 2.745, $p = 0.1$, (**B**) in the Elevated Plus Maze test in comparison with the reference compound citalopram (10 mg/kg i.p), Kruskal-Wallis test, Chi-square = 8.39, df = 3, $p = 0.04$ followed by Dunn's post-hoc test (citalopram-treated vs. saline-treated group, $p = 0.018$), and (**C**) in the Forced Swim Test, one-way ANOVA, F (3, 23) = 9.6, $p = 0.0003$ followed by Dunnett's post-hoc test comparing AMG9810-treated to vehicle-treated mice, $p = 0.012$, citalopram-treated compared to saline-treated group, Dunnett's test as post-hoc following one-way ANOVA, $p = 0.02$. Data are shown as individual dots; error bars represent the means ± SEM, $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

We present here 1) the first data for relatively selective *Trpv1* mRNA expression in the *Vglut2*-positive glutamatergic neurons of the mouse SuM, 2) functional results for the role of TRPV1 in anxiety and depression-like behaviors, as well as 3) the antidepressant potential of a TRPV1 antagonists.

Our results obtained by the ultrasensitive, fluorescent RNAScope in situ hybridization technique are supported by previous data using the *lacZ* reporter gene expression and radioactive ISH (Cavanaugh et al., 2011). Although TRPV1 has earlier been proposed to be broadly expressed in the mouse and rat CNS both at mRNA and protein levels using conventional in situ hybridization (ISH) and immunohistochemistry (Cristino et al., 2006; Mezey et al., 2000; Tóth et al., 2005), our results clearly showed relatively specific expression in the mouse SuM. These virtual contradictions might be attributed to species differences between mice and rats, differences between the traditional ISH and RNAScope sensitivity and specificity (Wang et al., 2012), post-translational protein degradation, as well as differences between mRNA and protein expression (Supplementary Table 1). We are aware of the limitation of demonstrating mRNA expression instead of the TRPV1 receptor protein, but the validity, specificity and reliability of most anti-TRPV1 antibodies have been questioned and challenged (Cavanaugh et al., 2011). The RNAScope technique with high degree of sensitivity and specificity (Anderson et al., 2016; Atout et al., 2022), provided reliable detection of *Trpv1* mRNA expression. Since mapping TRPV1 expression in the whole brain was beyond the scope of this study, there might be some other regions where this receptor is present.

Furthermore, as a novelty, we found that *Trpv1* in the mouse SuM is co-expressed with *Vglut2* suggesting its localization on cells having glutamate as the principal neurotransmitter. Since glutamatergic neurons in the SuM have different projections and connections towards a variety of brain regions involved in several functions, including anxiety, mood regulation and cognition (Ito et al., 2009; Kesner et al., 2021; Pedersen et al., 2017), we investigated the functional relevance of TRPV1 in the SuM on the basis of literature data ((Genro et al., 2012; Gobira et al., 2017; Gutiérrez-Guzmán et al., 2012; Ikemoto, 2005; Ikemoto et al., 2004, 2006; Nguyen et al., 2014; Shahidi et al., 2004; Shin and Ikemoto, 2010) Supplementary Table 2).

We showed that TRPV1 plays a role in anxiety and depression-like behaviors in mice, which is supported by previous data (Abdelhamid et al., 2014; Hayase, 2011; Kim et al., 2020; Manna and Umathe, 2012; Marsch et al., 2007b; Micale et al., 2008; Terzian et al., 2009; You et al., 2012). Despite the significant difference described between WT and TRPV1-deleted mice in the LDB, there was no difference in the EPM. Anxiety is a multifactorial and multidimensional condition (Ramos and Mormède, 1997) and these two tests measure different aspects (Ramos, 2008; Ramos et al., 2008; Ramos and Mormède, 1997). A potential explanation for this difference might be that TRPV1 is differently involved in the complexity of pathways and networks regulating the anxiety level. While both tests are appropriate to evaluate anxiety-like behaviors induced by lit and open spaces counteracting the curiosity and exploratory behavior of rodents (Cryan and Holmes, 2005), the EPM provides a more complex stressful environment. The narrow, elevated and lit platform of the EPM is more difficult to avoid compared to the lit compartment of the LDB. Furthermore, it was described that the movement pattern observed in the EPM depends not only on the anxiety level, but also on spontaneous locomotor activity and decision-making ability (Rodgers et al., 1995). These might also be considered as similar contributing factors in the LDB, but differences in the construction and complexity of the two test environments are likely to lead to different results.

As a limitation, the behavioral results obtained with the TRPV1 KO mice cannot be directly related to the specific

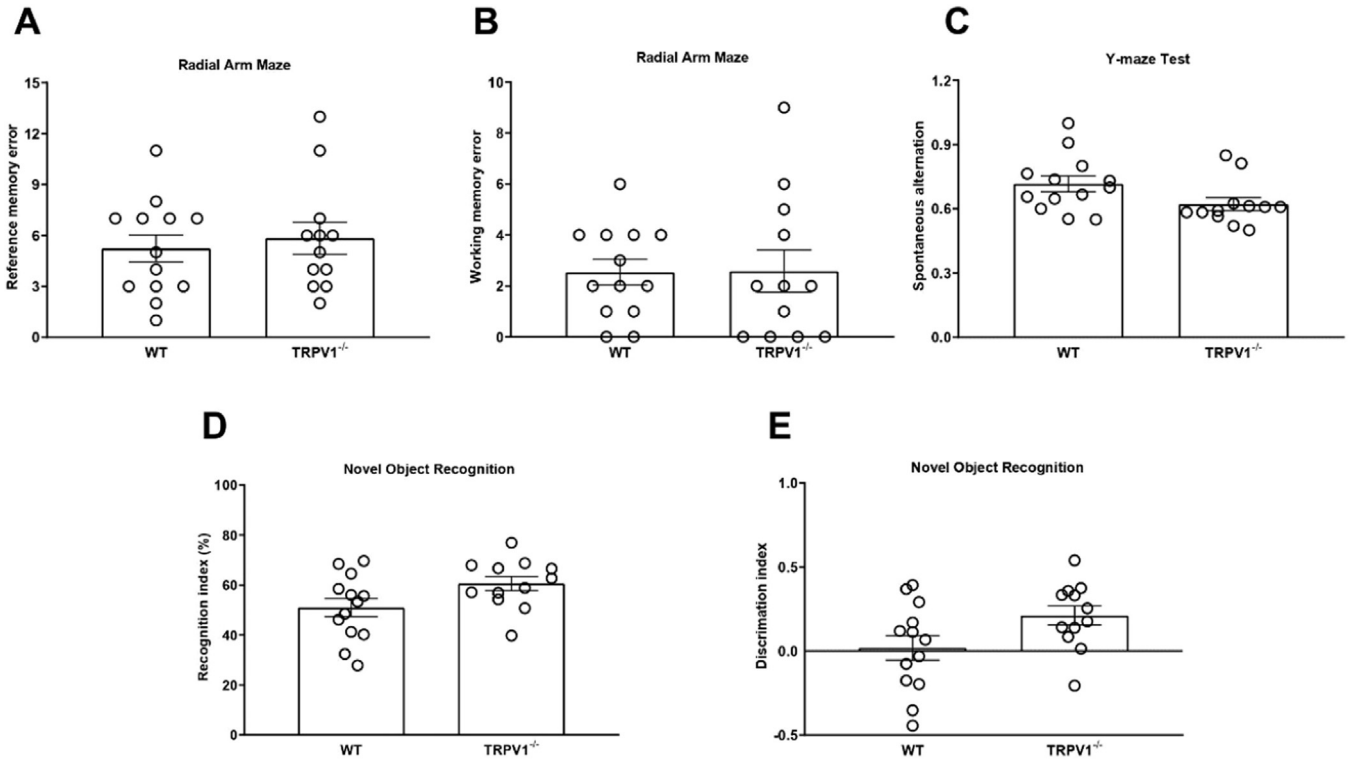

Fig. 5 TRPV1 deficiency does not influence learning and memory functions. Performance of WT and TRPV1$^{-/-}$ mice in (A) reference memory error in the Radial Arm Maze (RAM), unpaired Student's *t*-test, t(23) = 0.49, $p = 0.67$; (B) working memory error in RAM, Mann-Whitney test (medians, $U = 71$, N1 = 13, N2 = 12, $p = 0.72$); (C) spontaneous alternation index in Y-Maze Test, Mann-Whitney test (medians, $U = 42$, N1 = 13, N2 = 12, $p = 0.051$); (D) recognition index in the Novel Object Recognition test (NOR), unpaired Student's *t*-test, t(23) = 2.06, $p = 0.051$; (E) discrimination index in the NOR test, unpaired Student's *t*-test, t(23) = 2.06, $p = 0.051$. Data are shown as individual dots; error bars represent the means ± SEM.

location of TRPV1 activity in the brain. However, based on the relatively specific *Trpv1* mRNA expression restricted to the mouse SuM and evidence for the involvement of the SuM in anxiety-like behaviors (Aranda et al., 2006; López-Ferreras et al., 2020), we propose a potential functional relationship between these results. In addition, although the currently used simple tests indeed have limitations from the translational points of view, but they are the most commonly applied methods for providing proof-of-concept in drug effect evaluation with validated positive controls (Can et al., 2012; Petit-Demouliere et al., 2005).

The observed anti-depressant effect of the TRPV1 antagonist AMG9810 (Alawi et al., 2015; Gavva et al., 2005; Tékus et al., 2010) suggests the potential of TRPV1 antagonism as a novel mechanism for the treatment of mood disorders (Campos and Guimarães, 2009; Iglesias et al., 2022; Manna and Umathe, 2012). TRPV1 antagonists have long been suggested as potential analgesics (Bamps et al., 2021; Cui et al., 2006; Gomtsyan and Brederson, 2015; Premkumar and Sikand, 2008; Wong and Gavva, 2009). However, since TRPV1 is important in thermoregulation, its blockade by most candidates induced hyperthermia (Garami et al., 2010, 2020), including AMG9810 (Alawi et al., 2015), and some evoked hypothermia (Garami et al., 2018), which prevented their registration despite promising efficacy data. There are still candidates under clinical development, which do not influence thermoregulation. These thermo-neutral TRPV1 antagonists include e.g., A-1,165,442 (Reilly et al., 2012; Voight et al., 2014), AS1928370 (Watabiki et al., 2011) and NEO6860, a modality selective TRPV1 antagonist (Arsenault et al., 2018a; Brown et al., 2017) was tested in osteoarthritic patients and showed an analgesic trend (ClinicalTrials.gov Identifier: NCT02712957, (Arsenault et al., 2018b)). Furthermore, SYL1001 (Tivanisiran, (Moreno-Montañés et al., 2018)), which is a small interfering RNA targeting TRPV1, seems to be effective and is investigated in different eye diseases (ClinicalTrials.gov Identifiers: NCT04819269, NCT01438281, NCT01776658, NCT02455999 and NCT05310422 (Benitez-Del-Castillo et al., 2016)), SAF312 (Libvatrep, (Stasi et al., 2022)) is under development in eye drop formulation in patients with post-operative corneal pain (ClinicalTrials.gov Identifier: NCT04630158). These ongoing studies clearly show a drug development potential for TRPV1 antagonism.

In contrast to previous studies (Bashiri et al., 2018; Kim et al., 2020; You et al., 2012), our results did not show significant difference in memory and learning capability of young TRPV1-deficient mice versus the WTs under healthy conditions. These virtual contradictions can be attributed to differences in behavioral tests, experimental models and

designs (Iglesias et al., 2023; Kim et al., 2020; You et al., 2012), species (mice and rats (Bashiri et al., 2018)).

In conclusion, we demonstrated relatively selective *Trpv1* expression on glutamatergic neurons in the SuM of the mouse brain. TRPV1 is suggested to play a role in anxiety- and depression-like behavior without affecting spontaneous locomotor activity, memory and learning functions. Since TRPV1 blockade induces antidepressant-like effects, TRPV1 antagonists not influencing thermoregulation might provide perspectives for novel anti-depressant drug development besides their analgesic potential.

Contributors

Khai Huynh Ngoc: Conceptualization, Methodology, Experiment, Investigation, Formal analysis, Visualization, Writing – Original draft, Writing – review & editing. **Angéla Kecskés**: Conceptualization, Methodology, Experiment, Investigation, Supervision, Formal analysis, Visualization, Writing – review & editing. **Eszter Kepe**: Methodology, Experiment, Formal analysis. **Liza Nabi**: Methodology, Experiment, Formal analysis. **Julie Keeble**: Methodology, Experiment, Supervision, Investigation. **Éva Borbély**: Conceptualization, Methodology, Experiment, Investigation, Supervision, Formal analysis, Visualization, Writing – review & editing. **Zsuzsanna Helyes:** Conceptualization, Methodology, Investigation, Supervision, Formal analysis, Writing – review & editing, Resources, Project administration, Funding acquisition.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2023.04.017.

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