Peptidergic neurons of the Edinger–Westphal nucleus express TRPA1 ion channel that is downregulated both upon chronic variable mild stress in male mice and in humans who died by suicide

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Introduction

Transient receptor potential ankyrin 1 (TRPA1), a cation channel, is expressed predominantly in primary sensory neurons, but its central distribution and role in mood control are not well understood. We investigated whether TRPA1 is expressed in the urocortin 1 (UCN1)–immunoreactive centrally projecting Edinger–Westphal nucleus (EWcp), and we hypothesized that chronic variable mild stress (CVMS) would reduce its expression in mice. We anticipated that TRPA1 mRNA would be present in the human EWcp, and that it would be downregulated in people who died by suicide.

Methods: We exposed Trpa1 knockout and wild-type mice to CVMS or no-stress control conditions. We then performed behavioural tests for depression and anxiety, and we evaluated physical and endocrinological parameters of stress. We assessed EWcp Trpa1 and Ucn1 mRNA expression, as well as UCN1 peptide content, using RNAscope in situ hybridization and immunofluorescence. We tested human EWcp samples for TRPA1 using reverse transcription polymerase chain reaction. Results: Trpa1 mRNA was colocalized with EWcp/UCN1 neurons. Non-stressed Trpa1 knockout mice expressed higher levels of Ucn1 mRNA, had less body weight gain and showed greater immobility in the forced swim test than wild-type mice. CVMS downregulated EWcp/Trpa1 expression and increased immobility in the forced swim test only in wild-type mice. We confirmed that TRPA1 mRNA expression was downregulated in the human EWcp in people who died by suicide. Limitations: Developmental compensations and the global lack of TRPA1 may have influenced our findings. Because experimental data came from male brains only, we have no evidence for whether findings would be similar in female brains. Because a TRPA1-specific antibody is lacking, we have provided mRNA data only. Limited access to high-quality human tissues restricted sample size. Conclusion: TRPA1 in EWcp/UCN1 neurons might contribute to the regulation of depression-like behaviour and stress adaptation response in mice. In humans, TRPA1 might contribute to mood control via EWcp/UCN1 neurons.
TRPA1 ion channel is downregulated in chronic stress

We have studied the peptidergic, centrally projecting division of the Edinger–Westphal nucleus (EWcp).18–20 Urocortin 1 (UCN1) is a neuropeptide related to corticotropin-releasing hormone (CRH); recruitment of the UCN1-containing division of the EWcp in stress (mal)adaptation has been shown in mice,21,22 rats23–25 and nonhuman primates.26 It is noteworthy that depressed men who died by suicide showed EWcp/UCN1 expression that was 9 times higher than in controls.27 The importance of UCN1 in the stress adaptation response has been further corroborated in Ucn1 knockout mice.28 More recently, we have proven that EWcp/UCN1 neurons are recruited in chronic variable mild stress (CVMS) models of depression29 in rats30 and mice,31 and in complex models for mood disorders.32

Although the Allen Mouse Brain Atlas33 has indicated considerable Trpa1 mRNA in the Edinger–Westphal nucleus, it does not distinguish the centrally projecting division. We investigated the mRNA expression of Trpa1 in the EWcp and the role of TRPA1 in chronic stress adaptation using Trpa1 knockout mice and a CVMS model. We carried out behavioural tests for anxiety- and depression-like behaviour, and we tested physical and endocrine parameters to assess the stress adaptation response. Using RNAscope ISH combined with a semiquantitative immunofluorescence technique, we evaluated alterations in Trpa1 and Ucn1 mRNA expression, as well as UCN1 peptide content, in the EWcp as a result of CVMS in wild-type mice and mice lacking a functional TRPA1 receptor. To reveal translational relevance, we also tested TRPA1 mRNA expression in the human EWcp using reverse transcription polymerase chain reaction (RT-PCR). Finally, we compared EWcp/TRPA1 mRNA expression in controls and people who had died by suicide using the TaqMan assay.

Methods

Animals

Animals were housed in a temperature- and humidity-controlled environment with a 12-hour light-dark cycle (lights on at 6 am). They were kept in standard polycarbonate cages (365 mm × 207 mm × 144 mm) in groups of 4–6 mice per cage at the animal facility of the Department of Pharmacology and Pharmacotherapy at the University of Pécs. Mice were provided standard rodent chow and tap water ad libitum. All procedures were approved by the Animal Welfare Committee at Pécs University, National Scientific Ethical Committee on Animal Experimentation in Hungary (permission no: BA02/2000/33/2018), in agreement with the directive of the 1986 European Communities Council, and with the 1998 Law of XXCIII on Animal Care and Use in Hungary.

Trpa1 knockout mice were raised and characterized as described previously.34 For this experiment, Trpa1 knockout mice were bred on a C57BL/6j background as described earlier.31

Experimental design

To assess Trpa1 mRNA and UCN1 peptide expression in the EWcp, we used intact 12-week-old male Trpa1 wild-type mice (n = 6).

In another experiment, we used 12-week-old male Trpa1 knockout mice (n = 30) and their wild-type counterparts (n = 30) and the CVMS model. We assigned the mice to 4 experimental groups: Trpa1 knockout mice (n = 16) and wild-type mice (n = 16) were exposed to CVMS for 21 days, and another set of Trpa1 knockout (n = 14) and wild-type (n = 14) mice were not exposed to CVMS (non-stressed controls). Control mice were evaluated using a behavioural test battery in the first week. Then they were left undisturbed for the last 2 weeks before perfusion to avoid bias from the stress effect of behavioural testing (Figure 1).

CVMS paradigm

For 2 weeks before the CVMS experiments, the mice were handled twice a day. The 3-week CVMS paradigm was applied as published earlier.31 Briefly, the paradigm consisted of midday stressors (tilted cage, shaker stress, restraint stress, dark room exposure) and overnight stressors (social isolation, wet bedding, group holding). After the midday stress exposure, animals were placed back in their home cages. We measured each mouse’s body weight twice per week. We performed behavioural tests in the CVMS group in the final week and considered these to be midday stressors (Figure 1).

Behavioural test battery

In the marble burying test, we counted the number of marbles hidden in 30 minutes, a measure that is proportional to the animal’s anxiety state.34 In the open field test, we assessed locomotor activity (duration of locomotion and travelled distance) and anxiety (time spent in the periphery zone), as described earlier.22 We analyzed video recordings using SMART Junior tracking software (Panlab).

We used the sucrose preference test to measure anhedonia.35 We registered water and consumption of sucrose solution and calculated sucrose preference as follows: [consumption of sucrose solution/(consumption of water + consumption of sucrose solution)] × 100.

In the tail suspension test36 and the forced swim test,31 we measured the duration of immobility, which is directly proportional to depression-like behaviour; the longer the mouse stays immobile in the test, the higher the depression level.

Perfusion and tissue collection

On day 23 (24 hours after any manipulation), 30 mice were killed with urethane (intraperitoneal, 2.4 g/kg). All mice in the same cage became unconscious within 2 minutes. Then they were weighed and their tails were clipped. We collected blood (500 µL) by cardiac puncture into a syringe with 50 µL of 7.5% wt/wt EDTA solution (Sigma). After centrifugation, we collected plasma samples to determine
adrenocorticotropic hormone (ACTH) and corticosterone by radioimmunoassay.

The mice were then perfused transcardially with 20 mL of ice-cold 0.1 M phosphate-buffered saline (pH 7.4), followed by 150 mL of 4% paraformaldehyde solution in Millonig buffer (pH 7.4) for 15 minutes.

The adrenal glands and thymuses were removed and weighed using a Sartorius analytic scale (Sartorius AG). Data were corrected for body weight.

The brains were dissected and post-fixed in the same fixative for 72 hours at 4°C. We collected 4 series of 30 µm sections (Leica VT1000S vibratome; Leica Biosystems) and stored them in phosphate-buffered saline containing 0.01% sodium azide at 4°C. We selected 4 representative sections of the EWcp for each animal (Bregma −2.92 mm to −4.04 mm) for RNAscope.

We collected the trigeminal ganglia from the remaining 30 mice after quick cervical dislocation and decapitation. The trigeminal ganglia were snap-frozen on dry ice and stored at −80°C for later RNA extraction.

RNAscope ISH combined with immunohistochemistry

The RNAscope ISH pretreatment procedure was optimized for 30 µm paraformaldehyde-fixed sections, as previously published. We performed the additional steps of the RNAscope protocol based on the manufacturer’s instructions for the RNAscope Multiplex Fluorescent Reagent Kit (version 2; ACD).

In the intact mice, we used a mouse Trpa1 probe (cat. no. 400211; ACD), which was visualized by cyanine 3 dye (1:750). After channel development, slides were subjected to immunofluorescence using polyclonal rabbit anti-UCN1 antisera (1:20 000; RRID AB 2315527) for 24 hours at 24°C. After washes, we used Alexa 647-conjugated donkey anti-rabbit serum (1:500; RRID AB 2492288; cat. no. 711-605-152; Jackson Immunoresearch Europe Ltd) for 3 hours.

In the CVMS model, we performed Trpa1 mRNA labeling combined with UCN1 immunohistochemistry as described above. We performed another RNAscope for Ucn1 (cat. no. 466261; visualized with fluorescein 1:3000; ACD) on another series of sections.

We counterstained sections with 4’,6-diamidino-2-phenylindole (DAPI; ACD) and covered them with ProLong Gold Antifade mounting medium (Thermo Fisher Scientific).

The specificity of the widely trusted rabbit UCN1 antibody (RRID: AB 2315527) has been tested previously in mice. Omitting or replacing primary and secondary antibodies with nonimmune sera abolished labeling in both wild-type and knockout mice (images not shown).

We tested mouse 3-plex positive (Polr2a mRNA [fluorescein], Ppib mRNA [cyanine 3] and Ubc mRNA [cyanine 5]; cat. no. 320881; ACD) and 3-plex negative control probes to bacterial dabP mRNA (cat. no. 320871; ACD) on the EWcp (Appendix 1, Figure S1, available at www.jpn.ca/lookup/doi/10.1503/jpn.210187/tab-related-content).

Figure 1: Timeline of animal experiments. Behavioural tests (MBT, OFT, SPT, TST, FST) and CVMS protocol with midday (SHAKE, REST, TILT, DARK, WD) and overnight (WET, SI, GH) stressors. CVMS = chronic variable mild stress; DARK = dark room exposure; FST = forced swim test; GH = group holding; MBT = marble burying test; OFT = open field test; REST = restraint stress; SHAKE = shaker stress; SI = social isolation; SPT = sucrose preference test; TILT = tilted cage; TST = tail suspension test; WD = water deprivation; WET = wet bedding.
**Microscopy, digital imaging and morphometry**

We obtained digital images using a FluoView 1000 confocal microscope (Olympus) and sequential scanning. We used an 80 µm confocal aperture (optical thickness 3.5 µm) with a resolution of 1024 x 1024 pixels and a 40x objective for scanning. We selected the excitation and emission spectra for the respective fluorophores using the built-in settings of the FluoView software (FV10-ASW, version 0102; Olympus). DAPI was excited at 405 nm, fluorescein at 488 nm, cyanine 3 at 550 nm, and cyanine 5 and Alexa 647 at 650 nm.

We scanned sections for their respective wavelengths at 4 channels. We assessed colocalization on digital images that showed virtual blue (DAPI), green (fluorescein), red (cyanine 3) and white (cyanine 5, Alexa 647), representing the fluorescent signals of the 4 channels.

Ub1 mRNA showed a confluent or cluster-like pattern. Because counting of individual fluorescent dots was not possible, we measured Ub1 mRNA fluorescence in cell bodies with Image J software (version 1.42; US National Institutes of Health). We manually determined the region of interest at cytoplasmic areas of the neurons. We corrected the signal density for the background signal quantified outside (but next to) the perikarya; the specific signal density was expressed in arbitrary units. We determined the average of the specific signal densities of 10 neurons from 4 nonedited images, and the average of these 4 values represented the specific signal density value for 1 mouse. We quantified Ub1 peptide specific signal density the same way.

The Trpa1 mRNA signal appeared as distinguishable scattered fluorescent dots. We counted the number of puncta in 10 neurons, in 4 sections per animal and averaged these values as described above.

**Radioimmunoassay for ACTH and corticosterone**

We measured plasma ACTH and corticosterone titres by radioimmunoassay as described previously. The intra-assay coefficients of variation were 4.7% and 6.2%, respectively.

**Presence of TRPA1 mRNA in human EWcp**

**Postmortem human brain tissue samples**

Tissue samples of mesencephalic ventral periaqueductal grey matter containing the EWcp were microdissected within 1 hour after the person’s death (ethical approval numbers: ETT TUKEB 5912–2/2018/EKU; 55699–2/2017/EKU; 2446–2/2016/EKU). Samples were snap-frozen and stored at −80°C in the Human Brain Tissue bank, Semmelweis University, Budapest, Hungary, until further use. Those who provided the samples studied here (n = 3, Table 1) died suddenly from extracranial disease and did not show any brain neuropathologies.

**RT-PCR**

Brain and mouse trigeminal ganglia samples were quickly homogenized using disposable nuclease-free pestle homogenizers (Merck) in TRI Reagent (Zymo Research). Total RNA was extracted using a Direct-zol RNA Miniprep kit (Zymo Research). The RNA samples were treated with DNase I (Zymo Research) on a column, according to the manufacturer’s instructions. We assessed the concentration and purity of the total RNA with an ND-1000 Spectrophotometer V3.5 (NanoDrop Technologies, Inc.). Samples were stored at −80°C.

We reverse-transcribed 1 µg RNA using a Maxima First Strand CDNA Synthesis Kit (Thermo). We used the Applied Biosystems QuantStudio 5 real-time PCR system (Thermo) with the SensiFast SYBR Lo-ROX Kit (Bioline) according to the manufacturer’s instructions. The gene of interest was Trpa1. The reference gene for the human samples was DNA-directed RNA polymerase II subunit RPBI (POLR2A); the reference genes for the mouse trigeminal ganglia samples were β-actin (Actb) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh); Table 2). We measured Ucn1 gene expression to validate that the tissue punch contained the EWcp. For a positive control, we used cDNA from a TRPA1 mRNA–expressing human oral squamous cell carcinoma cell line (PECA: PE/CA-PJ41 [clone D2]; cat. no. 98020207–1VL; Sigma-Aldrich), which was extracted and transcribed as described for the human EWcp samples.

We performed RT-PCR experiments in technical replicates and included a melt curve analysis to ensure specificity. Reverse transcription minus control showed a lack of genomic DNA contamination. The PCR products were identified using agarose gel electrophoresis with the GeneRuler Low Range DNA Ladder (Thermo). Statistical comparison of the averaged Actb and Gapdh cycle threshold values revealed that expression of the housekeeping genes was not affected by CVMS exposure (control: 16.33 ± 0.155; CVMS: 16.50 ± 0.093, H0; p = 0.35).

**Comparison of EWcp/TRPA1 mRNA expression in human controls and people who died by suicide**

For this experiment, we obtained more samples from the Human Brain Tissue bank: 3 from controls and 3 from males who had died by suicide (Table 3).

**TaqMan quantitative RT-PCR**

We determined relative gene expression ratios of human TRPA1 using the Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo) and SensiFast Probe Lo-ROX mix (Meridian Bioscience) according to the manufacturer’s instructions. The gene of interest was Trpa1 and the reference gene was ribonuclease P protein subunit p29 (POP4). We used FAM conjugated TaqMan Gene Expression Assays (Thermo) to amplify the target loci (TRPA1: Hs00175798_m1; POP4: Hs00198357_m1).

**Table 1: Characteristics of humans from whom brain samples were taken**

| Case | Sex | Age (yr) | RIN | Cause of death |
|------|-----|----------|-----|----------------|
| 1    | Male | 55       | 6.6 | Acute myocardial infarction |
| 2    | Male | 74       | 6.1 | Acute heart failure |
| 3    | Male | 65       | 6.2 | Acute heart failure |

RIN = RNA integrity number.
We determined the means of the cycle threshold values of 3 parallel measurements and calculated gene expression using $\Delta\Delta^Ct$ method.\(^{41}\) POP4 cycle threshold values in control samples (25.59 ± 0.439) did not differ from those obtained from people who died by suicide (25.44 ± 0.187; $t_{4} = 0.321$, $p = 0.763$). For a positive control, we used cDNA from a Chinese hamster ovary cell line overexpressing human TRPA1 mRNA.\(^{42}\) We performed mRNA extraction and cDNA transcription as described for human samples. Reverse transcription minus control showed a lack of genomic DNA contamination.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean for each experimental group. We tested the data sets for normality\(^{43}\) and homogeneity of variance.\(^{44}\) Outlier data beyond the 2 $\sigma$ range were excluded. We evaluated data by 2-way analysis of variance (ANOVA). We performed Fisher post hoc tests based on first- or second-order effects in the ANOVA. We used a Student $t$ test for independent samples to compare the Trpa1 receptor mRNA expression of CVMS-exposed versus control mice, and to compare tissue samples from human controls and people who died by suicide. We conducted analyses using Statistica 8.0 (StatSoft; $\alpha = 5\%$).

**Results**

Trpa1 mRNA expression was localized to EWcp/UCN1 neurons in mice

Nearly all UCN1-immunoreactive cells coexpressed Trpa1 transcripts in the EWcp, but we detected no remarkable Trpa1 mRNA in other neurons (i.e., neurons that were not UCN1-immunoreactive) of this brain area (Figure 2).

TRPA1 receptors might contribute to modulation of mood and stress adaptation response via EWcp/UCN1 neurons

Findings for the modulation of mood and stress adaptation response are summarized in Table 4.

**Behavioural tests**

In the marble burying test, CVMS exposure increased the number of marbles hidden in both wild-type and Trpa1 knockout mice (main stress effect). Trpa1 knockout mice buried fewer marbles than wild-type mice (main genotype effect) without influencing the effect of CVMS (no interaction; Figure 3A).

In the open field test, the peripheral locomotor activity of Trpa1 knockout mice was lower than that of wild-type mice ($p < 0.001$; Figure 3B). In contrast, after CVMS exposure,
Trpa1 knockout mice moved more in the periphery than wild-type mice ($p = 0.008$). Time spent on the periphery was the main anxiety parameter of the open field test, and CVMS had different effects in the 2 genotypes (interaction). Control Trpa1 knockout mice spent less time next to the walls of the device than wild-type mice ($p < 0.001$; Figure 3C); interestingly, CVMS exposure increased anxiety levels in Trpa1 knockout mice only ($p < 0.001$).

CVMS exposure affected preference for sweetened water ($F_{1,56} = 15.7, p < 0.001$): sucrose preference was reduced in stressed wild-type ($p = 0.01$) and Trpa1 knockout ($p = 0.01$) mice, without any genotype effect or interaction (Figure 3D).

In the tail suspension test, only CVMS exposure affected immobility time ($F_{1,56} = 15.74, p < 0.001$; Figure 3E), with a significant increase both in wild-type mice ($p = 0.003$) and Trpa1 knockout mice ($p = 0.014$).
In the forced swim test, CVMS increased immobility time (the main effect of stress) differently in the 2 genotypes (interaction): non-stressed Trpa1 knockout mice had higher immobility scores than their wild-type counterparts ($p = 0.02$). This augmented depression-like behaviour was not altered by CVMS exposure in Trpa1 knockout mice ($p = 0.82$); however, in wild-type mice CVMS exposure dramatically increased such behaviour ($p < 0.001$; Figure 3F).

**Endocrine and physical parameters**

ANOVA revealed that CVMS influenced ACTH levels differently in the 2 genotypes (interaction; $F_{1,26} = 11.63$, $p = 0.004$; Figure 4A). Post hoc tests showed that CVMS effectively increased ACTH in wild-type mice ($p = 0.001$). The baseline ACTH level in Trpa1 knockout mice was markedly higher than that in wild-type mice ($p = 0.015$), and CVMS exposure failed to induce any further increase ($p = 0.26$).
CVMS significantly enhanced corticosterone concentrations (main effect of stress; $F_{1,26} = 5.00, p = 0.03$) without a difference in genotypes (Figure 4B). Accordingly, relative adrenal weight was higher after exposure to CVMS (main effect of stress; Figure 4C). However, unlike corticosterone, the effect of CVMS on adrenal weight was different in wild-type and Trpa1 knockout mice (interaction): adrenal weight in stressed Trpa1 knockout mice was significantly higher ($p = 0.004$) than in stressed wild-type mice.

CVMS exposure significantly reduced thymus weight (main effect of stress; Figure 4D). However, this effect was significant in wild-type mice only (i.e., interaction). In contrast,
the relative thymus weight remained constant in stressed Trpa1 knockout mice (p = 0.48).

An assessment of body weight gain revealed that CVMS exposure stopped weight gain both in wild-type mice (p < 0.001) and Trpa1 knockout mice (p < 0.001; main effect of stress; Figure 4E). Trpa1 knockout mice gained significantly less weight than their wild-type counterparts (main effect of genotype). A comparison of body weight data across experimental groups before CVMS exposure revealed no statistical differences (wild-type control: 25.51 g ± 0.75 g; Trpa1 knockout control: 25.59 g ± 0.71 g; wild-type CVMS: 26.21 g ± 0.44 g; Trpa1 knockout CVMS: 24.43 g ± 0.35 g; genotype: F1,56 = 2.02, p = 0.16; stress: F1,56 = 0.17 p = 0.68; interaction: genotype F1,56 = 2.458 p = 0.12). Comparison of body weight data after CVMS exposure (Figure 4F) revealed a significant main effect of stress and genotype, but not their interaction (Table 2). Post hoc tests confirmed that CVMS-exposed Trpa1 knockout mice had lower body weights than control Trpa1 knockout mice (p = 0.013), but in wild-type mice this difference appeared to be a tendency (p = 0.1). Absolute body weight in stressed wild-type and Trpa1 knockout mice did not differ significantly (p = 0.12).

**CVMS influences Trpa1 and UCN1 expression**

To assess how CVMS affected the number of Trpa1 transcripts in EWcp/UCN1 cells, we performed RNAscope ISH for Trpa1 and anti-UCN1 immunohistochemistry in wild-type mice only. We further confirmed the presence of Trpa1 mRNA in UCN1-positive neurons. Semi-quantitation revealed that CVMS exposure reduced the number of Trpa1 transcripts in UCN1 neurons by 40% (t9 = 5.09; p = 0.003; Figure 5A to C). We assessed the cells’ Ucn1 mRNA content using RNAscope ISH and UCN1 peptide content with immunohistochemistry in both genotypes. In control Trpa1 knockout mice, we observed elevated Ucn1 mRNA content compared to control wild-type mice (Figure 5D to H). With CVMS, the Ucn1 mRNA content in wild-type mice showed a strong tendency to increase (p = 0.059), but we observed no considerable change in Ucn1 mRNA specific signal density in Trpa1 knockout mice (p = 0.31). At the peptide level, genotype modified the effect of stress (i.e., interaction). Post hoc comparisons revealed that with CVMS exposure, the EWcp of Trpa1 knockout mice stored more UCN1 peptide than the EWcp of control Trpa1 knockout mice (p = 0.047; Figure 5I to M).

To test whether CVMS had a global influence on Trpa1 expression, we examined trigeminal ganglion samples using quantitative RT-qPCR (RT-qPCR). We detected no difference between the control and CVMS-exposed groups (1.0323 ± 0.106 v. 1.019 ± 0.043; t1 = 0.111, p = 0.91).

**TRPA1 mRNA is expressed in the human EWcp**

We detected TRPA1 and the housekeeping gene POLR2A in all 3 human EWcp samples. The presence of UCN1 transcripts confirmed that all samples contained the EWcp area (Figure 6).

**EWcp/TRPA1 mRNA is downregulated in people who died by suicide**

In the EWcp samples from 3 controls and 3 people who died by suicide, we detected both TRPA1 and the housekeeping gene POP4. Compared to controls, TRPA1 mRNA was significantly downregulated in those who had died by suicide. (t5 = 2.88; p = 0.044; Figure 7).

**Discussion**

We provide, to our knowledge, the first evidence for Trpa1 mRNA expression in EWcp/UCN1 neurons in both mice and humans. The finding that nearly all mouse EWcp/UCN1 cells contained Trpa1 mRNA transcripts without detectable Trpa1 mRNA expression outside the UCN1 cells strongly suggests that TRPA1 may have a specific regulatory function in this cell type. Taking into account the fact that we used a global knockout strain in this study, we cannot exclude the possibility that the loss of the functional TRPA1 receptor outside the EWcp may have contributed to the observed alterations in behaviour. Nevertheless, our finding that Trpa1 expression was not altered in the trigeminal ganglia of mice exposed to CVMS suggests that Trpa1 is differentially regulated in the EWcp. Considering the importance of EWcp/UCN1 neurons in stress regulation23,24,31 and stress-related psychopathologies,24 a similar role for TRPA1 receptors could also be assumed. Indeed, in a well-known animal model of depression,29,32,45 as well as in humans who died by suicide, lower expression of Trpa1 or TRPA1 was detected in the EWcp.

The lack of TRPA1 in (control) knockout mice resulted in Ucn1 upregulation, together with less body weight gain, higher resting ACTH levels and a depressive phenotype in the forced swim test, but not in the tail suspension test or the sucrose preference test. Increased Ucn1 in association with and unaltered EWcp/UCN1 peptide content may suggest increased UCN1 release. UCN1 has been shown to have an anorexigenic effect.46 This may explain the reduced body weight gain we found in non-stressed Trpa1 knockout mice, and their increased depression-like phenotype might be explained by increased UCN1 and CRH1 receptor signalling via the dorsal raphe nucleus.47–49

The fact that we detected no basal genotype-related difference in the tail suspension test further supports the concept that these tests do not give consistent results in all cases,9 or that effects might be test-specific. Moreover, Trpa1 knockout mice were less (not more) anxious in both tests used (open field test, marble burying test). Although anxiety might be a depression-related symptom, in some cases anxiety and depression are differentially regulated by gene mutations (as we have shown in PACAP knockout mice32), or anxiety might have an adaptive role (i.e., in mothers during the postpartum period).39 In this sense, the reduced anxiety we found in Trpa1 knockout mice might have been part of their maladaptive repertoire. Nevertheless, enhanced UCN1 tone in the knockout mice may have contributed to their reduced anxiety levels, probably via increased UCN1/CRH2 receptor signalling.39,52 An additional interpretation of our open field test findings could be that Trpa1 knockout mice were more...
sensitive to stress in this test. This was possible because EWcp/UCN1 neurons project to the extended amygdala, and infusion of UCN1 into the amygdala increases anxiety via CRH receptors. This possibility should be confirmed with further experimentation. It is possible that stress exposure results in increased UCN1 release that contributes to higher anxiety levels in stressed Trpa1 knockout mice.

We used the CVMS model because it is widely used and well characterized in mice, and because EWcp/UCN1 neurons are recruited in this model. CVMS exposure induced typical somatic, hormonal (ACTH and corticosterone) and behavioural (anxiety-, anhedonia- and depression-like) changes as signs of increased activity in the hypothalamic-pituitary-adrenal (HPA) axis.

Figure 5: Trpa1, Ucn1 mRNA and UCN1 peptide expression in EWcp neurons upon CVMS. (A and B) Trpa1 mRNA (red) was downregulated in wild-type mice exposed to CVMS, as also shown in (C) the histogram. (A' and B') Trpa1 mRNA transcripts (red) were localized to cells containing UCN1 peptide (white). (D to H) Ucn1 mRNA (green) was expressed at higher levels in control Trpa1 knockout mice (black bars). CVMS increased Ucn1 mRNA expression in wild-type mice only (grey bars). (I to M) UCN1 peptide content (white) of EWcp neurons. The UCN1 peptide SSD was elevated in Trpa1 knockout mice upon exposure to CVMS. DAPI (blue) labelling was performed to mark the nuclei of cells. *p < 0.05, Fisher post hoc test following 2-way analysis of variance. CVMS = chronic variable mild stress; DAPI = 4',6-diamidino-2-phenylindole; EWcp = centrally projecting division of the Edinger–Westphal nucleus; SSD = specific signal density; UCN1 = urocortin 1.
The stress of anesthetic injection before perfusion may have caused acute ACTH and corticosterone levels. Nevertheless, our findings of increased adrenal weight, reduced thymus size and lack of body weight gain strongly suggest that the HPA axis was constitutively active for a longer period, confirming the reliability of our CVMS model. The higher relative adrenal weight of stressed \textit{Trpa1} knockout mice compared to wild-type mice may reflect a remarkable long-term increase in activity in the HPA axis. However, the immunosuppressive effect of glucocorticoids should be associated with reduced thymus weight. We did not find \textit{Trpa1}-related alterations in thymus weight, which could be explained by altered differentiation of lymphocytes, or by disturbed HPA axis regulation in \textit{Trpa1} knockout mice. Further studies are needed to determine whether \textit{Trpa1} modulates HPA axis response at the level of the anterior pituitary or adrenal cortex to explain the discrepancy between basal ACTH and corticosterone levels.

Knocking out \textit{Trpa1} prevented the development of a CVMS-induced depressive-like phenotype in the forced swim test, but it aggravated anxiety (open field test), along with exaggerated adrenal enlargement and elevated levels of EWcp/UCN1 peptide. The observed changes might be attributable to increased basal depression and reduced resting anxiety levels in \textit{Trpa1} knockout mice (i.e., the depressive-like phenotype prevented further increase in depressive parameters, and the original less anxious phenotype left more space for a greater increase in anxiety). The CVMS-induced up-regulation of \textit{Ucn1} expression in wild-type mice but not in \textit{Trpa1} knockout mice might also underline the observed behavioural phenotype. The lack of functional \textit{Trpa1} might result in a lower number of cation channels in the cell membrane of UCN1 neurons, leading to diminished depolarization ability in the cells. Thus, CVMS-induced EWcp/UCN1 activation might be limited.

The elevated UCN1 peptide content associated with unaltered \textit{Ucn1} mRNA expression in stressed \textit{Trpa1} knockout mice may also suggest reduced peptide production and release or enhanced storage. This may lead to downregulated UCN1/CRHR2 signalling and increased activity in the HPA axis, supported by our finding of increased relative weight of the adrenal glands of stressed \textit{Trpa1} knockout mice. Because the instability of the neuropeptide content of a cell depends on the ratio of production, breakdown and release, the intensity of the immunosignal does not necessarily correlate with the secretory activity of the cell. Increased rate of production, transport and release might be...
associated with a reduced immunosignal, and strong label-ling might refer to inhibited cellular function.24 Measurements at the mRNA level provide further evidence for tran-scriptional activity, but considering our lack of information on the rate of translation and peptide release, further electrophysiological and microdialysis experiments are required to determine how the EWcp/UCN1 neurons contribute to mood control.

EWcp/UCN1 mRNA upregulation in depression-related suicide in humans has been demonstrated previously.27 To prove the translational relevance of our mouse results, we also measured TRPA1 mRNA using RT-qPCR in human EWcp obtained from frozen human brain samples. Because of a lack of cryoprotection, we could not perform RNAscope ISH, and in our recent study lipofuscin made it difficult to interpret fluorescence signals.29 We found abundant UCN1 mRNA expression in these tissue punches, together with TRPA1 mRNA, similar to our findings for the mouse EWcp. In line with our findings in CVMS-exposed mice, TRPA1 mRNA was also downregulated in people who died by suicide. This observation suggests that TRPA1 signalling in the human EWcp may affect mood status.

Taking the excitatory effect of this ion channel into consideration, we assume that reduced TRPA1 mRNA expression and presumably fewer cation channels in the membrane may lead to reduced excitability of UCN1-expressing neurons, contributing to maladaptive processes in depression. Further experiments using pharmacological tools and electrophysiological recordings are needed to determine how TRPA1 signalling contributes to maladaptation via UCN1 neurons. Given the relatively restricted and low expression of TRPA1 in the brain,60,61 we propose that its pharmacological modulation will act mainly on the EWcp, with valuable therapeutic significance and limited side effects in the central nervous system.

Limitations

Possible developmental compensations may have contributed to the behavioural phenotype of our global knockout mouse strain. Because the functional receptor was deleted both in the periphery and in some brain areas where limited60,61 neuronal Trpa1 expression is also found, we could not exclude the possibility that other peripheral or central mechanisms that we did not examine here may have contributed to the behavioural phenotype of knockout mice. Because no reliable TRPA1 receptor antibody is available, we are unable to present protein-level data. We did not examine female tissue samples in this study. Because EWcp/UCN1 neurons express estrogen receptor β62 results in female mice could have been influenced by the estrus cycle phase. Future studies need to examine whether our findings in males are also applicable to females. The limited availability of brain samples from neuropathology-free controls and people who died by suicide (with short post mortem delay) explains the relatively low sample size in our human studies.

Conclusion

In this study we identified EWcp/UCN1 neurons as important Trpa1 receptor mRNA expression sites in the mouse brain. We proved the regulatory role of the TRPA1 receptor in the stress adaptation of urocortinergic neurons using a CVMS model in mice. The presence of TRPA1 mRNA expression in human EWcp, which was downregulated in brain samples from men who died by suicide, supports the translational value of these findings. Further research is needed to clarify the exact physiologic role of TRPA1 in the EWcp and bring to light its therapeutic potential in the management of mood disorders.

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