Research Paper

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

Viktória Kormos, MD, PhD; Angéla Kecskés, MSc, PhD; József Farkas, MD, PhD; Tamás Gaszner, MD; Valér Csernus, MD, PhD, DSc; Ammar Alomari, PharmD; Dániel Hegedüs, BSc; Éva Renner, MSc, PhD; Miklós Palkovits, MD, PhD, DSc; Dóra Zelena, MD, PhD, DSc; Zsuzsanna Helyes, MD, PhD, DSc; Erika Pintér, MD, PhD, DSc; Balázs Gaszner, MD, PhD

Background: 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.

Introduction

Transient receptor potential ankyrin 1 (TRPA1), a nonselective cation channel, is expressed mainly by nociceptive primary sensory neurons of the dorsal root and trigeminal ganglia.[1] It is activated by painful cold,[2] heat and mechanical stimuli[3] and reactive electrophilic ligands (e.g., free radicals, fatty acids), and it is released during inflammatory and degenerative processes.[4,5] Upon activation, calcium influx triggers several intracellular pathways.[6] The role of TRPA1 in nociception and inflammatory responses has been well established.[7,8]

Trpa1 knockout mice have shown reduced response to specific acute and chronic TRPA1-mediated nociceptive stimuli[2,9–13] attributed to functional changes in the peripheral sensory nervous system.[14] However, little is known about the potential role of TRPA1 receptors in long-term complex central adaptation responses such as adaptation to chronic stress.

The data are contradictory with respect to the central nervous system distribution of TRPA1.[15,16] In the in situ hybridization (ISH) database of the Allen Mouse Brain Atlas,[17] we found relatively high levels of *Trpa1* expression next to the

Correspondence to: V. Kormos, Department of Pharmacology and Pharmacotherapy, and B. Gaszner, Department of Anatomy, University of Pécs, Szigeti u. 12, H-7624, Pécs, Hungary; viktoria.kormos@aok.pte.hu, balazs.b.gaszner@aok.pte.hu

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midsagittal plane, in the Edinger–Westphal nucleus (http://mouse.brain-map.org/gene/show/93201).

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] rats[23–25] 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 depression[29] in rats[30] and mice,[31] and in complex models for mood disorders.[32]

Although the Allen Mouse Brain Atlas[17] 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.[33] 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 test[36] 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

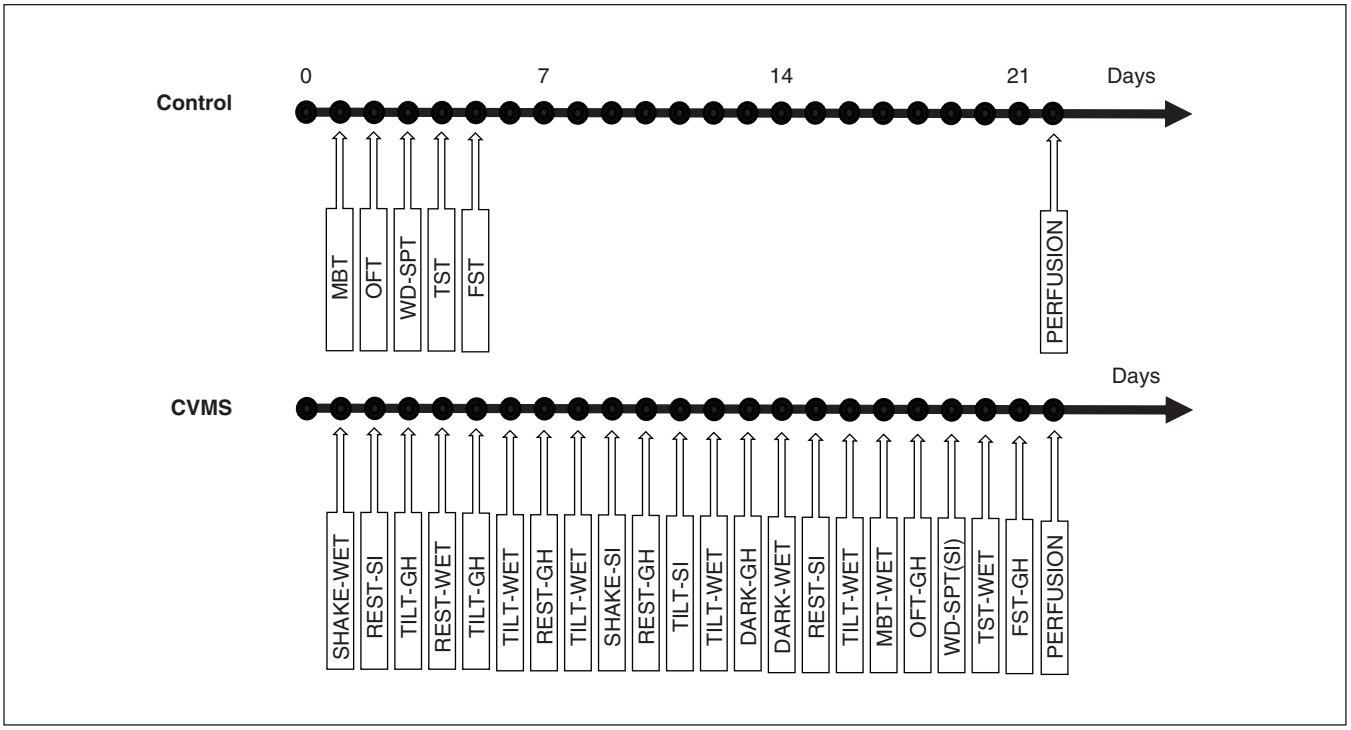

Figure 1: Timeline of animal experiments. Behavioural tests (MBT, OFT, SPT, TST, FST) and CVMS protocol with midday (SHAKE, REST, TILT, DARK, WD) and overnight (WET, SI, GH) stressors. CVMS = chronic variable mild stress; DARK = dark room exposure; FST = forced swim test; GH = group holding; MBT = marble 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.

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[37]) 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[38] pretreatment procedure was optimized for 30 µm paraformaldehyde-fixed sections, as previously published.[39] 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 antiserum (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 labelling 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.[31] Omitting or replacing primary and secondary antibodies with nonimmune sera abolished labelling 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).

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 × 1024 pixels and a 40× 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.

Ucn1 mRNA showed a confluent or cluster-like pattern. Because counting of individual fluorescent dots was not possible, we measured *Ucn1* 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 UCN1 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[40] and corticosterone titres[23,24,31] 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.

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.

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-transcripted 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 RPB1 (*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: 16.332 ± 0.155; CVMS: 16.503 ± 0.093; $t_{13} = -0.968$, $p = 0.35$).

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 2: Primers used to amplify target loci for RT-PCR

Gene amplified	Nucleotide sequence of primer	Primer type	Product length (bp)	NCBI reference sequence
Hs *TRPA1*	TCCTCTCCATCTGGCAGCAAAG	Forward	115	NM_007332.3
	GGACGCATGATGCAAAGCTGTC	Reverse		
Hs *POLR2A*	GAGAGCGTTGAGTTCCAGAACC	Forward	152	NM_000937.5
	TGGATGTGTGCGTTGCTCAGCA	Reverse		
Hs *UCN1*	CTTCTCTGTCCATTGACCTCACC	Forward	123	NM_003353.4
	ATCACTTGCCCACCGAGTCGAA	Reverse		
Mm *Trpa1*	ATCCAAATAGACCCAGGCACG	Forward	101	NM_177781.5
	CAAGCATGTGTCAATGTTTGGTACT	Reverse		
Mm *Actb*	CTGTATGCCTCTGGTCGTAC	Forward	214	NM_007393.5
	TGATGTCACGCACGATTTCC	Reverse		
Mm *Gapdh*	TTCACCACCATGGAGAAGGC	Forward	237	NM_008084.3
	GGCATGGACTGTGGTCATGA	Reverse		

Hs = *Homo sapiens*; Mm = *Mus musculus*; NCBI = National Center for Biotechnology Information; RT-PCR = reverse transcription polymerase chain reaction.

Table 3: Characteristics of human controls and people who died by suicide from whom brain samples were taken

Case	Sex	Age (yr)	RIN	Cause of death	Post mortem time (h)
1	Male	55	6.7	Acute myocardial infarction	1
2	Male	37	6.1	Accidental electric shock	8
3	Male	81	6.0	Acute heart failure	5
4	Male	31	6.2	Suicide	8
5	Male	41	6.5	Suicide	4
6	Male	49	6.1	Suicide	4.5

RIN = RNA integrity number.

We determined the means of the cycle threshold values of 3 parallel measurements and calculated gene expression using ΔΔCt 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 σ 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; α = 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,

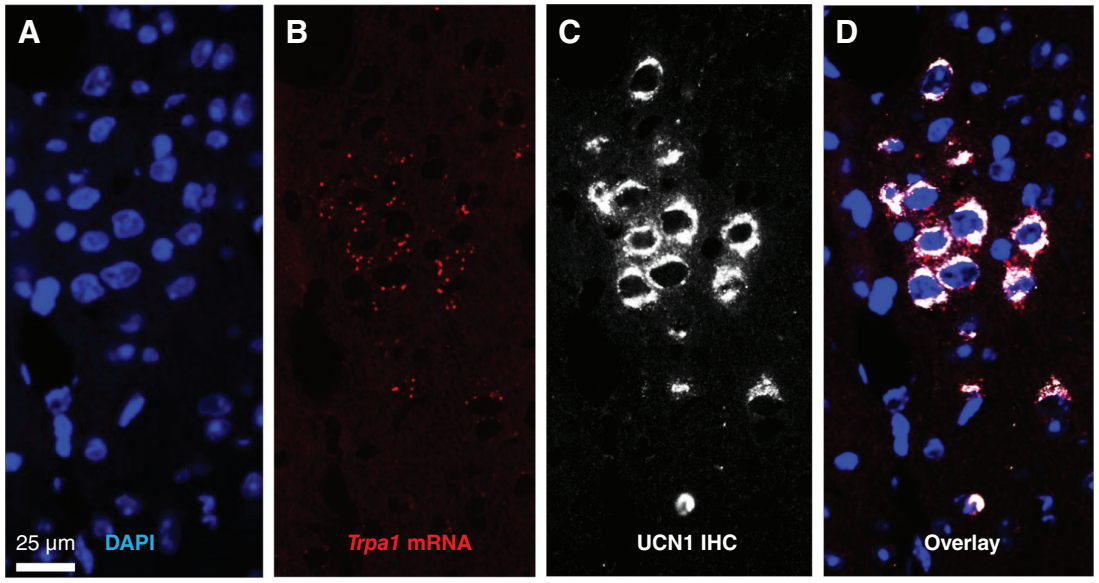

Figure 2: Representative images of *Trpa1* mRNA–expressing urocortinergic cells from the mouse Edinger–Westphal nucleus. (A) Sections were counterstained with DAPI (blue) for nuclei. (B) *Trpa1* mRNA (red) was visualized by RNAscope. (C) The UCN1 peptide (white) was detected by immunofluorescence (IHC). (D) Note the colocalization of *Trpa1* mRNA and UCN1 peptide. DAPI = 4',6-diamidino-2-phenylindole; IHC = immunohistochemistry; UCN1 = urocortin 1.

Table 4: Summary of the statistical assessment of data by analysis of variance

	CVMS		Genotype		CVMS × genotype	
Variable	$F_{1,56}$	*p* value	$F_{1,56}$	*p* value	$F_{1,56}$	*p* value
Marble burying test, marbles buried	178.9	< 0.001	4.81	0.03	0.04	0.82
Open field test, periphery distance	14.81	< 0.001	1.20	0.27	23.29	< 0.001
Open field test, periphery time	10.52	0.002	42.31	< 0.001	33.18	< 0.001
Sucrose preference test, sucrose preference	15.70	< 0.001	0.01	0.97	0.03	0.87
Tail suspension test, immobility	15.74	< 0.001	3.01	0.08	0.16	0.68
Forced swim test, immobility	15.23	< 0.001	0.66	0.42	7.4	0.01
Plasma ACTH	2.75	0.11	0.23	0.63	11.63	0.004
Plasma corticosterone	5.00	0.03	0.09	0.92	0.01	0.90
Relative adrenal weight	113.84	< 0.001	9.13	0.03	4.46	0.039
Relative thymus weight	7.31	0.009	24.59	< 0.001	15.79	0.002
Change in body weight	100.85	< 0.001	4.71	0.03	3.72	0.06
Body weight	11.68	0.001	4.53	0.037	1.003	0.32
Ucn1 mRNA	5.30	0.02	4.5	0.04	0.43	0.51
UCN1 peptide	0.94	0.34	0.45	0.50	5.22	0.03

ACTH = adrenocorticotropic hormone; CVMS = chronic variable mild stress; UCN1 = urocortin 1.

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 ($p = 0.014$).

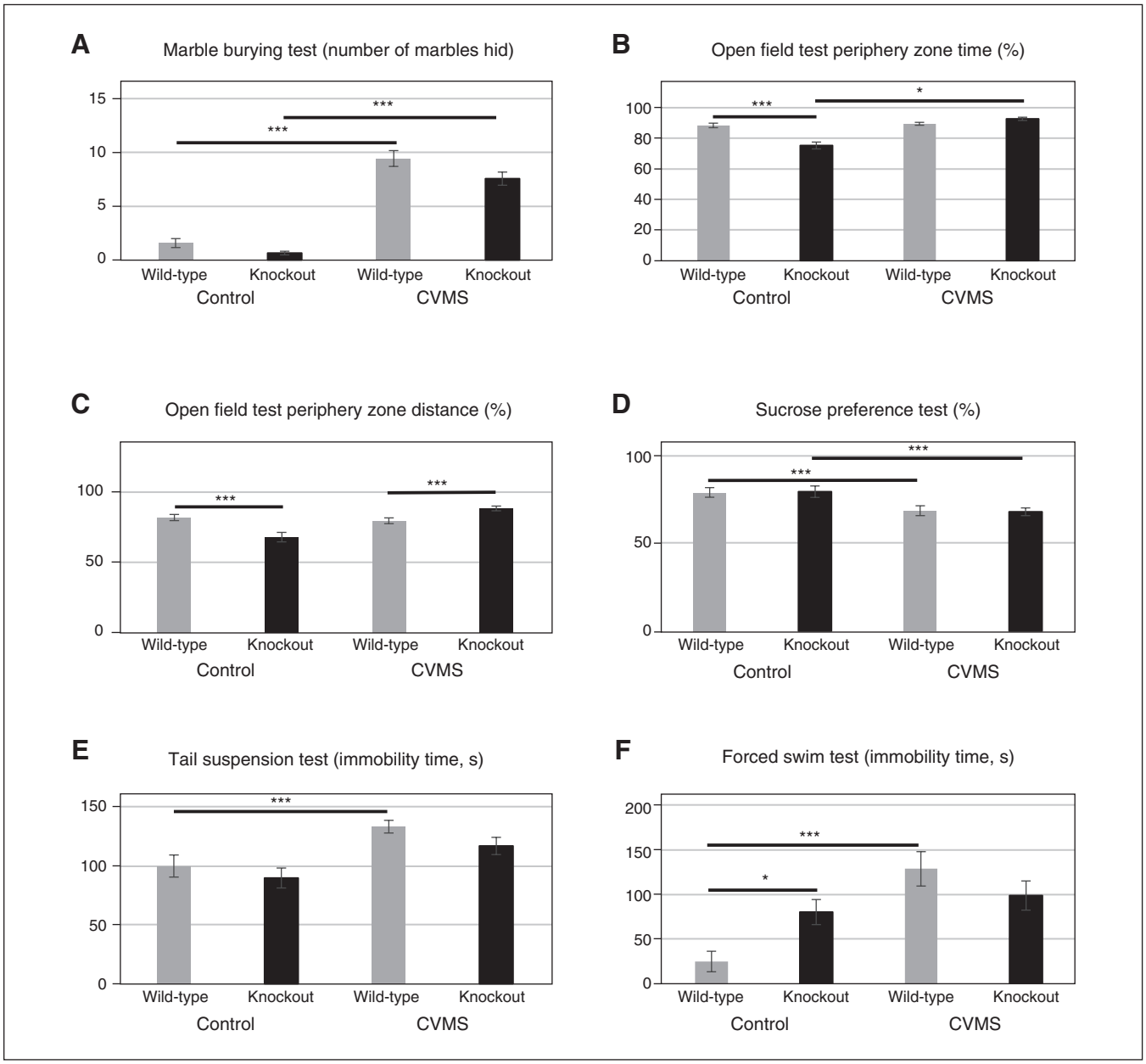

Figure 3: Findings from behavioural assessments. (A) To assess anxiety levels, we calculated the number of hidden marbles in the marble burying test. (B) We evaluated the ratio of time spent in the peripheral part of the arena in the open field test. (C) To reveal locomotor effects, we evaluated distance travelled during the open field test. (D) We determined anhedonia levels with the sucrose preference test. (E and F) We evaluated depression-like behaviour as immobility time in the tail suspension test and the forced swim test. Two-way analysis of variance followed by a Fisher post hoc test: *$p < 0.05$, ***$p < 0.001$. n = 14–16 per group. Grey bars represent wild-type mice; black bars represent *Trpa1* knockout mice. CVMS = chronic variable mild stress.

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 then 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).

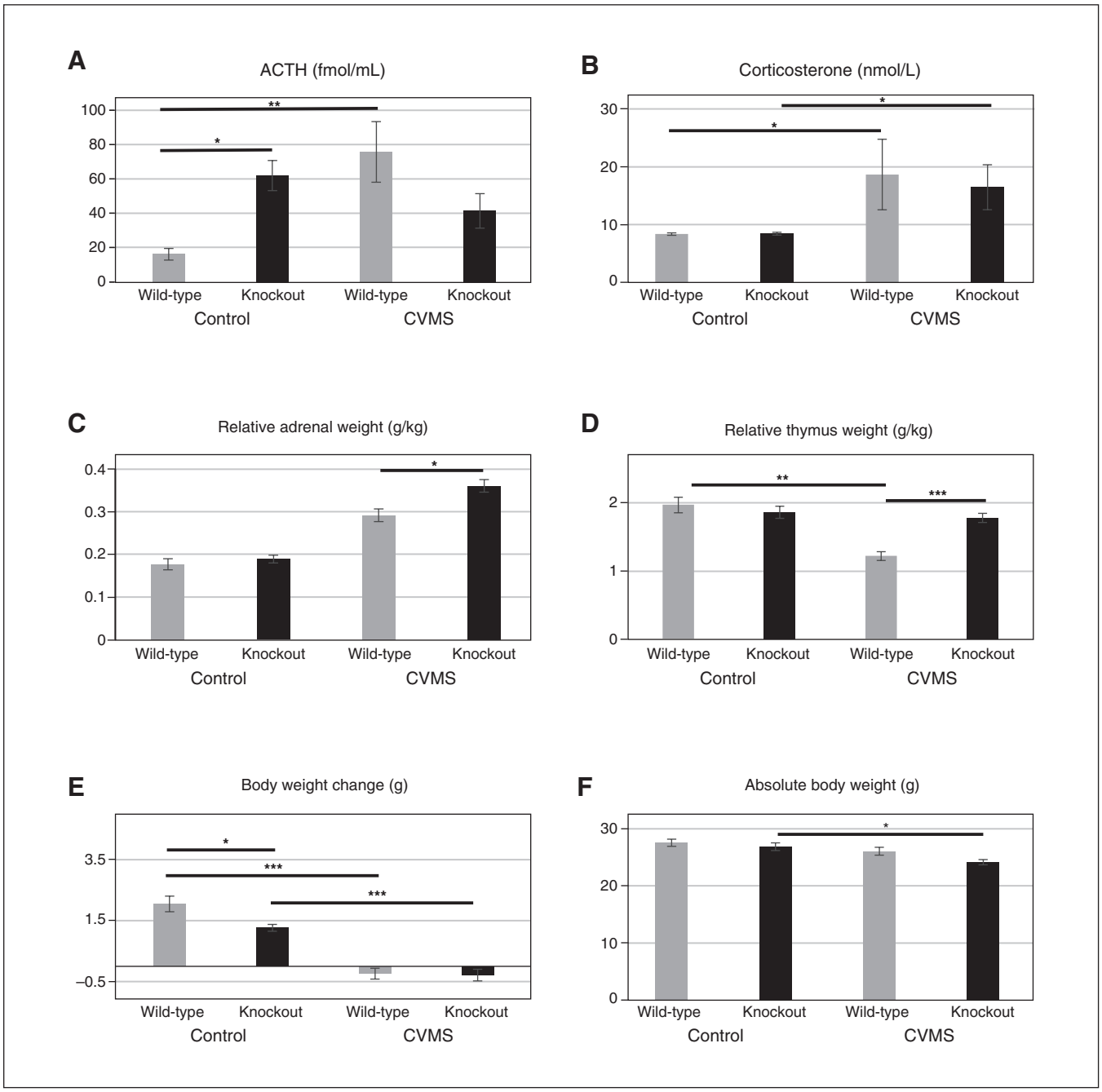

Figure 4: Efficacy of exposure to CVMS. We assessed the activity of the hypothalamic–pituitary–adrenal axis by determining (A) serum ACTH and (B) corticosterone titres. We found that (C) relative total adrenal weight, (D) relative thymus weight and (E) body weight change mirrored somatic changes induced by chronic stress. (F) Absolute body weight of mice at end of the in vivo experiment. Two-way analysis of variance followed by a Fisher post hoc test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n = 14–16 per group. Grey bars represent wild-type mice; black bars represent *Trpa1* knockout mice. ACTH = adrenocorticotropic hormone; CVMS = chronic variable mild stress.

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: $F_{1,56} = 2.02$, $p = 0.16$; stress: $F_{1,56} = 0.17$ $p = 0.68$; interaction: genotype $F_{1,56} = 2.458$ $p = 0.12$). 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. Semiquantitation revealed that CVMS exposure reduced the number of *Trpa1* transcripts in UCN1 neurons by 40% ($t_{11} = 5.09$; $p = 0.003$; 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-PCR (RT-qPCR). We detected no difference between the control and CVMS-exposed groups (1.0323 ± 0.106 v. 1.019 ± 0.043; $t_{13} = 0.111$, $p = 0.91$).

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. ($t_4 = 2.88$; $p = 0.044$; 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 regulation[23,24,31] and stress-related psychopathologies,[26] 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,[50] 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 mice[22]), or anxiety might have an adaptive role (i.e., in mothers during the postpartum period).[51] 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.[19,52] An additional interpretation of our open field test findings could be that *Trpa1* knockout mice were more

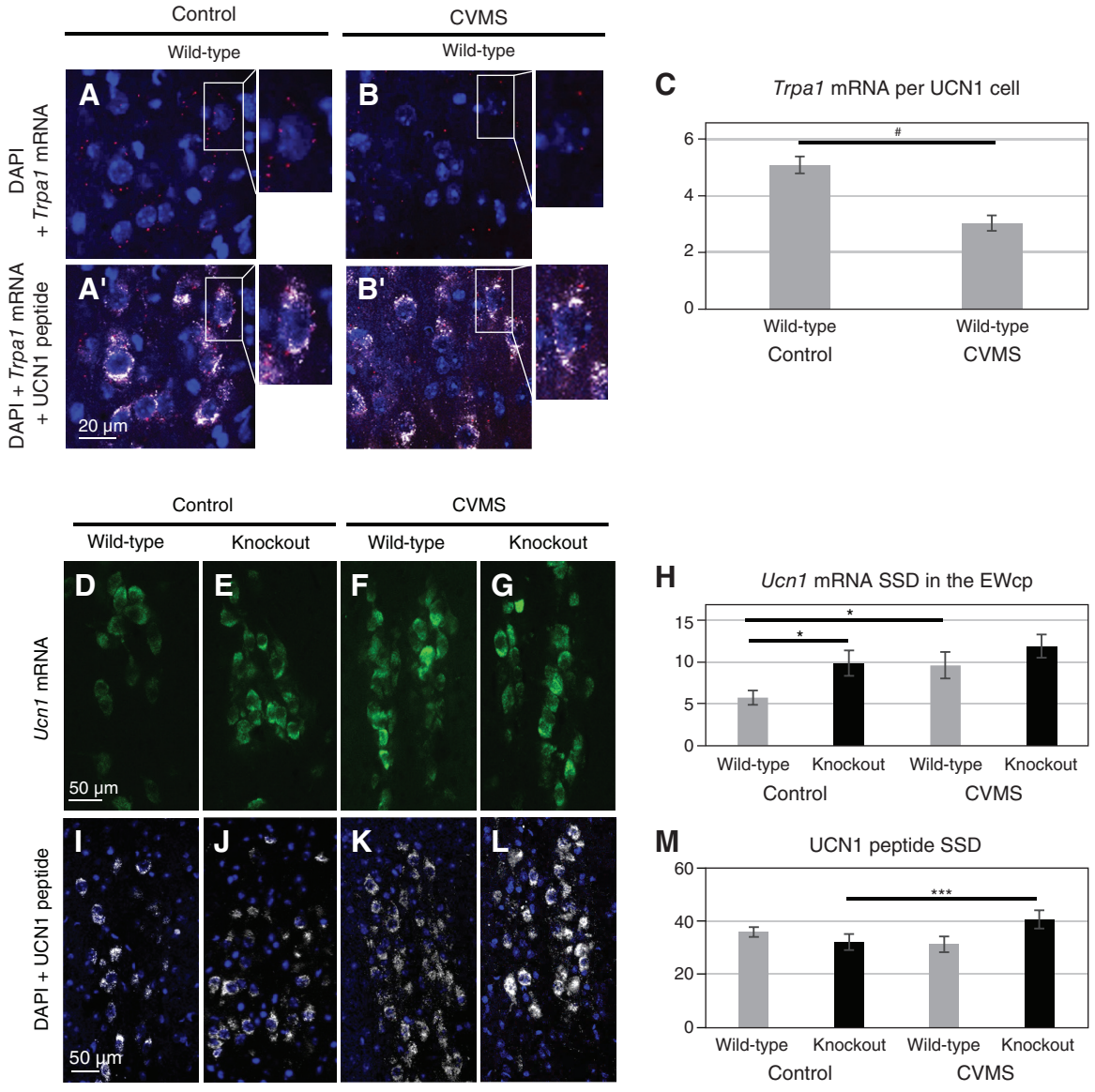

Figure 5: *Trpa1*, *Ucn1* mRNA and UCN1 peptide expression in EWcp neurons upon CVMS. (A and B) *Trpa1* mRNA (red) was downregulated in wild-type mice exposed to CVMS, as also shown in (C) the histogram. (A' and B') *Trpa1* mRNA transcripts (red) were localized to cells containing UCN1 peptide (white). (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. #$p < 0.05$ in a Student *t* test. *$p < 0.05$, 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.

sensitive to stress in this test. This was possible because EWcp/UCN1 neurons project to the extended amygdala,[53] and infusion of UCN1 into the amygdala increases anxiety via CRH receptors.[54] 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,[45] and because EWcp/UCN1 neurons are recruited in this model.[31,32] CVMS exposure induced typical somatic,[55–57] hormonal (ACTH and corticosterone) and behavioural (anxiety-, anhedonia- and depression-like) changes as signs of increased activity in the hypothalamic–pituitary–adrenal (HPA) axis.[31,32]

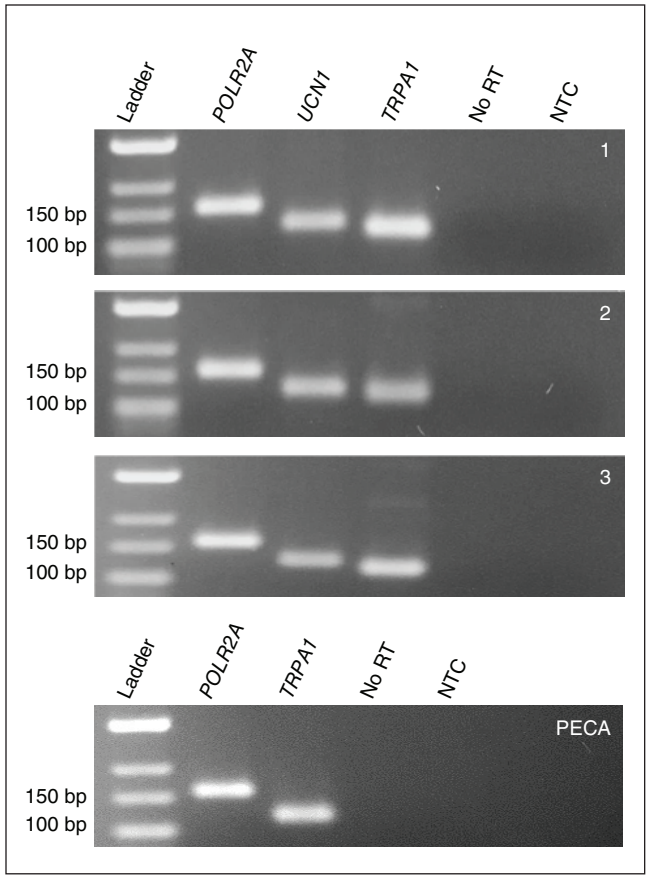

Figure 6: Electrophoretograms of RT-PCR products. We studied 3 human EWcp samples (1 to 3) and used a TRPA1-expressing human oral squamous cell carcinoma culture (PECA) as a positive control. The housekeeping gene (DNA-directed RNA polymerase II subunit RPB1; *POLR2A*, size 152 bp) and the gene of interest (*TRPA1*, size 115 bp) were expressed in all samples, including the PECA cell culture. The *UCN1* (size 123 bp) RT-PCR products proved that all brain samples contained the EWcp area. We also used a no reverse transcription control and a no template control. EWcp = centrally projecting Edinger–Westphal nucleus; no RT = no reverse transcription control; NTC = no template control; PECA = human oral squamous cell carcinoma cell line (PE/CA-PJ41; clone D2); RT-PCR = reverse transcription polymerase chain reaction; TRPA1 = transient receptor potential ankyrin 1; UCN1 = urocortin 1.

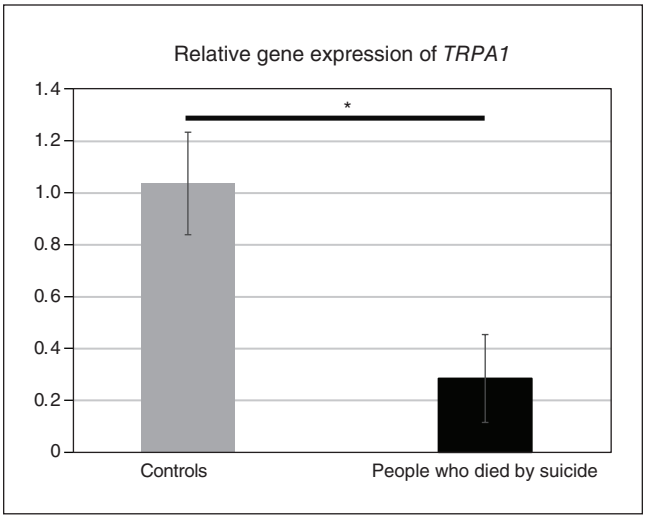

Figure 7: Relative *TRPA1* gene expression in human centrally projecting Edinger–Westphal nucleus samples from male controls (n = 3) and people who died by suicide (n = 3) as determined by TaqMan quantitative reverse transcription polymerase chain reaction (*$p < 0.05$; Student t test).

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 *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 TRPA1-related alterations in thymus weight, which could be explained by altered differentiation of lymphocytes,[58] or by disturbed HPA axis regulation in *Trpa1* knockout mice. Further studies are needed to determine whether 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 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 *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 upregulation of *Ucn1* expression in wild-type mice but not in *Trpa1* knockout mice might also underline the observed behavioural phenotype. The lack of functional 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[31,32] might be limited.

The elevated UCN1 peptide content associated with unaltered *Ucn1* mRNA expression in stressed *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,[19,52] supported by our finding of increased relative weight of the adrenal glands of stressed *Trpa1* knockout mice. Because the stainability 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 labelling might refer to inhibited cellular function.[24] Measurements at the mRNA level provide further evidence for transcriptional 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.[59] 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 limited[60,61] 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 β,[62] 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.

Affiliations: From the Department of Pharmacology and Pharmacotherapy, Medical School and Molecular Pharmacology Research Group, Centre for Neuroscience, Szentágothai Research Centre, University of Pécs, Pécs, Hungary (Kormos, Kecskés, Alomari, Hegedüs, Helyes, Pintér); the Department of Anatomy, Medical School and Research Group for Mood Disorders, Centre for Neuroscience, Szentágothai Research Centre, University of Pécs, Pécs, Hungary (Farkas, T. Gaszner, Csernus, Hegedüs, B. Gaszner); Human Brain Tissue Bank and Laboratory, Semmelweis University, Budapest, Hungary (Renner, Palkovits); the Department of Physiology, Medical School, Szentágothai Research Centre, Centre for Neuroscience, University of Pécs, Pécs, Hungary (Zelena); PharmInVivo Ltd., Pécs, Hungary (Helyes, Pintér).

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Contributors: V. Kormos, A. Kecskés, Z. Helyes and B. Gaszner designed the study. J. Farkas, T. Gaszner, A. Alomari and D. Hegedüs acquired the data, which A. Kecskés, J. Farkas, V. Csernus, A. Alomari, E. Renner, M. Palkovits, D. Zelena and E. Pintér analyzed. V. Kormos, A. Kecskés, J. Farkas, A. Alomari, D. Hegedüs, Z. Helyes and B. Gaszner wrote the article, which A. Kecskés, J. Farkas, T. Gaszner, V. Csernus, A. Alomari, E. Renner, M. Palkovits, D. Zelena and E. Pintér reviewed. All authors approved the final version to be published and can certify that no other individuals not listed as authors have made substantial contributions to the paper.

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