SUPPLEMENTAL DATA

SUPPLEMENTAL DATA MATERIALS AND METHODS

Culture of pineal glands

After sacrifice, pineal glands were immediately sampled and dipped in ice cold RPMI 1640 medium without phenol red and complemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and glutamine (2mM), as detailed elsewhere (1). At the laboratory, the culture was pursued using 24-well culture dishes (NunclonTM Surface; VWR International, Fontenay-sous-Bois, France), one organ per well in 500 µL of medium. The culture plates were placed in a MIR-154 incubator (Sanyo; Osaka, Japan)

Melatonin quantification

Melatonin in the culture medium was quantified by High Performance Liquid Chromatography (HPLC) using either (1) a 100 x 4.6 mm C8 reversed-phase analytic column (Waters Spherisorb; Milford, MA) with particles length of 3 µm and an Agilent fluorescence detector (1100 series; Santa Clara, CA, USA) or (2) a 125 x 4.6 mm C18(2) reversed-phase analytic column (Luna, Phenomenex; Le Pecq, France) with particles length of 5µm and a Dionex™ ULTIMATE™ 3100 fluorescence detector (Thermo Scientifique™, Villebon-sur-Yvette, FR). The excitation and emission wavelengths were of 280 nm and 340nm, respectively. The mobile phase consisted of 0.1 M Na_2HPO_4 containing 10% (C8 column) or 20% (C-18 column) acetonitrile; pH was adjusted at 6.5 with orthophosphoric acid. The flow was from 1 (protocol 1) to 1.5 ml/min (protocol 2), (for more details on the HPLC procedures see (2)). Quantifications were occasionally performed using a commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) according to the manufacturer’s instructions. All the assays used gave similar results.

RNA extraction and cDNA synthesis

After sampling tissues and organs were placed in RNA later®, for 24 h at +4 °C and then stored at -80 °C until they were processed. Total RNA was extracted using the TRIzol® reagent according to the manufacturer's recommendations and treated with DNase Ambion DNA-free™. Total RNA was quantified (NanoDrop® 2000 Spectrophotometer; Thermo scientific, Waltham, MA) and

integrity was checked by electrophoresis and wavelength ratio. Reverse transcription was performed using the PrimeScript™ reverse transcriptase. The quality of cDNA was checked by polymerase chain reaction (PCR) amplification using β-actin degenerated primers (Table 1).

Reverse transcription (RT)-PCR and cloning of TRPV1 and TRPV4

The PCR reaction was performed using the GoTaq polymerase and the following conditions: initialization at 94 °C (2 min) followed by 10 cycles of denaturation (94 °C, 30 sec), annealing (55.3 °C, 30 sec) and extension (72 °C, 1.5 min), and by another 30 cycles of: denaturation (94 °C, 30 s), annealing (53.3 °C, 30 s) and extension (72 °C, 1.5 min). For the generation of full-length cDNA sequences, 3' and 5' RACE (Rapid Amplification of cDNA Ends) PCR was performed using the SMART RACE cDNA amplification kit. TRPV1 and TRPV4 3' extremities were obtained using trout specific primers (Table 1). The RACE-PCR and nested-RACE-PCR steps were: initialization (95 °C, 1 min) followed by 10 cycles of denaturation (95 °C, 30 sec), annealing (69.5 °C and 63.3 °C for TRPV1 and TRPV4 respectively, 1 min) and elongation (68 °C, 3 min), and 30 cycles of denaturation (95 °C, 30 sec), annealing (67.5 °C and 61.3 °C for TRPV1 and TRPV4 respectively, 1 min) and extension (68 °C, 3 min) 30 times. Amplification was performed with Taq Advantage polymerase-2.

After migration on a 1% agarose gel, PCR products were extracted and purified using a gel extraction kit and sub-cloned into a pGEM-T Easy vector to be transfected into DH5α electro-competent bacteria. After selection of the positive clones, the vectors were extracted and purified using minipreps according to the manufacturer's instructions for sequencing of the amplicons by Eurofins (Ebersberg, Germany).

Real time quantitative (q)PCR

Real-time qPCR was performed using a 1:50 cDNA dilution (n = 5) for the detection in different tissues, and a 1:5 cDNA dilution (n = 23-36) for the detection in pineal glands after the in vitro culture. The specificity of TRPV1 and TRPV4 probes produced (Table 1) was checked by PCR (see above). The qPCR analyses were performed using a Light-Cycler™ system version 2.0 and the LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche Diagnostics, Meylan, France). The qPCR conditions were as follows: enzyme activation at 95 °C for 3 min followed by 40 cycles of denaturation (95 °C, 30 sec), annealing (60 °C, 45 sec) and elongation (72 °C, 45 sec), ending with a

melting curve program (consisting in a stepwise of denaturation at 95°C for 15 sec, followed by a cooling into annealing temperature, 60°C for 1 min, ending on a rise of 0.3°C from 60 to 95°C in 15 sec). The elongation factor 1 alpha (EF1α) was used to normalize the results as already validated in rainbow trout (3). The relative expression of TRPV1 and TRPV4 was quantified according to the Pfaffl method (4) based on the real-time qPCR efficiencies for each gene.

Sequences analysis, Statistics and drawings

The Basic Local Alignment Search Tool (BLAST) at NCBI (5) was used to search the data bases for significant alignments. The search for conserved domains was performed using InterProScan v5 (6). Transmembrane domains were determined using Phobius (http://phobius.sbc.su.se/).

Compounds and chemicals

Capsaicin, capsazepine, dimethylsulfoxide (DMSO), gadolinium, glutamine, 4-α-phorbol-12,13-didecanoate (4αPDD), ruthenium red (RuR), streptomycin were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Stock solutions were dissolved in 100% ethanol for capsaicin, 100% methanol for capsazepine, distilled water for gadolinium chloride and in DMSO for 4αPDD. Solvent concentration did not exceed 0.2%; controls contained an equivalent amount of solvent. The melatonin ELISA kit was from IBL International^{GmbH} (Hamburg, Germany). RNA later®, TRIzol®, and DNase Ambion DNA-free™ reagent were from Life technologies SAS (Saint Aubin, France). The PrimeScript™ reverse transcriptase and the Taq Advantage polymerase-2 were from Ozyme (Saint Quentin en Yvelines, France). The GoTaq polymerase and the pGEM-T Easy vector were from Promega (Charbonnières, France). The SMART RACE cDNA amplification kit was from ClonTech (Saint-Germain-en-Laye, France). The gel extraction and mini-prep kits were from QIAGEN Sciences (Courtaboeuf, France).

SUPPLEMENTAL TABLE 1

Primers list

	primer	sequence	Length (bp)
RT-PCR	TRPV1d1-F*****	ATCAAGAGGCTGTTYGAGGC	879
	TRPV1d1-R*****	GGCGATTAGGAATSTCACTGC	
	TRPV1d2-F	GCAGTGASATTCCTAATCGCC	1156
	TRPV1d2-R	ACYTCCTCCACWCTGAAGCA	
	TRPV4d1-F	GGVCGCTTCTTCCAGCCC	1528
	TRPV4d1-R	CCACCCTGAAGCACCASC	
	β-actin-F	CTGGAGAAGAGCTAYGAGCTG	212
	β-actin-R	GTACATGGTGGTACCDCCAGA	
RACE-PCR	TRPV1om-3’	AGGACCTGGGGACCAGGGCTGGAGA	604
	TRPV4om-3’	GCCAGGTGTCCAAGGAGAGCAAGAAG	606
	TRPV4om-3’	GGGGAGATGGTGACAGTGGGGAAGAA	699
qPCR	TRPV1om-F	CGTCCTGCTGAAGGCTCTA	121
	TRPV1om-R	TGTCTGTGTATGCAGCATTTACTA	
	TRPV4om-F	GAGAATCGCCATGAGATGC	155
	TRPV4om-R	TGGGATGGGCGGTAGTA	
	EF1αom-F	AGCGCAATCAGCCTGAGAGGTA	299
	EFαom-R	GCTGGACAAGCTGAAGGCTGAG	
in situ* Hybridization	TRPV4omHIS3-F	ACCTGTTCAAACTGACCATCGG	700
	TRPV40mHIS3-R	TCATGCCGACAGAGTTTTGTGC	

The design of the TRPV1 primers was based on the alignment (ClustalW, BioEdit) of zebra fish *Danio rerio* (EU423314.1) and salmon *Salmo salar* (NM_001140498.1) cDNA sequences. The design of TRPV4 primers was based on nucleotide BLAST alignments of TRPV4 sequences from *Dicentrarchus labrax* (7), *D. rerio* (BC163540.1), *Canis lupus* (NM_001127315), *Mus musculus* (NM_022017.3) and *Homo sapiens* (BC117426.1). *****The TRPV1 primers for making the *in situ* hybridization probe were TRPV1d1-F and TRPV1d1-R (above).

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