In the Heat of the Night: Thermo-TRPV Channels in the Salmonid Pineal Photoreceptors and Modulation of Melatonin Secretion

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Abstract

Photoperiod plays an essential role in the synchronization of metabolism, physiology and behavior to the cyclic variations of the environment. In vertebrates, information is relayed by the pineal cells and translated into the nocturnal production of melatonin. The duration of this signal corresponds to the duration of the night. In fish, the pinealocytes are true photoreceptors in which the amplitude of the nocturnal surge is modulated by temperature in a species-dependent manner. Thus, the daily and annual variations in the amplitude and duration of the nocturnal melatonin signal provide information on daily and calendar time. Both light and temperature act on the activity of the penultimate enzyme in the melatonin biosynthesis pathway, the arylalkylamine *N*-acetyltransferase (AANAT: serotonin → *N*-acetylserotonin). While the mechanisms of the light/dark regulation of melatonin secretion are quite well understood, those of temperature remain unelucidated. More generally the mechanisms of thermoreception are unknown in ectotherms. Here we provide the first evidence that two thermo-TRP (transient receptor potential) channels, TRPV1 and TRPV4, are expressed in the pineal photoreceptor cells of a teleost fish, where they modulate melatonin secretion *in vitro*. The effects are temperature dependent, at least for TRPV1. Our data support the idea that the pineal of fish is involved in thermoregulation, and that the pineal photoreceptors are also thermoreceptors. In other nervous and non-nervous tissues, TRPV1 and TRPV4 display a ubiquitous but quantitatively variable distribution. These results are a fundamental step in the elucidation of the mechanisms of temperature transduction in fish.

INTRODUCTION

The pineal photoreceptor cells of ectothermic vertebrates transduce light and temperature information into a nervous and a neurohormonal output. The former, aspartate and/or glutamate, is released in the dark at the synaptic contacts with the pinealofugal neurons; the neurons then convey rapid (msec) electric information to the brain (1,2). The latter, melatonin, is produced at night and this 24h rhythm is an essential component of the fish time keeping system (3,4). The duration of the melatonin surge is dictated by photoperiod while temperature controls the amplitude (3,5). This melatonin rhythm contributes to synchronizing metabolism, physiology and behavior to the daily and annual variations of the environment (4). While the impact of photoperiod on the pineal outputs is relatively well understood, virtually nothing is known on how temperature operates. More generally, little progress has been made in the last decades concerning temperature regulation and transduction of the temperature information in ectothermic vertebrates (6-8). Temperature sensitive neurons have been identified in the lateral line (9), spinal cord (10,11), and in the epithalamus and preoptic area of the brain (12,13); the latter also receives thermal input from other unidentified photosensitive structures. Interestingly, the pineal organ of fish has been involved in behavioral thermoregulation mediated by both a rapid (nervous) and a slow (hormonal) mechanism (14-16). And, it has been established that both the nervous (17) and neurohormonal (18) information released by the organ reach the preoptic area, a major neuroendocrine center. Altogether, although clear direct evidence is still missing, there is strong support that the fish pineal organ is a photo-thermo-sensor. The questions then rise to know how and where is the temperature information captured and transduced within the organ?

A number of observations suggested to us that the pineal cone-like photoreceptor is also a thermoreceptor. (***i***) The nocturnal rise in melatonin production is a calcium- and cAMP-dependent process (3,19-21): photoreceptor depolarization in the dark induces the opening of voltage-gated Ca^{2+} channels (VGCC); the subsequent increase in $[Ca^{2+}]_i$ activates both the synthesis of cAMP and the activity of the arylalkylamine *N*-acetyltransferase (AANAT; EC: 2.3.1.87), the first step in the conversion of serotonin into melatonin (22). This dose-dependent process is reversed upon illumination (3). (***ii***) A similar involvement of Ca^{2+} and VGCC in the release of the neurotransmitter is

also suspected, as is the case in the retinal cone (23). (***iii***) In cultured pineal organs, both cAMP production and AANAT activity display the same pattern in response to temperature; this is a species-dependent process (5). It is also a cellular-mediated process because the response of AANAT activity to temperature variation differs if measured on the native (*i.e., in situ*) or recombinant enzymes (24,25). (***iv***) In mammals, temperature sensing is mediated by members of the Transient Receptor Potential (TRP) channels family (TRPA1, TRPV1-4 and TRPM8), which are permeable to Ca^{2+} (26,27), and activate at different ranges of temperature (28).

Altogether, it is not unreasonable to hypothesize that the fish cone-like photoreceptor cell is also a thermo-receptor utilizing both VGCC and TRPs to modulate $[Ca^{2+}]_i$ and, consequently, to control the nocturnal production of melatonin and the secretion of the excitatory neurotransmitter; in other words, one intracellular second messenger for two external cues and two outputs. In a first attempt to tackle this question we report here the cloning of two TRP channels (TRPV1 and TRPV4) in rainbow trout. We describe their tissue distribution and localization and bring the first evidence that TRPV are expressed in the retinal and pineal photoreceptors and that they contribute to controlling pineal melatonin secretion at night. Pharmacological experiments suggest that at least TRPV1 channels are involved in thermo-sensing.

MATERIALS AND METHODS

Animals

Two years-old rainbow trout, *Oncorhynchus mykiss,* were provided by "Les Viviers Cathares" (Chalabre, France). Animals were sacrificed at the hatchery by electrocution and the organs of interest were dissected out and placed either in ice-cold fixative, RNA later® or culture medium. All experiments were performed according to the European Union regulations (European directive 91/492/CCE).

Pineal organs culture

The organs were cultured as indicated in the *Supplemental Materials and Methods* section under conditions of photoperiod and temperature similar to those at which the animals were acclimated before sacrifice; medium was replaced once after 24 h of culture. The impact of TRP

agonists and antagonists was investigated after 48 h of culture. For this purpose, the glands were placed in the dark for 6 h (starting at 09:00 of the LD cycle) in the absence or presence of the compounds tested (concentrations are given in the Results section and figures). Controls included organs cultured in the presence of vehicle and placed either in the dark or maintained under light. At the end of the experiments, glands and media were then collected separately and stored at -80 °C.

Melatonin quantification

Melatonin in the culture medium was quantified using High Performance Liquid Chromatography (HPLC) or Enzyme-Linked Immuno-Sorbent Assay (ELISA) as indicated in the *Supplemental Materials and Methods* section.

RNA extraction, cDNA synthesis and sequences analysis

See *Supplemental Materials and Methods*.

Reverse transcription (RT)-PCR, cloning of TRPV1 and TRPV4, real time quantitative (q)PCR

The TRPV1 and TRPV4 sequences were obtained by PCR using degenerated primers (*Supplemental Table 1*). The PCR and qPCR conditions are detailed in *Supplemental Materials and Methods*.

***In situ* hybridization**

Tissues were fixed in 4% paraformaldehyde in 0.2 M Sorensen buffer, prepared and processed as detailed (29). The TRPV1 and TRPV4 riboprobes were generated from pituitary extracts and primers as indicated in *Supplemental table 1*.

Statistics and drawings

Statistical analyses and plotting were performed using Prism.v6 (GraphPad™ Software Inc., San Diego, CA). The pharmacological and qPCR data were analyzed using the one-way or two-ways ANOVA followed by a post-hoc Tukey's comparison of means, or by the unpaired Students *t*-test.

Compounds and chemicals

See *Supplemental Materials and Methods*.

RESULTS

Cloning of TRPV1 and TRPV4

O. mykiss TRPV1 and TRPV4 were cloned from pineal extracts. The TRPV1 mRNA sequence was 2807 bp long (GenBank Acc # KJ135121); the corresponding coding region was 2415 bp long and encoded a 805 aa sequence that displayed high identity with the TRPV1 sequences from *S. salar* (96%), and the predicted TRPV1 sequence from *Esox lucius* (80%) and lower identity with those from other fish (~60% with *O. niloticus* and *D. rerio*) and tetrapods (<53%) (*Supplemental Fig. 1*). The TRPV4 mRNA sequence was 3035 bp long (GenBank Acc # KJ135122); the corresponding ORF (2637 bp) encoded a 879 aa sequence which shared 98% identity with *S. salar* TRPV4, 89% with *D. labrax* TRPV4, 79% with *D. rerio* and *O. mossambicus* TRPV4 and 70-75% with tetrapods TRPV4 (*Supplemental Fig. 1*). The *InterProScan* search for conserved domains confirmed that trout TRPV1 and TRPV4 belong to the superfamily of TRPV channels, possessing the typical ankyrin repeats, the calcium moiety and the 6 transmembrane domains (*Supplemental Fig. 1*). In addition, aa residues known to be important for the function of the rat TRPV1 were also identified in the fish sequences, as discussed later and *Supplemental Fig. 1*. Finally, the phylogenetic reconstruction indicated TRPV1 and TRPV4 grouped with their respective orthologues (not shown).

Tissue distribution and relative expression of TRPV1 and TRPV4

TRPV1 and TRPV4 distribution was ubiquitous but the levels of expression varied from an organ to another (Fig. 1). TRPV1 expression in intestine and kidney was a two-fold higher than in the retina, brain, pineal organ, spleen, heart and blood cells. The pituitary gland, skin and liver had the lowest levels of expression. The highest expression of TRPV4 was found in extracts from the pineal organ, kidney, intestine and heart, while in extracts from all other nervous and peripheral tissues investigated the levels were a four-fold lower (Fig. 1).

Cellular localization of TRPV1 and TRPV4 in the pineal organ and retina

In the pineal organ, both TRPV1 and TRPV4 mRNA were specifically localized in the photoreceptor cells, identified by their typical shape and localization in the pineal epithelium (Fig. 2). Similarly, expression was also detected in the outer nuclear layer (ONL) of the retina (Fig. 3a, c). Differences were however observed in other layers. TRPV1 expression was also seen in the basal part of the inner nuclear layer (INL) as well as in the ganglion cell layer (GCL) and the corresponding axons that make the optic nerve (Fig. 3b). TRPV4 expression was seen in the central and basal parts of

the INL; it was detected neither in the GCL nor in the optic nerve (Fig. 3c).

Impact of temperature on melatonin secretion

Before investigating the effects of TRPV analogs *in vitro*, it was necessary to know what the best conditions for culturing the pineal organs are. For this purpose, pineal glands were cultured at different times and different temperatures in the dark as indicated in figure 4. The highest levels of secretion were found after incubation times of 6 and 12 h with peak values at temperatures between 15 and 20°C (Fig. 4A). It is noteworthy that at the long incubation times (6 and 12 h) melatonin secretion increased from 0 to 5°C, then marked a plateau between 5 and 10°C, and rose again from 10 to 15°C.

Figure 4B shows the release of melatonin as a function of time and temperature expressed as a percent of the value found after 1 h of incubation. The different profiles obtained indicate that melatonin secretion increased continuously up to 12 h of culture at 0°C, while between 5 and 20°C it increased up to 6 h and then initiated a decrease. Conversely at temperatures above 20/25°C melatonin secretion decreased continuously, either progressively (25°C) or dramatically (30 and 35°C).

Impact of TRPV agonists and antagonists on pineal melatonin

In view of the above results we decided to investigate the effects of the TRPV analogs at the temperature at which the animals were adapted in their natural habitat at the time the experiments were done (*i.e.*, between 10 and 20°C) and for a 6 h incubation. Under these conditions, capsaicin, a TRPV1 agonist, induced a dose-dependent effect on melatonin release, stimulatory at low, and inhibitory at high, concentrations; both effects were neutralized in the presence of capsazepine, a TRPV1 antagonist, (1 µM; Fig. 5a). No effect was detected with the agonist 4-α-phorbol-12,13-didecanoate (4αPDD) alone (Fig. 5b). This suggested to us that the TRPV4 channel expressed in the photoreceptor cells (Fig. 2), did not respond to the agonist challenge or were already maximally active. The later explanation remains possible because in the presence of ruthenium red (RuR; 10 µM), a TRPV4 blocker, 4αPDD induced inhibition at low concentrations and stimulation at high concentrations (Fig. 5b).

The later experiment and the role devoted to TRPV channels in mammals (see introduction) suggested to us that TRPV1 and TRPV4 channels might be under different states of activity depending

on the ambient temperature. To investigate this point, pineal glands were cultured at different temperatures and in the absence or presence of either 1 µM for capsazepine, the TRPV1 antagonist, or 10 µM gadolinium, the TRPV4 antagonist. Figure 6 shows the data obtained from two independent experiments performed in spring. We found that in the presence of the TRPV1 antagonist melatonin secretion was ~40 % lower at 16°C and ~20% lower at 20°C, compared to controls. The results were however significant only at 16°C. The TRPV4 antagonist gadolinium was found to inhibit melatonin secretion at 8°C by ~30% (Fig. 6); the effect was however not statistically significant.

Impact of temperature and TRPV agonists on the pineal expression of TRPV channels *in vitro*

We took advantage of the previous experimental set up to investigate whether temperature and the TRPV antagonists used had an impact on the TRPV1 and TRPV4 gene expression. We found that neither the temperature of incubation nor the pharmacological treatments affected significantly the amount of TRPV1 and TRPV4 mRNA (not shown). However, we found that TRPV1 and TRPV4 mRNA expression varied with the month at which the experiments were performed, *i.e.*, March and June (in which water temperature was 8°C and 15°C, respectively; Fig. 7).

DISCUSSION

The present study adds to the very short list of TRPV sequences available in fish. Our analyses of the cloned sequences confirmed that they belong to the TRPV1 and TRPV4 families. Both displayed a ubiquitous distribution, as described for mammals (30), zebrafish and sea bass (31,32). Whether the TRP channels have the same function in all the tissues where they are expressed remains an open question. One main function of TRPV1 and TRPV4 is to transduce thermal stimuli (28,33); but they may also be sensitive to chemical, mechanic and ionic stimuli (31,34,35). It is thus possible that the quantitative differences observed between the organs and tissues where they are expressed reflect specific requirements to either one or several of these stimuli (see (31,36) for extensive discussion). It is interesting that the kidney and intestine exhibited high TRPV mRNA levels. A previous investigation in the sea bass also identified a TRPV4-like compound by immunocytochemistry in the kidney and intestine (31). The immunocytochemical labeling in the intestine was interpreted as non-specific; the present demonstration of the presence of mRNA

encoding both TRPV1 and TRPV4 would indicate the channels are indeed present in this tissue.

The main focus of this study was to investigate the possible involvement of thermo-TRPs in the temperature-dependent control of melatonin production in trout. Two studies in the zebrafish indicated TRPV1 and TRPV4 are involved in temperature sensing (32,37). It was therefore of interest that both thermo-TRPs were expressed in the pineal gland and retina, the two main sites where melatonin is produced. As emphasized in the introduction, the pineal organ deserves special attention because of its capacity to sense both light and temperature (5), and because it is involved in behavioral thermoregulation (15,16). We bring here the first and unequivocal evidence that TRPV1 and TRPV4 are co-expressed exclusively in the melatonin producing cells of the pineal organ, *i.e.*, the cone photoreceptors (5). This contrasts with previous studies in the zebrafish where pineal expression was reported neither for TRPV1 nor for TRPV4, although both were expressed in all other sensory organs (32,36). It is noteworthy, however, that a TRPV1-like protein was immuno-detected in the rat pinealocyte (38). This cell type is a homologue of the fish pineal photoreceptor; it produces melatonin but has lost its direct photosensitive properties (4). The presence of TRPV1 in the melatonin producing cells of the fish and mammalian pineal glands appears thus as an ancestral character, shared by these homologous cells. In the mammalian pinealocyte the TRPV1-immuno-detected protein was associated with the synaptic ribbons, which function is enigmatic. Indeed, in contrast with the fish pineal photoreceptor the mammalian pinealocyte does not establish synaptic transmission *sensu stricto*; the gland has lost the second-order neurons, which in fish convey the light information to the brain (4,39). Interestingly however, TRPV1 has also been observed in the synaptic ribbons of the retinal photoreceptors of the zebrafish and goldfish (40). It is well established that the fish pineal photoreceptors display structural and functional analogies with the vertebrates' retinal cones; both derive from a common diencephalic origin (39). For this reason we felt interesting to explore TRPV1 and TRPV4 sites of expression also in the retina. Our study confirms the presence of TRPV1 in the retinal photoreceptors and thus extends the already high number of analogies between the retinal and pineal photoreceptors. We also extend these analogies to TRPV4. The identification of TRPV4 in trout, contrasts with previous observations in the zebrafish retina (36,41). Perhaps these discrepancies result from the use of immunocytochemistry *vs. in situ* hybridization. The former is probably less

specific particularly when it comes to detect a membrane bound protein, and with an antibody generated against TRP from other more distant species. The presence of TRPVs in trout pineal and retinal photoreceptors is consistent with the idea that TRPVs modulate the Ca^{2+}-dependent release of the excitatory neurotransmitter at the ribbons synapses, as is the case in cerebral neurons (26,37). We may speculate that the TRPVs expressed in the synaptic pedicles of the pineal photoreceptors are involved in the regulation of the described effects of temperature on the nervous discharges of the trout pineal neurons (2). We also found that TRPV1 and TRPV4 were expressed in the INL of the trout retina, as is the case in zebrafish (36,42). In contrast the GCL of trout expressed TRPV1 only, while the opposite was found in the zebrafish. It is beyond the scope of this work to speculate on the discrepancies and roles played by TRPV in the different layers of the retina that may be due to species-dependent characteristics, or to methodological and technical issues.

We had reasons to believe that TRPV1 and TRPV4 could be involved in the control of melatonin secretion in the pineal photoreceptor as detailed in the introduction. Also, the study in the mammalian pinealocyte indicated that the TRPV1 agonist capsaicin can stimulate melatonin release by cultured glands *in vitro* (38). We thus decided to investigate the impact of TRPV1 and TRPV4 agonists and antagonists on melatonin secretion by trout pineal glands in culture. As a first step we examined which where the best *in vitro* conditions to perform these experiments. We found that at temperatures above 20°C melatonin secretion decreased with the duration of the incubation, progressively at 25°C and dramatically at 30° and 35°C. Considering the aerobic scope of the trout, we believe that the survival of the organs was challenged at temperatures above 20°C. At 20° and below, melatonin secretion increased with time up to 6 h of incubation. After 6 h a decrease was observed except at 4°C; this probably reflects a feed-back inhibition of melatonin production by the increasing concentrations of melatonin released in the media as previously shown (43,44). We concluded that a 6 h incubation in the dark, at temperatures ranging from 15 to 20°C, were the best conditions to investigate the effects of the TRPV analogs were. Under these conditions, it appeared unambiguously that the TRPV1 agonist capsaicin induced a bimodal effect on melatonin release that was suppressed in the presence of the TRPV1 antagonist capsazepine. In the rat the stimulation needed higher concentrations of capsaicin while no inhibition was seen (38). The increase in melatonin secretion

observed at low capsaicin concentrations is consistent with the fact that Ca^{2+} entry into the photoreceptor stimulates synthesis. The inhibition observed at higher concentrations of capsaicin might result from a feed-back inhibition by high melatonin levels, as commented above (43,44). Alternatively, it might reflect an effect of the agonist at other sites; indeed, the pharmacological classification of TRP agonists and antagonists is based on studies in mammals only, with no warrantee that this classification applies to fish. The effect of capsaicin is however consistent with the observation that the trout TRPV1 sequence possesses a conserved aa residue that mediates the effects of capsaicin in the TRPV1 sequence of the rat (45,46). It is to note that the zebrafish TRPV1 sequence displays a mutated aa residue at the similar position, and the channel does not respond to capsaicin (32). In contrast to TRPV1, the involvement of TRPV4 in the control of melatonin secretion was less clear because the TRPV4 agonist 4αPDD had no visible effect at the concentrations tested. However, a dose-dependent inhibition of melatonin secretion was observed in the presence of the antagonist ruthenium red. Several possibilities non-mutually exclusive might explain these observations: (i) all or part of the channels were already activated stimulating melatonin secretion, and the addition of the antagonist counteracted these effects; (ii) 4αPDD is not a TRPV4 agonist in trout while ruthenium red acts as an agonist (this situation is not unusual as drugs characterized in mammals may not have the same pharmacological profile in fish); (iii) the antagonist ruthenium red was acting at other TRP channels than TRPV4 as already observed (47).

If the hypothesis is valid that the fish TRPV1 and TRPV4 channels are thermo-sensors, the use of antagonists of these channels alone should induce visible effects at some, but not all, temperatures. Indeed we found that the TRPV1 antagonist capsazepine had a significant impact on melatonin secretion at 16°C. This would suggest that the increase in melatonin secretion observed at 16°C was due in part to the activation of TRPV1 channels, which became inactive in the presence of capsazepine. A further support to the idea that trout TRPV1 is a temperature receptor comes from the analysis of the rat and trout sequences. Both possess conserved aa sequences in the pore and C-terminal regions of the channel (33,45,48); *Supplemental Fig. 1*). These include a tyrosine residue Y637, located in the pore region, which corresponding residue Y652 in rat is specifically required for temperature activation; this tyrosine is located close to other residues required for temperature and

chemical activation (48). It is also found in the zebrafish sequence (Y659). Interestingly, one study in the zebrafish indicated that TRPV1, which is required for normal heat-induced locomotion, activates at a temperature ≥ 25°C (32). This is above the suspected range of temperature sensitivity found here in the trout, but below the activation threshold of the rat TRPV1 (42°C). It has been suspected that differences in the C-terminal tail length of TVPV1 are responsible for the observed differences in threshold activation of TRPV1 in vertebrates (49). They may reflect specific adaption to the environmental requirements of the species considered.

Experiments aiming at investigating the impact of the TRPV4 antagonist gadolinium at different temperatures did not bring clear-cut information. Although not statistically significant, our data suggest a temperature activation at the low range of temperatures (± 8°C). The unequivocal demonstration that trout TRPV4 is activated in the cold range of temperatures needs further experimental support by investigating a narrower temperature interval and/or different times of the year. Indeed, the data presented in figure 6 are the mean of two independent experiments performed in March of two successive years (with an identical adaptation to an environmental temperature of 8°C). Our observation that the relative abundance of TRPV1 and TRPV4 varied from March to June leads us to suspect possible annual variations and/or a possible effect of previous temperature history on their expression levels. Another possibility could be that the TRPV4 activation depends on both the ambient temperature and salinity as previously shown (50).

Conclusions

The present study brings the first demonstration that thermos-TRPV channels are specifically expressed in the cone photoreceptors of a fish pineal gland were they contribute to controlling the nocturnal rise in melatonin secretion. We also bring evidence that TRPV1 activation is temperature dependent. Thus, the well establish effect of temperature on the amplitude of the nocturnal rise in melatonin secretion by the fish pineal gland (3,5) is likely to involve TRPV1 channels. These data strengthen the hypothesis that the fish pineal photoreceptor is a photo-thermo-receptor where light and temperature are likely to act through the same intracellular messenger, Ca^{2+}. The ubiquitous distribution of thermos-TRP channels support the hypothesis that thermoregulation in fish is achieved through a hierarchically organized temperature-sensing network (8). In view of the importance the

pineal gland and its outputs play in behavioral thermoregulation and synchronization of rhythmic metabolic and neuroendocrine processes, we believe it occupies a crucial position in this network. It might also be a sensor of the internal medium properties. Indeed, TRPV can also integrate subtle variations in membrane stretching, pH, ionic and chemical components; and, photoreceptors are in direct contact with the cerebrospinal fluid (CSF) (51). An old hypothesis, never investigated so far, suggested that the pineal gland of fish could sense variations in CSF pressure (52). Finally, the channels might also be part of the yet unidentified bridge that links the pineal glial cells to the photoreceptors; indeed, arachidonic acid a metabolite produced mainly by the glial cells (53), is known to modulate TRPVs activity (54,55).

Fish have adapted for thousands of years to temperature changes occurring in synchrony with the daily and annual variations of photoperiod. This harmony is being challenged now by the current global climate change: temperature is changing while photoperiod remains the same. This may in part explain the recently observed perturbations of timed biological processes in vertebrates. More information is thus urgently needed now, including a functional characterization of all thermo-TRPs expressed in fish, in order to better understand how factors of the external and internal environment affect the production of the time-keeping hormone melatonin.

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LEGEND OF FIGURES

Figure 1. **Relative expression of TRPV1 and TRPV4 in trout tissues and organs**

One-way ANOVA was followed by Tukey's multi-comparison test. Columns bearing different letters are significantly different. Mean ± SEM (n = 5). Details in Supplemental Materials and Methods.

Figure 2. Localization of TRPV1 and TRPV4 mRNA in trout pineal organ.

In situ hybridization of 8 µm sections. The antisense probes directed against TRPV1 (**a**) and TRPV4 (**b, c**) mRNA gave similar results. A labeling was seen exclusively in the cells that border the pineal lumen (L) as shown by the red arrows. The other cell types of the epithelium (glial and ganglion cells) as well as the surrounding blood vessels (bv) were not stained. Some areas of the pineal epithelium remained unstained or weakly stained. At a high magnification (**c**) the typical segmented and elongated shape of the photoreceptors cells is clearly distinguished. Hybridization with either sense probe gave no labeling (**d**). Bars = 50 µm (**a**, **b**, **c**) and 200 µm (**d**).

Figure 3. Localization of TRPV1 and TRPV4 mRNA in trout retina.

Eight µm sections were hybridized with the antisense probes directed against TRPV1 (**a, b**) and TRPV4 (**c**). The photoreceptors in the outer nuclear layer (ONL) are labeled by both probes (**a, c**). Yellow arrow (**b, c**): position of the amacrine cells in the inner nuclear layer (INL). White arrow (**c**): position of the bipolar and Müller cells nuclei in the INL. Red arrow (b, c): ganglion cells. Double arrow (**b**): axons of the ganglion cells that will form the optic tract. GCL: ganglion cell layer; IPL: inner plexiform layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium. Bars = 50 µm (**a**, **b**, **c**).

Figure 4. Effects of time and temperature on pineal melatonin secretion *in vitro*.

The glands were placed in the dark at 12:00 and cultures for 12 h. the media were sampled at the times indicated. **a**: Effects of temperature at different incubation times. One way ANOVA indicated the variations were statistically significant at each temperature investigated; Mean ± SEM (n = 6, from one representative experiment). **b**: Kinetics of melatonin secretion at different temperatures. The data are replotted from (**a**) and express as a % of the secretion measured after 1 h of culture. The standard errors are omitted for clarity of the graph.

Figure 5. Impact of capsaicin (a) and 4αPDD (b) on pineal melatonin secretion *in vitro*.

Melatonin was measured after 6 h of culture in the dark and in the absence or presence of the drugs as indicated. **a, capsaicin**: One-way ANOVA indicated significant effects of capsaicin (one arrow). Two-way ANOVA indicated a significant interaction of both drugs (two arrows). **b, 4αPDD**: The variations were not statistically significant as analyzed by the two-way ANOVA. One-way ANOVA indicated no significant effects of 4αPDD alone. In the presence of the antagonist ruthenium red the inhibitory effect of 4αPDD was significant (one-way ANOVA; one arrow). Mean ± SEM (n = 13-14) from two independent experiments performed respectively, in January and May (**a**), and May and December (**b**).

Figure 6. Effects of TRPV1 and TRPV4 antagonists on pineal melatonin secretion *in vitro* at different temperatures.

The pineal organs were cultured for 6 h in the dark and at different temperatures as indicated in the abscissae, either in the presence of the TRPV1 antagonist capsazepine (1 µM) or of vehicle (methanol 0.01% in the case of capsazepine). The data are the mean from 2 independent experiments performed in March and June (capsazepine), and March from 2 successive years (gadolinium); for each experiment melatonin values were normalized to the values measured at 5°C. Mean ± SEM (n = 13-14). Two-way ANOVA indicated significant effects of capsazepine ($P<0.05$), which interacted significantly with temperature ($P<0.005$). The multiple comparison of means indicated that the effect was significant at 16°C; this was confirm by using the multiple *t* test for comparison of means ($P<0.0009$). In the case of gadolinium, neither the two-way ANOVA nor the multiple *t* test for comparison of means indicated any statistically significant difference.

Figure 7. Relative expression of TRPV1 and TRPV4 at two times of the year.

The pineal organs cultured as indicated in figure 6 were used to investigate the relative amounts of TRPV1 and TRPV4 mRNA in March (water temperature: 8°C) and June (water temperature: 15°C). In both cases, the relative mRNA abundance was higher in June compared to March. Mean ± SEM (n = 23 in March and 36 in June). Two-tailed Student's *t* test: $P<0.0001$.

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Figure 1

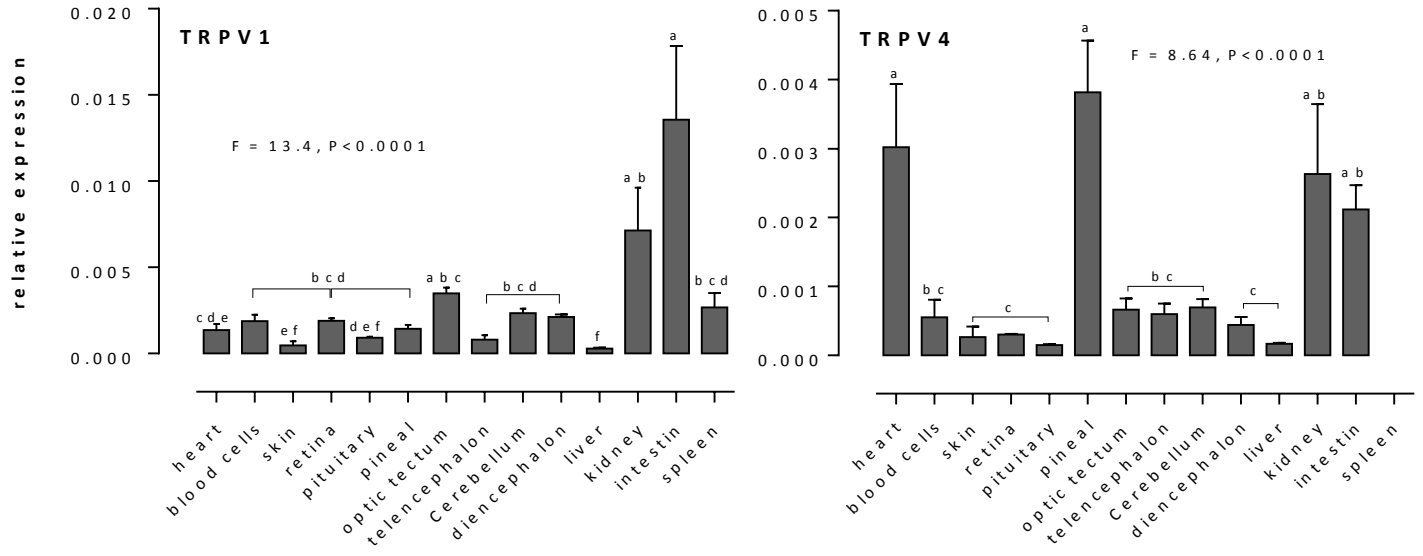

relative expression
0.020
0.015
0.010
0.005
0.000
TRPV1
F = 13.4, P<0.0001
c d e
e f
b c d
d e f
a b c
b c d
f
a b
a
b c d
heart
blood cells
skin
retina
pituitary
pineal
optic tectum
telencephalon
Cerebellum
diencephalon
liver
kidney
intestin
spleen
0.005
0.004
0.003
0.002
0.001
0.000
TRPV4
F = 8.64, P<0.0001
a
b c
c
a
b c
c
a b
a b
heart
blood cells
skin
retina
pituitary
pineal
optic tectum
telencephalon
Cerebellum
diencephalon
liver
kidney
intestin
spleen

Figure 2

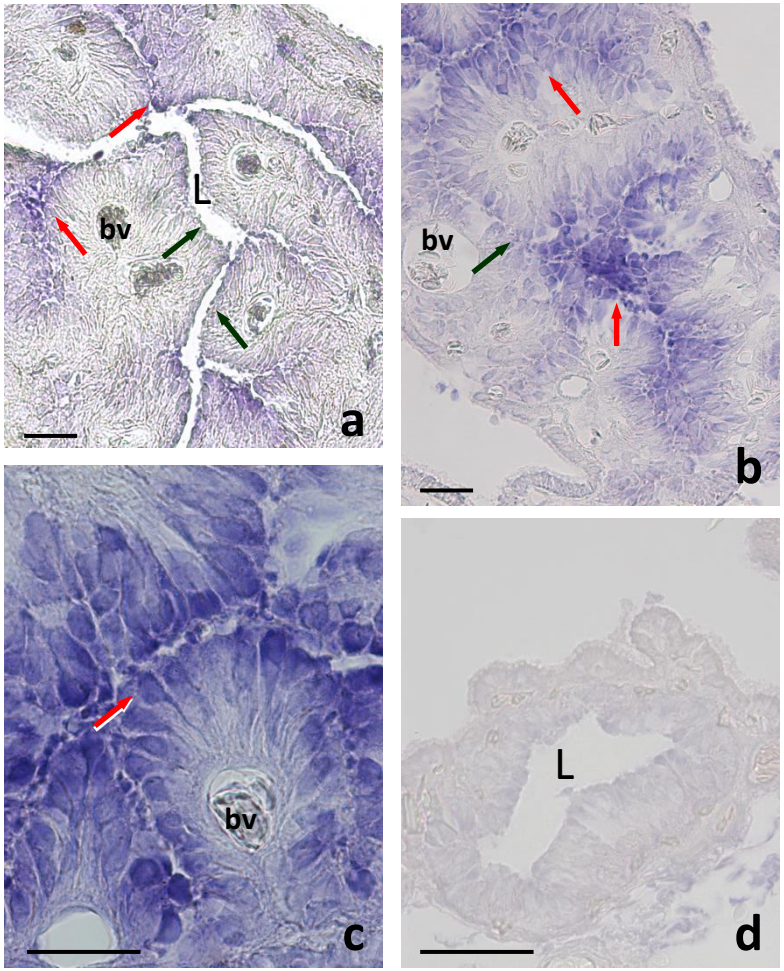


Figure 3

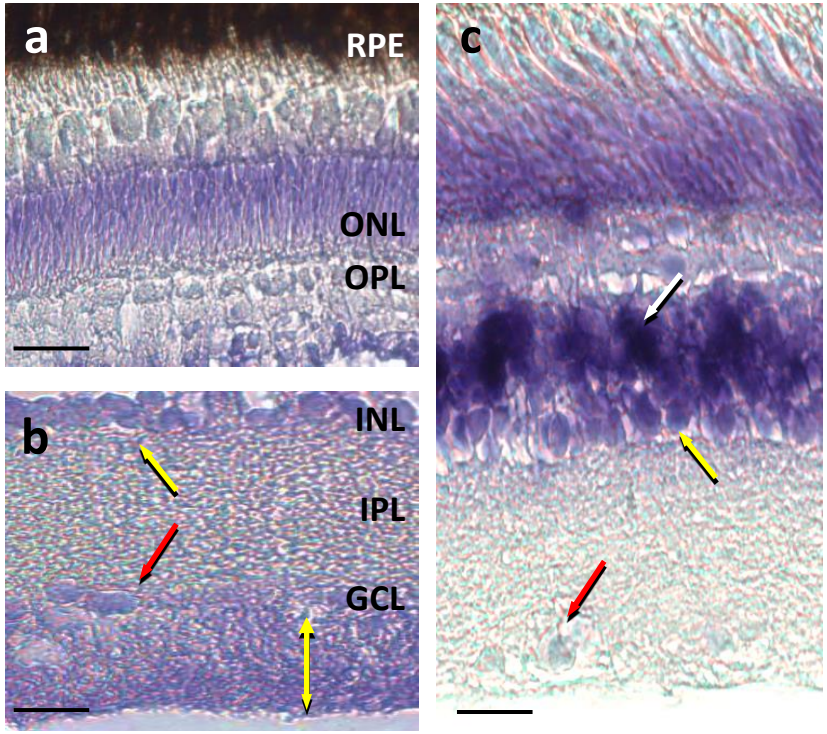


Figure 4

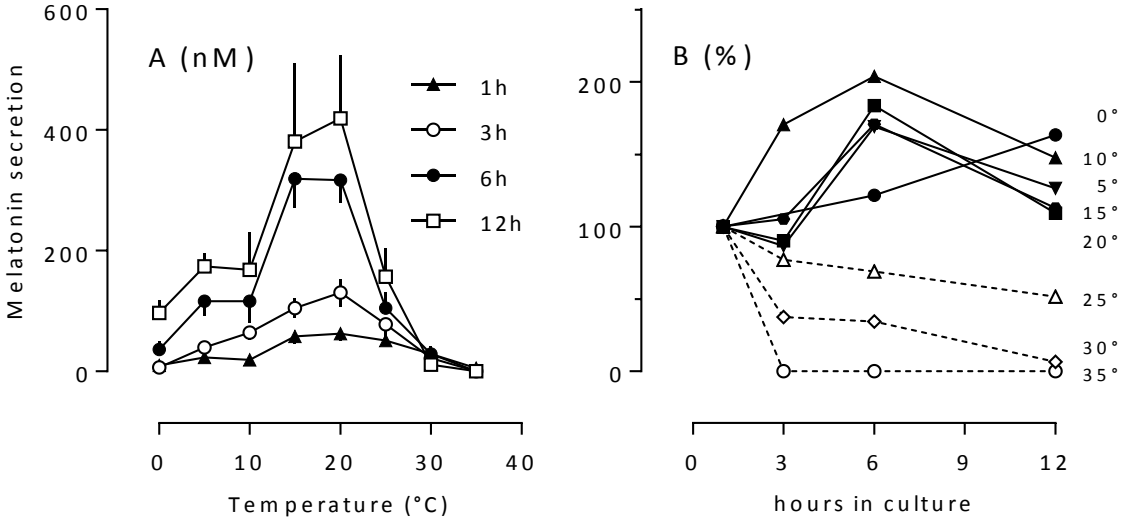

A (nM)
Melatonin secretion
600
400
200
0
1h
3h
6h
12h
0
10
20
30
40
Temperature (°C)
B (%)
200
100
0
0°
10°
5°
15°
20°
25°
30°
35°
0
3
6
9
12
hours in culture

Figure 5

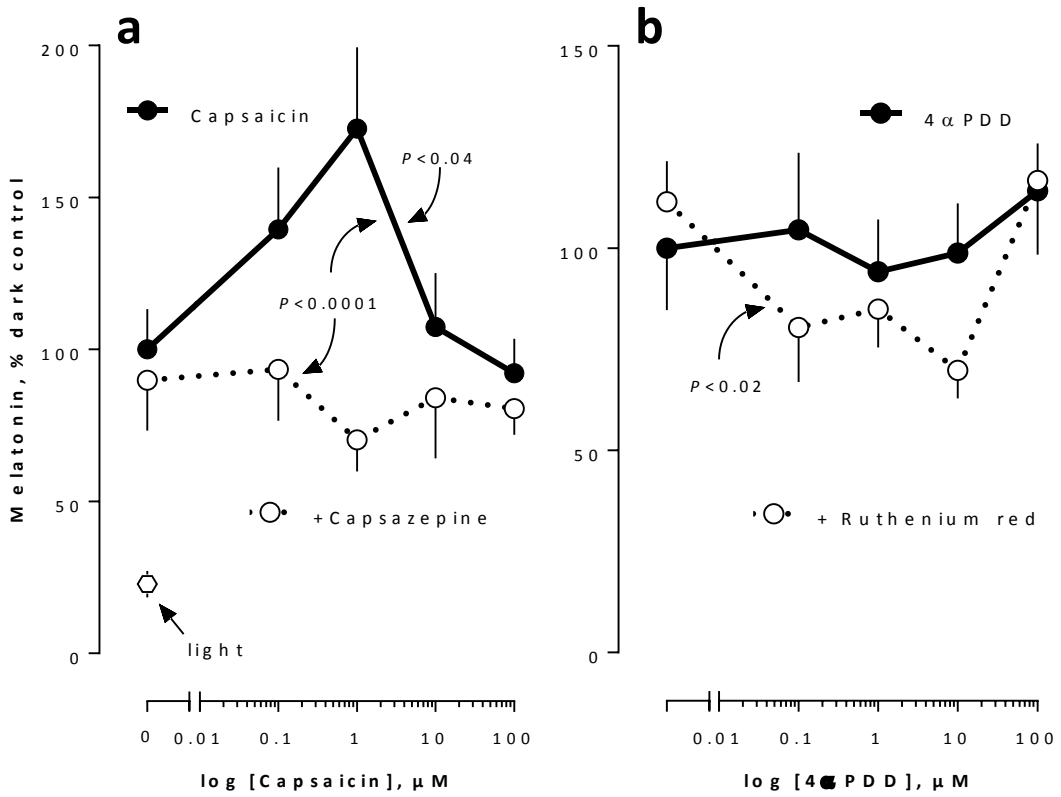

a
b
Capsaicin
4α PDD
P < 0.04
P < 0.0001
P < 0.02
+ Capsazepine
+ Ruthenium red
light
Melatonin, % dark control
200
150
100
50
0
150
100
50
0
0
0.01
0.1
1
10
100
0.01
0.1
1
10
100
log [Capsaicin], µM
log [4α PDD], µM

Figure 6

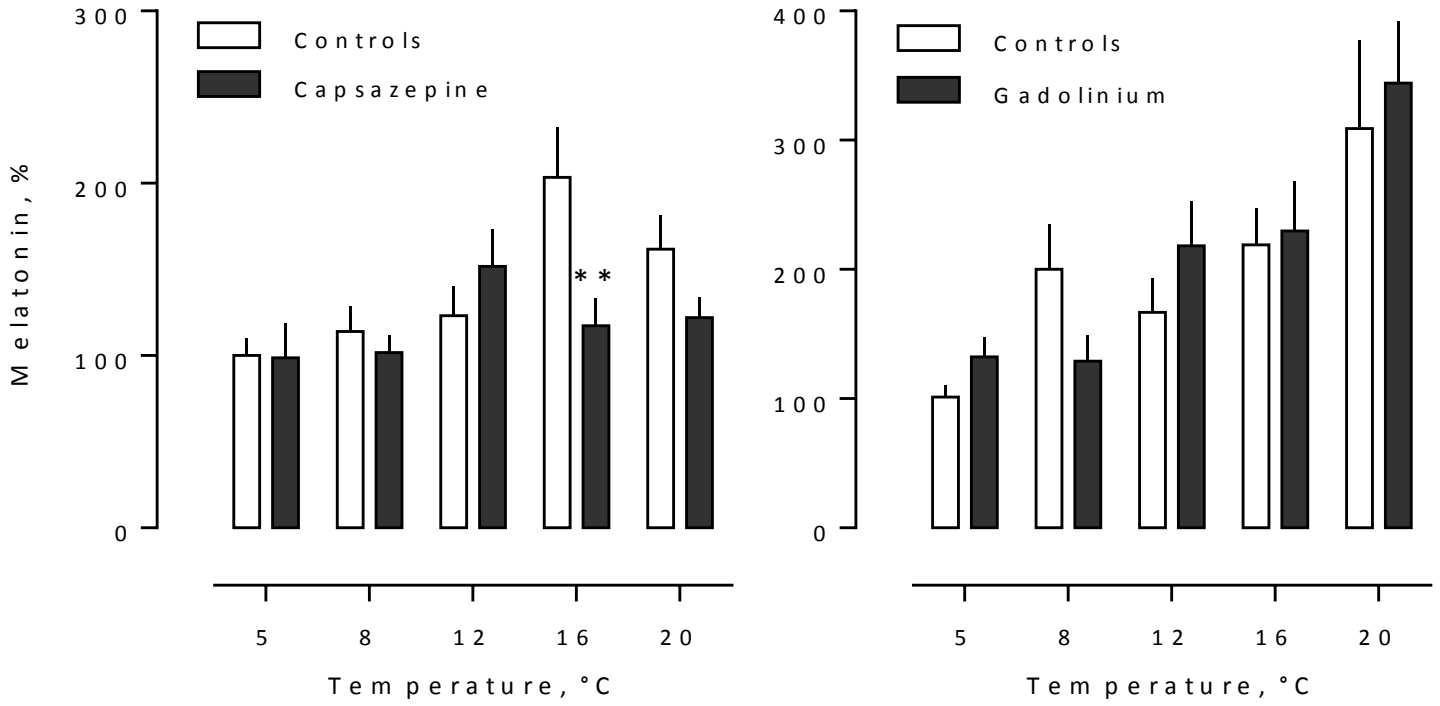
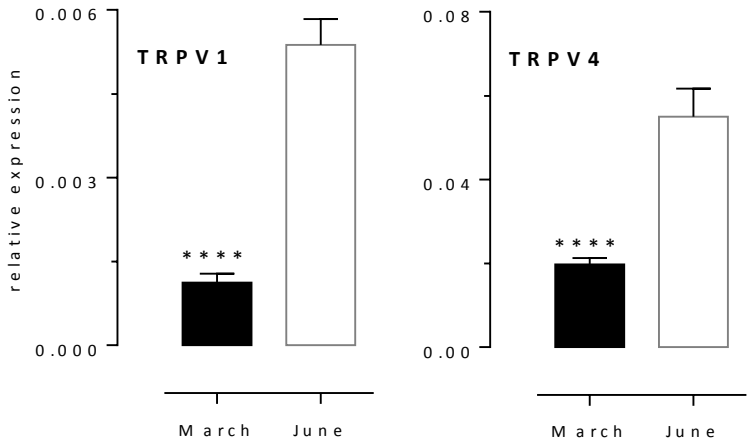

300
200
100
0
Melatonin, %
Controls
Capsazepine
**
5
8
12
16
20
Temperature, °C
400
300
200
100
0
Controls
Gadolinium
5
8
12
16
20
Temperature, °C

Figure 7


relative expression
0.006
0.003
0.000
TRPV1
****
March
June
0.08
0.04
0.00
TRPV4
****
March
June