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

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1 **Title:** In the heat of the night: Thermo TRPV channels in the salmonid pineal photoreceptors and
2 modulation of melatonin secretion

3

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11

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13

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20

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26

27

28 **Abstract**

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

47

48

49 INTRODUCTION

50 The pineal photoreceptor cells of ectothermic vertebrates transduce light and temperature
51 information into a nervous and a neurohormonal output. The former, aspartate and/or glutamate, is
52 released in the dark at the synaptic contacts with the pinealofugal neurons; the neurons then convey
53 rapid (msec) electric information to the brain (1,2). The latter, melatonin, is produced at night and this
54 24h rhythm is an essential component of the fish time keeping system (3,4). The duration of the
55 melatonin surge is dictated by photoperiod while temperature controls the amplitude (3,5). This
56 melatonin rhythm contributes to synchronizing metabolism, physiology and behavior to the daily and
57 annual variations of the environment (4). While the impact of photoperiod on the pineal outputs is
58 relatively well understood, virtually nothing is known on how temperature operates. More generally,
59 little progress has been made in the last decades concerning temperature regulation and transduction of
60 the temperature information in ectothermic vertebrates (6-8). Temperature sensitive neurons have been
61 identified in the lateral line (9), spinal cord (10,11), and in the epithalamus and preoptic area of the
62 brain (12,13); the latter also receives thermal input from other unidentified photosensitive structures.
63 Interestingly, the pineal organ of fish has been involved in behavioral thermoregulation mediated by
64 both a rapid (nervous) and a slow (hormonal) mechanism (14-16). And, it has been established that
65 both the nervous (17) and neurohormonal (18) information released by the organ reach the preoptic
66 area, a major neuroendocrine center. Altogether, although clear direct evidence is still missing, there is
67 strong support that the fish pineal organ is a photo-thermo-sensor. The questions then rise to know
68 how and where is the temperature information captured and transduced within the organ?

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

76 also suspected, as is the case in the retinal cone (23). (iii) In cultured pineal organs, both cAMP
77 production and AANAT activity display the same pattern in response to temperature; this is a species-
78 dependent process (5). It is also a cellular-mediated process because the response of AANAT activity
79 to temperature variation differs if measured on the native (*i.e., in situ*) or recombinant enzymes
80 (24,25). (iv) In mammals, temperature sensing is mediated by members of the Transient Receptor
81 Potential (TRP) channels family (TRPA1, TRPV1-4 and TRPM8), which are permeable to Ca^{2+}
82 (26,27), and activate at different ranges of temperature (28).

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

92

93 **MATERIALS AND METHODS**

94 **Animals**

95 Two years-old rainbow trout, *Oncorhynchus mykiss*, were provided by “Les Viviers Cathares”
96 (Chalabre, France). Animals were sacrificed at the hatchery by electrocution and the organs of interest
97 were dissected out and placed either in ice-cold fixative, RNA later® or culture medium. All
98 experiments were performed according to the European Union regulations (European directive
99 91/492/CCE).

100 **Pineal organs culture**

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

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

109 **Melatonin quantification**

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

113 **RNA extraction, cDNA synthesis and sequences analysis**

114 See *Supplemental Materials and Methods*.

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

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

119 **In situ hybridization**

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

123 **Statistics and drawings**

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

127 **Compounds and chemicals**

128 See *Supplemental Materials and Methods*.

129

130 **RESULTS**

131 **Cloning of TRPV1 and TRPV4**

132 *O. mykiss* TRPV1 and TRPV4 were cloned from pineal extracts. The TRPV1 mRNA sequence
133 was 2807 bp long (GenBank Acc # KJ135121); the corresponding coding region was 2415 bp long
134 and encoded a 805 aa sequence that displayed high identity with the TRPV1 sequences from *S. salar*
135 (96%), and the predicted TRPV1 sequence from *Esox lucius* (80%) and lower identity with those from
136 other fish (~60% with *O. niloticus* and *D. rerio*) and tetrapods (<53%) (*Supplemental Fig. 1*). The
137 TRPV4 mRNA sequence was 3035 bp long (GenBank Acc # KJ135122); the corresponding ORF
138 (2637 bp) encoded a 879 aa sequence which shared 98% identity with *S. salar* TRPV4, 89% with *D.*
139 *labrax* TRPV4, 79% with *D. rerio* and *O. mossambicus* TRPV4 and 70-75% with tetrapods TRPV4
140 (*Supplemental Fig. 1*). The *InterProScan* search for conserved domains confirmed that trout TRPV1
141 and TRPV4 belong to the superfamily of TRPV channels, possessing the typical ankyrin repeats, the
142 calcium moiety and the 6 transmembrane domains (*Supplemental Fig. 1*). In addition, aa residues
143 known to be important for the function of the rat TRPV1 were also identified in the fish sequences, as
144 discussed later and *Supplemental Fig. 1*. Finally, the phylogenetic reconstruction indicated TRPV1
145 and TRPV4 grouped with their respective orthologues (not shown).

146 **Tissue distribution and relative expression of TRPV1 and TRPV4**

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

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

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

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

161 **Impact of temperature on melatonin secretion**

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

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

173 **Impact of TRPV agonists and antagonists on pineal melatonin**

174 In view of the above results we decided to investigate the effects of the TRPV analogs at the
175 temperature at which the animals were adapted in their natural habitat at the time the experiments were
176 done (*i.e.*, between 10 and 20°C) and for a 6 h incubation. Under these conditions, capsaicin, a TRPV1
177 agonist, induced a dose-dependent effect on melatonin release, stimulatory at low, and inhibitory at
178 high, concentrations; both effects were neutralized in the presence of capsazepine, a TRPV1
179 antagonist, (1 µM; Fig. 5a). No effect was detected with the agonist 4- α -phorbol-12,13-didecanoate
180 (4 α PDD) alone (Fig. 5b). This suggested to us that the TRPV4 channel expressed in the photoreceptor
181 cells (Fig. 2), did not respond to the agonist challenge or were already maximally active. The later
182 explanation remains possible because in the presence of ruthenium red (RuR; 10 µM), a TRPV4
183 blocker, 4 α PDD induced inhibition at low concentrations and stimulation at high concentrations (Fig.
184 5b).

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

187 on the ambient temperature. To investigate this point, pineal glands were cultured at different
188 temperatures and in the absence or presence of either 1 μ M for capsazepine, the TRPV1 antagonist, or
189 10 μ M gadolinium, the TRPV4 antagonist. Figure 6 shows the data obtained from two independent
190 experiments performed in spring. We found that in the presence of the TRPV1 antagonist melatonin
191 secretion was ~40 % lower at 16°C and ~20% lower at 20°C, compared to controls. The results were
192 however significant only at 16°C. The TRPV4 antagonist gadolinium was found to inhibit melatonin
193 secretion at 8°C by ~30% (Fig. 6); the effect was however not statistically significant.

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

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

201

202 **DISCUSSION**

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

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

216 The main focus of this study was to investigate the possible involvement of thermo-TRPs in
217 the temperature-dependent control of melatonin production in trout. Two studies in the zebrafish
218 indicated TRPV1 and TRPV4 are involved in temperature sensing (32,37). It was therefore of interest
219 that both thermo-TRPs were expressed in the pineal gland and retina, the two main sites where
220 melatonin is produced. As emphasized in the introduction, the pineal organ deserves special attention
221 because of its capacity to sense both light and temperature (5), and because it is involved in behavioral
222 thermoregulation (15,16). We bring here the first and unequivocal evidence that TRPV1 and TRPV4
223 are co-expressed exclusively in the melatonin producing cells of the pineal organ, *i.e.*, the cone
224 photoreceptors (5). This contrasts with previous studies in the zebrafish where pineal expression was
225 reported neither for TRPV1 nor for TRPV4, although both were expressed in all other sensory organs
226 (32,36). It is noteworthy, however, that a TRPV1-like protein was immuno-detected in the rat
227 pinealocyte (38). This cell type is a homologue of the fish pineal photoreceptor; it produces melatonin
228 but has lost its direct photosensitive properties (4). The presence of TRPV1 in the melatonin producing
229 cells of the fish and mammalian pineal glands appears thus as an ancestral character, shared by these
230 homologous cells. In the mammalian pinealocyte the TRPV1-immuno-detected protein was associated
231 with the synaptic ribbons, which function is enigmatic. Indeed, in contrast with the fish pineal
232 photoreceptor the mammalian pinealocyte does not establish synaptic transmission *sensu stricto*; the
233 gland has lost the second-order neurons, which in fish convey the light information to the brain (4,39).
234 Interestingly however, TRPV1 has also been observed in the synaptic ribbons of the retinal
235 photoreceptors of the zebrafish and goldfish (40). It is well established that the fish pineal
236 photoreceptors display structural and functional analogies with the vertebrates' retinal cones; both
237 derive from a common diencephalic origin (39). For this reason we felt interesting to explore TRPV1
238 and TRPV4 sites of expression also in the retina. Our study confirms the presence of TRPV1 in the
239 retinal photoreceptors and thus extends the already high number of analogies between the retinal and
240 pineal photoreceptors. We also extend these analogies to TRPV4. The identification of TRPV4 in
241 trout, contrasts with previous observations in the zebrafish retina (36,41). Perhaps these discrepancies
242 result from the use of immunocytochemistry *vs.* *in situ* hybridization. The former is probably less

243 specific particularly when it comes to detect a membrane bound protein, and with an antibody
244 generated against TRP from other more distant species. The presence of TRPVs in trout pineal and
245 retinal photoreceptors is consistent with the idea that TRPVs modulate the Ca²⁺-dependent release of
246 the excitatory neurotransmitter at the ribbons synapses, as is the case in cerebral neurons (26,37). We
247 may speculate that the TRPVs expressed in the synaptic pedicles of the pineal photoreceptors are
248 involved in the regulation of the described effects of temperature on the nervous discharges of the
249 trout pineal neurons (2). We also found that TRPV1 and TRPV4 were expressed in the INL of the
250 trout retina, as is the case in zebrafish (36,42). In contrast the GCL of trout expressed TRPV1 only,
251 while the opposite was found in the zebrafish. It is beyond the scope of this work to speculate on the
252 discrepancies and roles played by TRPV in the different layers of the retina that may be due to
253 species-dependent characteristics, or to methodological and technical issues.

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

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

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

299 chemical activation (48). It is also found in the zebrafish sequence (Y659). Interestingly, one study in
300 the zebrafish indicated that TRPV1, which is required for normal heat-induced locomotion, activates
301 at a temperature $\geq 25^{\circ}\text{C}$ (32). This is above the suspected range of temperature sensitivity found here
302 in the trout, but below the activation threshold of the rat TRPV1 (42°C). It has been suspected that
303 differences in the C-terminal tail length of TRPV1 are responsible for the observed differences in
304 threshold activation of TRPV1 in vertebrates (49). They may reflect specific adaptation to the
305 environmental requirements of the species considered.

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

317 **Conclusions**

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

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

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

343

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351

352 **LEGEND OF FIGURES**

353

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

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

358

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

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

367

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

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

376

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

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

384

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

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

394

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

397 The pineal organs were cultured for 6 h in the dark and at different temperatures as indicated in the
398 abscissae, either in the presence of the TRPV1 antagonist capsazepine (1 μ M) or of vehicle
399 (methanol 0.01% in the case of capsazepine). The data are the mean from 2 independent
400 experiments performed in March and June (capsazepine), and March from 2 successive years
401 (gadolinium); for each experiment melatonin values were normalized to the values measured at
402 5°C. Mean \pm SEM (n = 13-14). Two-way ANOVA indicated significant effects of capsazepine
403 ($P < 0.05$), which interacted significantly with temperature ($P < 0.005$). The multiple comparison of
404 means indicated that the effect was significant at 16°C; this was confirm by using the multiple *t* test
405 for comparison of means ($P < 0.0009$). In the case of gadolinium, neither the two-way ANOVA nor
406 the multiple *t* test for comparison of means indicated any statistically significant difference.

407

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

409 The pineal organs cultured as indicated in figure 6 were used to investigate the relative amounts of
410 TRPV1 and TRPV4 mRNA in March (water temperature: 8°C) and June (water temperature:
411 15°C). In both cases, the relative mRNA abundance was higher in June compared to March. Mean
412 \pm SEM (n = 23 in March and 36 in June). Two-tailed Student's *t* test: $P < 0.0001$.

413

414

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Figure 1

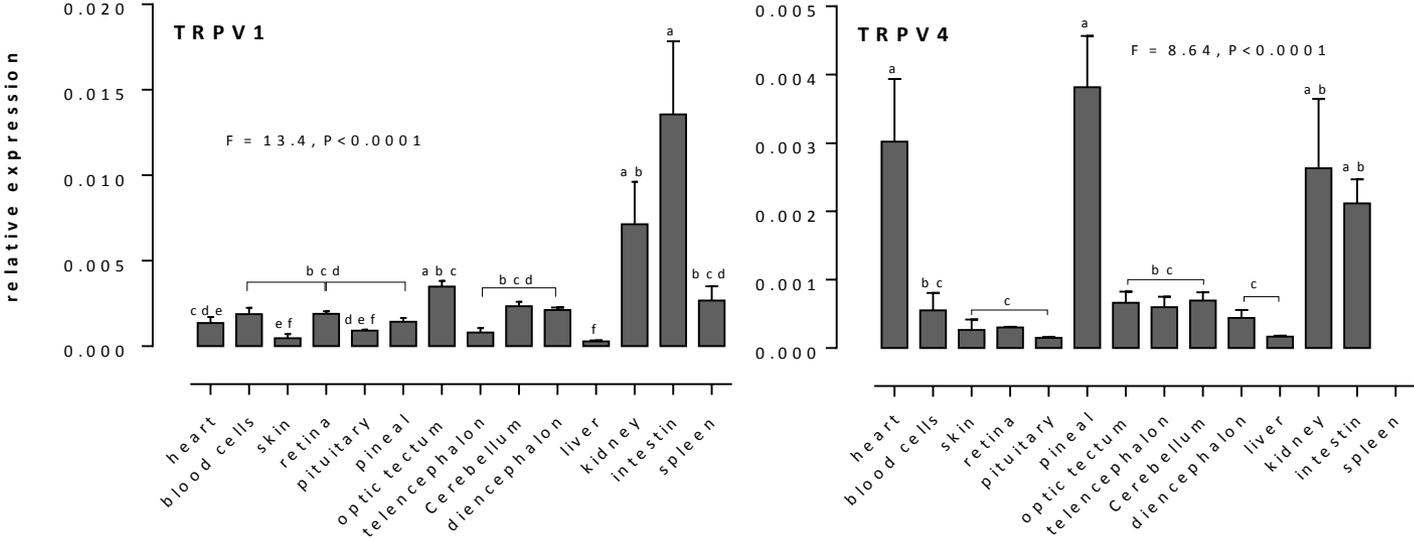


Figure 2

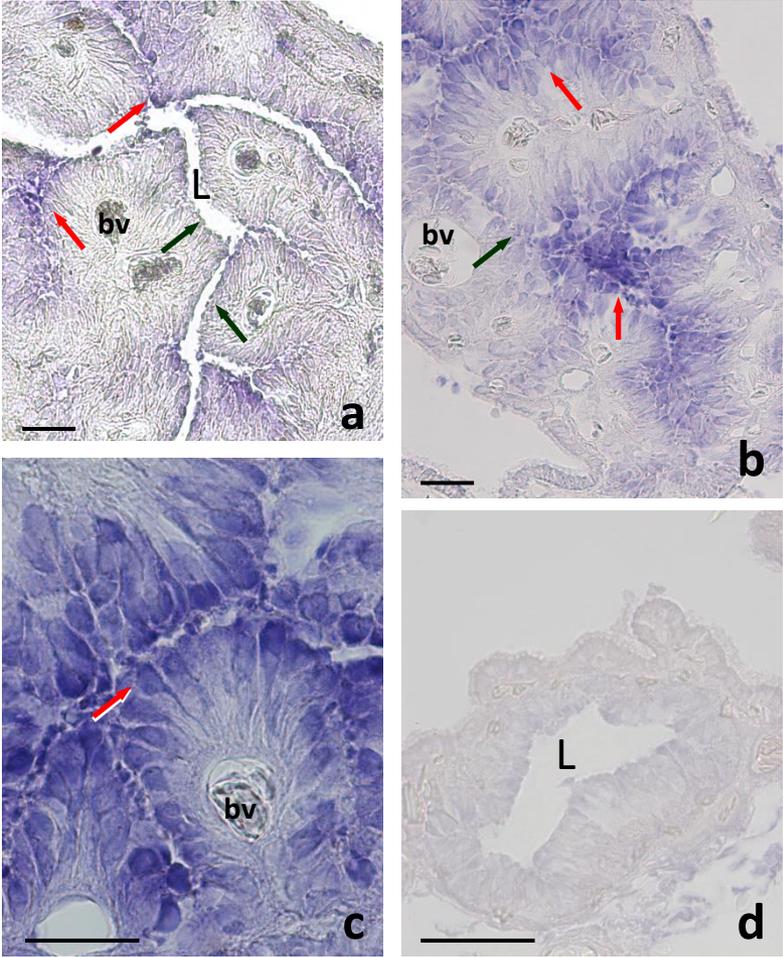


Figure 3

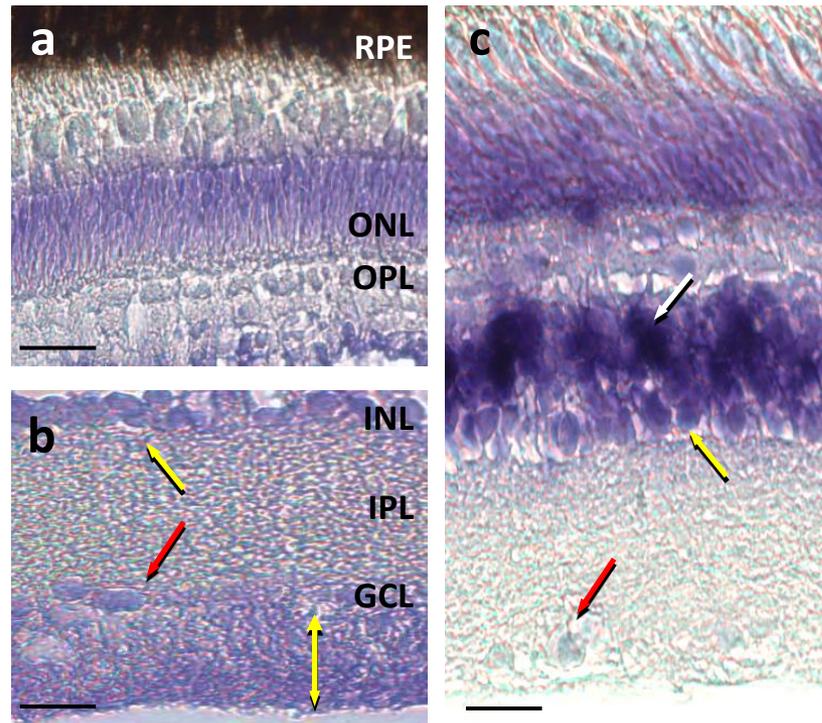


Figure 4

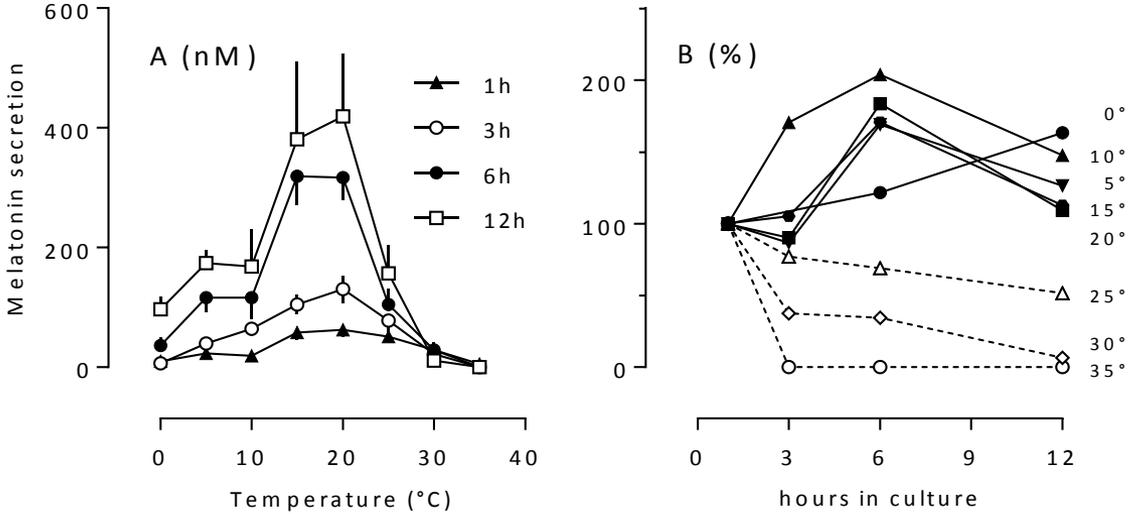


Figure 5

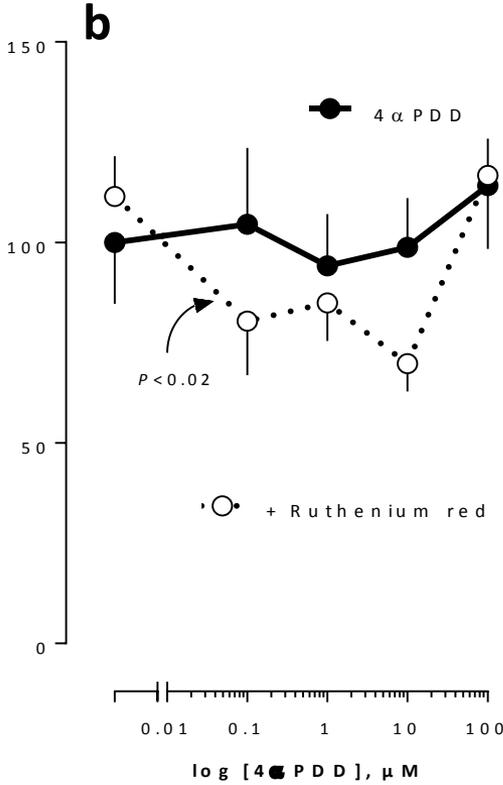
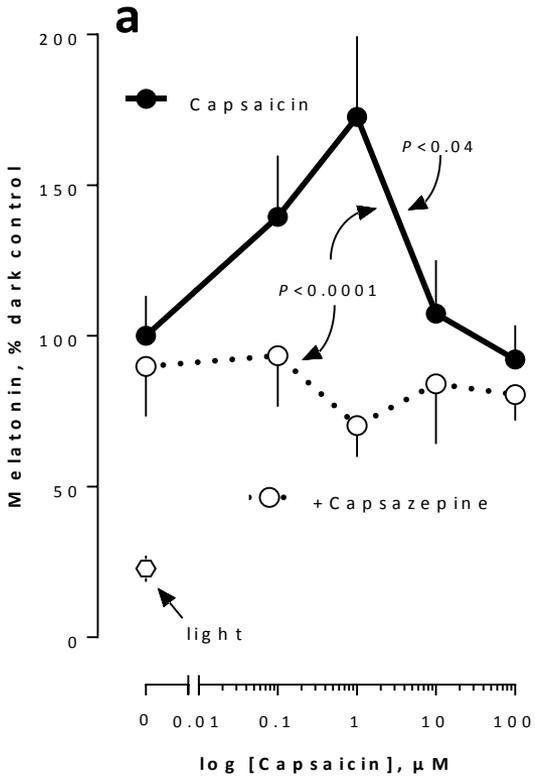


Figure 6

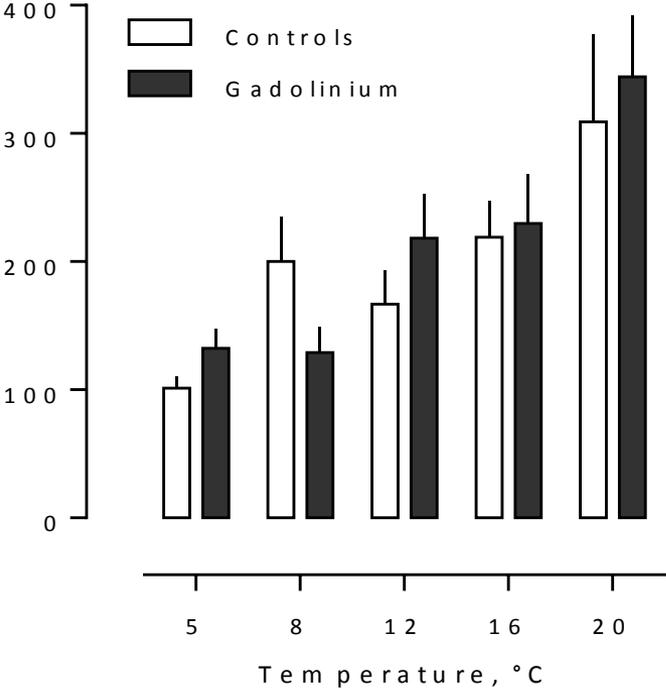
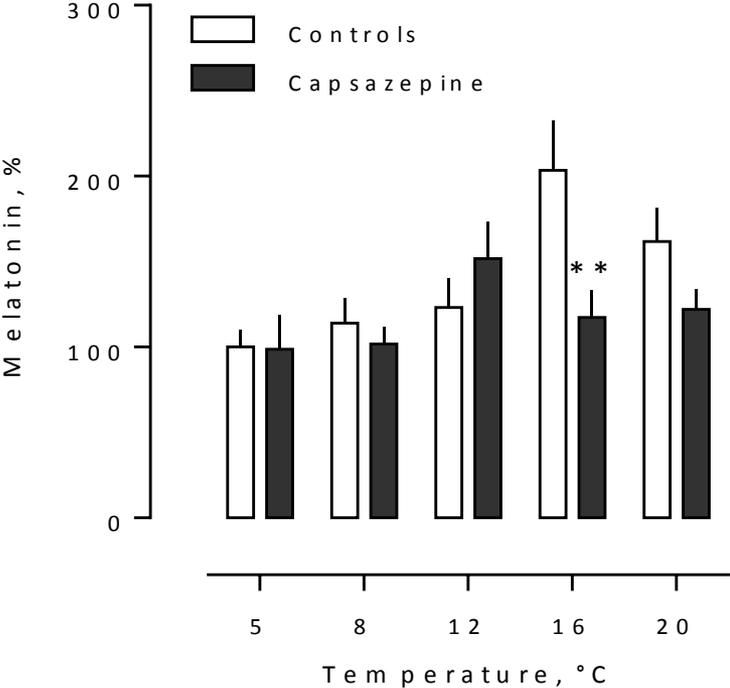


Figure 7

