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Melatonin Modulates Secretion of Growth Hormone and Prolactin by Trout Pituitary Glands and Cells in Culture

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In Teleost fish, development, growth, and reproduction are influenced by the daily and seasonal variations of photoperiod and temperature. Early *in vivo* studies indicated the pineal gland mediates the effects of these external factors, most probably through the rhythmic production of melatonin. The present investigation was aimed at determining whether melatonin acts directly on the pituitary to control GH and prolactin (PRL) secretion in rainbow trout. We show that 2-[^{125}I]-iodomelatonin, a melatonin analog, binds selectively to membrane preparations and tissue sections from trout pituitaries. The affinity was within the range of that found for the binding to brain microsomal preparations, but the number of binding sites was 20-fold less than in the brain. In culture, melatonin inhibited pituitary cAMP accumulation induced by forskolin, the adenyl cyclase stimulator. Forskolin also induced an increase in GH release, which was reduced in the presence of picomolar concentrations of melatonin. At higher concentrations, the effects of melatonin became stimulatory. In the absence of forskolin, melatonin induced a dose-dependent increase in GH release, and a dose-dependent decrease in PRL release. Melatonin effects were abolished upon addition of luzindole, a melatonin antagonist. Our results provide the first evidence that melatonin modulates GH and PRL secretion in Teleost fish pituitary. Melatonin effects on GH have never been reported in any vertebrate before. The effects result from a direct action of melatonin on pituitary cells. The complexity of the observed responses suggests several types of melatonin receptors might be involved. (*Endocrinology* 144: 4648–4658, 2003)

IN VERTEBRATES, LIGHT IS a major environmental factor regulating the daily and seasonal rhythms of melatonin production (1). Melatonin is an internal timekeeping molecule that plays a role in the timing and control of a number of physiological processes and behaviors (2–4). Retinal melatonin is produced, used, and metabolized locally, whereas melatonin from the pineal gland is released into the blood and acts as a neuroendocrine hormone (1). In all vertebrate species examined thus far, pineal and blood melatonin levels are high during nighttime and low during daytime, and this daily pattern changes on an annual basis as a consequence of the seasonal variations in day length.

In Teleost fish, the pineal gland and melatonin have been involved in the control of daily variations of locomotor activity, sleep-like state, skin pigmentation, or demand feeding; and crucial annual functions, such as growth and reproduction, are also influenced by melatonin from the pineal gland (2, 4). However, the available data differ with gender, photoperiod, temperature, and reproductive stage, so that no clear-cut picture arises. Moreover, we have no idea of how and where melatonin might act to mediate the effects of photoperiod. This is further complicated by the observation that expression of melatonin receptors and 2-[^{125}I]-iodomelatonin (^{125}I-Mel) binding sites display a widespread distribution in the fish brain (5–7), in marked contrast with the situation described in mammals (8).

For a number of reasons, we have suspected that melatonin might act directly on the hypothalamus-pituitary-gonadal axis of fish to modulate the secretion of GH and prolactin (PRL). First, GH and PRL are members of the same hormone family presumably derived from a common ancestor molecule (9). Second, changes in photoperiod modulate fish growth and development (4, 10), and adaptation to salinity (11, 12). Third, pinealectomy and/or melatonin administration affect the size of pituitary PRL-producing cells (13) as well as a number of GH- and PRL-related processes, including plasma electrolyte balance (fresh water Teleost fish only), body fat content, liver glycogen levels (2). Moreover, there is increasing evidence that GH and PRL act in opposite directions to modulate water and electrolyte balance in fish (14). Fourth, melatonin affects hypothalamic processes known to be involved in the control of GH secretion, including serotonin (goldfish, snakehead) and dopamine (trout) metabolism (2, 15); the preoptic nuclei might mediate part of these effects because they contain dopaminergic neurons and express ^{125}I-Mel binding sites (5, 7). Fifth, we have shown expression of melatonin receptors cDNA and ^{125}I-Mel binding sites in the pike pituitary (16, 17). Finally, melatonin modulates cAMP content in cultured pike pituitaries (16), and GH release is a cAMP-dependent process (18). Altogether, these observations suggest that melatonin may act directly on pituitary cells to modulate GH and PRL secretion.

The present study was designed to determine whether melatonin could act directly on the trout pituitary to modulate GH and PRL secretion. For this purpose, we examined the binding of ^{125}I-Mel on crude membrane preparations and

Abbreviations: 3-D, Three-dimensional; ECL, enhanced chemiluminescence; ^{125}I-Mel, 2-[^{125}I]-iodomelatonin; LA, labeled; NLA, nonlabeled; PPD, proximal pars distalis; PRL, prolactin; anti-rtGH, rainbow trout anti-GH; anti-rtPRL, rainbow trout anti-PRL.

frozen tissue sections. We also investigated the effects of melatonin on cAMP accumulation as well as GH and PRL secretion, in trout pituitary glands and cells in culture.

Materials and Methods

Fish

Female rainbow trout (*Oncorhynchus mykiss*, Teleost) originated from two commercial hatcheries: trout from La pisciculture de Piedelance (Vienne, France) weighed 250–300 g (^{125}I-Mel binding; cAMP quantification); trout from Les Viviers Cathares (Aude, France) weighed 1000–1200 g (all other experiments). All animal experimentation was conducted in accord with accepted standards of humane animal care. Fish were killed by decapitation. The pituitaries were removed, and either frozen in dry ice and stored at −78 C (binding assays on membranes), or frozen in isopentane at −50 C (binding assays on tissue sections), or dipped in culture medium at +4 C (all other experiments).

Binding assays on tissue sections

Serial pituitary sections were obtained on a cryostat at −20 C and processed for autoradiographic binding as previously described (16). In brief, sections were mounted on gelatin-coated slides and kept at −20 C. Sections were then preincubated at +4 C for 15 min in Tris-HCl buffer (100 mM, pH 7.4) containing 4 mM $CaCl_2$. They were then incubated in the same buffer containing 100 pM ^{125}I-Mel, with or without an excess of cold melatonin (1 μM) for 60 min at room temperature. After washing, sections were air-dried and exposed to BioMax film (Kodak, Rochester, NY) for 10 d. Six trout pituitaries were used for this experimental series, which were processed in three independent experiments.

Binding assays on pituitary membranes

Preparation of membranes and binding assays were as detailed elsewhere (16). In brief, 60 pooled pituitaries were homogenized by means of an Ultraturax in ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 4 mM $CaCl_2$. The homogenate was centrifuged at 800 × *g* (10 min, +4 C), and the pellet was discarded. The supernatant was then centrifuged again at 80,000 × *g* (20 min, +4 C). The resulting pellet was suspended by sonication in Tris-HCl buffer at the concentration of 2–5 mg proteins/ml. The suspension was stored at −78 C. Protein concentrations were determined using the method of Bradford with BSA as standard. Aliquots containing 50–100 μg of membrane proteins were incubated in a final volume of 60 μl of Tris-HCl buffer in the presence of increasing concentrations of ^{125}I-Mel. The conditions were those set up for the binding on membrane preparations from trout brain (6). Nonspecific binding was determined in the presence of cold melatonin in excess (50 μM). After a 1-h incubation at 21 C, membranes were collected by vacuum filtration through Whatman GF/C glass fiber filters. The filters were washed three times with ice-cold buffer and radioactivity was measured (Wallac γ counter, PerkinElmer, Cogniere, France). Each curve corresponds to data obtained from 60 pooled pituitaries, and each plot corresponds to triplicate determinations. This experiment was duplicated. Data were fitted to the equation of a rectangular hyperbola.

Organ and cell cultures

The culture medium was HEPES-buffered RPMI 1640 supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Fetal calf serum (10%) was added only in experiments where cAMP was measured. A serum-free culture medium was used when GH and PRL were measured. Culture was at 14 ± 1 C in a 5% CO_2/95% O_2 atmosphere.

After dissection, pituitary glands were cultured in 24-well culture plates (1 pituitary/well/ml), and medium was changed 24 h later. The pharmacological treatments were performed in a final volume of 600 μl/well (see below). Pituitary cells were obtained by first cutting 20 organs into pieces by means of a razor blade. The pieces were then incubated for 30 min (under gentle stirring, at room temperature) in 30 ml of Ca^{2+}/Mg^{2+}-free Hanks' solution, containing 0.08 U/ml trypsin. The undigested fragments were decanted (5 min at +4 C), and the suspension was centrifuged for 5 min at 800 × *g* (+4 C). The cells in the pellet were washed in a fresh Ca^{2+}/Mg^{2+}-free Hanks' solution for 5 min, centrifuged again, and finally suspended in culture medium. The decanted undigested fragments were incubated in a fresh trypsin solution containing 0.004% deoxyribonuclease, and a second cell suspension was obtained as indicated above. The cells from the first and second suspensions were pooled and counted. Viability was greater than 95% as assessed by the trypan blue extrusion method. Typically, cells were seeded at the density of 350,000-cells/well/300 μl (96-wells poly-lysine coated plates), or 750,000 cells/well/600 μl (24-well collagen-coated plates, or polycarbonate inserts; Transwells, Costar, Corning, NY).

The pharmacological treatments were performed 2 d after initiating the culture. The organs and cells were cultured in the presence of the drugs and for the durations indicated in the results section and legends of the figures. For treatment durations longer than 3 h, medium was changed every 3 h. At the end of the incubation period, the pituitary glands were frozen (cAMP measurements) or immersed in fixative (immunocytochemistry). The culture media were sampled and frozen at −20 C.

cAMP measurements

cAMP was quantified using a commercially available kit (Amersham Pharmacia Biotech, Orsay, France) using [^{125}I]-cAMP as a tracer.

Immunochemical detection of GH

GH and PRL detections were performed according to the indirect method, using specific rainbow trout anti-GH (anti-rtGH) and anti-PRL (anti-rtPRL) antibodies raised against the corresponding recombinant proteins (19, 20).

Immunocytochemistry. At the end of the culture, rainbow trout pituitaries were fixed for 12 h in Halmi fixative and then embedded in paraffin (21). Horizontal 5 μm thick pituitary sections were mounted onto precoated (3-aminopropyl triethoxysilane) slides and air-dried overnight at 32 C. The sections were deparaffinized, re-hydrated in graded ethanol series, rinsed in distilled water and then in Tris-BSA buffer. The following steps consisted of: 1) 30 min in a 3% H_2O_2 solution; 2) 1 h, or overnight at +4 C, in the primary antibody dilution (anti-rtGH, 1/500; anti-rtPRL, 1/300); 3) 30 min in a 1/500 solution of biotinylated goat antirabbit IgG; 4) 1 h 30 min in the avidin-biotin peroxidase complex diluted 1/50; 5) 5 min in 3,3′-diaminobenzidine. Each of these steps was followed by three 10 min washes in buffer. The primary antibody (step 2) was omitted in the control sections. Finally, the sections were dehydrated, and mounted in Depex (EMS, Fort Washington, PA).

Western and dot blots. For the Western blots, proteins extracted from trout pituitaries or released in the culture medium were resolved on 15% SDS-PAGE gels (22). Gels were run at 100 mV for 2 h. Prestained SDS-PAGE molecular weight standards were used to determine the molecular weight of the proteins. The gels were equilibrated in transfer buffer (Tris-glycine-20% methanol) for 5 min, and the proteins were transferred overnight to 0.45-μm Immobilon-P membranes using an electroblotting transfer system, according to the manufacturer's protocol instructions. The transfer current was 25 mA. For the dot blots, 20–40 μl of culture medium were directly layered, under vacuum, on Immobilon-P membranes using a dot-blot apparatus. All the membranes were air-dried overnight and then blocked for 2 h in PBS (pH 7.4) containing 0.2% Tween-20 and 0.05% thimerosal (TPBS), and 10% nonfat dry milk. They were then incubated 2 h (room temperature), or overnight (+4 C), in the primary antibody dilution (anti-rtGH, 1/600,000; anti-rtPRL, 1/120,000); in PBS containing 1 mg/liter BSA fraction V). The membranes were then washed in TPBS (twice for 10 min each followed by twice for 5 min each), then in PBS (twice for 5 min each) before a 1-h exposure to horseradish peroxidase conjugated goat antirabbit IgG (0.00083 μg/ml in TPBS containing 0.1% normal goat serum). Finally, the membranes were washed in TPBS (three times for 10 min each), then in PBS (three times for 5 min each). Immunodetection was performed using the enhanced chemiluminescence (ECL) system, and the blots were exposed to Biomax films.

Quantification of GH immunopositive areas in tissue sections

The immunostained sections were observed on a Leica (Rueil Malmaison, France) microscope, connected to a video system (SSC-C108P, Sony,

Tokyo, Japan) and a computer (Power Macintosh G3; Microsoft, Redmond, WA). Images (640 × 960 pixels) were recorded using the Strata VideoShop software (Apacabar, Lyon, France) and analyzed using the National Institutes of Health Image software. The surfaces of the labeled (LA) and unlabeled (NLA) areas were calculated for each pituitary section; the ratio $R = \Sigma_{LA}/\Sigma_{NLA}$ was then deduced for each pituitary gland, where Σ_{LA} and Σ_{NLA} are the sum the LA and NLA areas, respectively. A representative R-value was obtained when taking one out of nine serial sections in each pituitary gland (~300 sections). The R values from a same experimental group of glands were then averaged (n = 3).

Quantification of GH and PRL on dot blots

Preliminary investigations indicated there was a linear relationship between the amount of medium layered and the intensity of the immunochemical reaction for a 1/600,000 dilution of anti-rtGH and 10- to 30-μl deposits of undiluted culture medium, providing that exposure to ECL did not exceed 5 min (not shown). The same held true with an anti-rtPRL dilution of 1/120,000 and 5- to 50-μl deposits of undiluted culture medium (not shown). Typically, deposits were of 20 μl undiluted medium for GH detection and 40 μl undiluted medium for PRL detection. In some experiments, serial dilutions of recombinant GH protein (19) were immunodetected in parallel. This was not possible with recombinant PRL, which was not available. However, we were mainly interested in relative variations, so that for each membrane, data were normalized to the controls values. The spots density in the dot blots was measured using the TotalLab (Phoretix) software (Nonlinear Dynamics, Newcastle, UK). All data correspond to the mean of 6–12 determinations, layered each in duplicate. All experiments were done at least two times, more often four to five times.

Statistics

Data were analyzed using the one-way or two-way ANOVA, or by the Student's *t* test. Analysis and data plotting was made using the GraphPad (San Diego, CA) Prism software.

Chemicals and other products

The 3,3′-diaminobenzidine kit was from Vector Laboratories (Peterborough, UK); the cAMP ([^{125}I]-labeled) quantification kit, ^{125}I-Mel and ECL were from Amersham Pharmacia Biotech. RPMI 1640-HEPES buffered, penicillin-streptomycin, and trypsin were from Life Technologies, Inc./Invitrogen (Cergy Pontoise, France). SDS-PAGE molecular weight standards were from Bio-Rad (Marnes-la-Coquette, France). Immobilon-P membranes were from Millipore (St-Quentin-Yvelynes, France). All other products were from Sigma (St-Quentin-Fallavier, France).

For the pharmacological treatments, a 10^{-2} M melatonin solution was prepared in absolute ethanol. Further dilutions were made in culture medium. The final ethanol concentrations never exceeded 0.01%, and this had no effect on the release of GH or PRL.

Results

2-[^{125}I]-iodomelatonin (^{125}I-Mel) binds specifically to sections and membranes from trout pituitaries

^{125}I-Mel bound in a saturable fashion to pituitary membranes (Fig. 1A). Scatchard re-plot of the data indicate that maximal binding was 1.96 ± 0.3 fmol/mg protein, and dis-

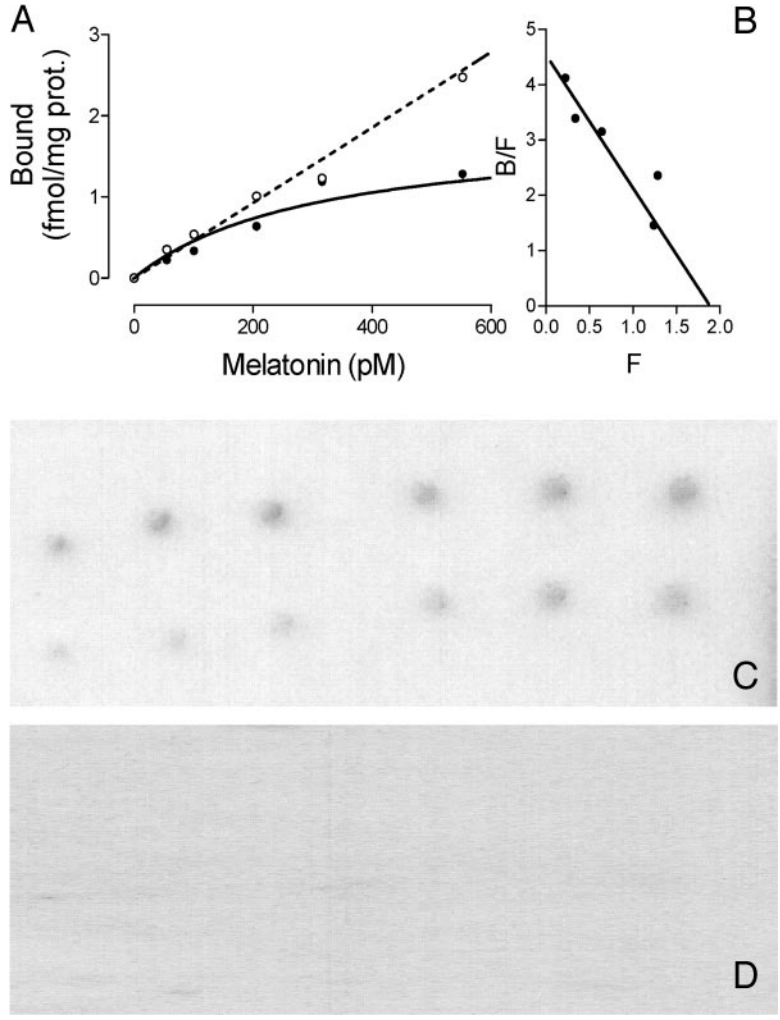

FIG. 1. Binding of 2-[^{125}I]iodomelatonin to trout pituitary membranes and sections. A and B, Membranes. A, Binding was performed in the presence of increasing concentration of 2-[^{125}I]-iodomelatonin. The specific binding (*solid circles*) is defined as total binding minus nonspecific binding (*open circles*), determined in the presence of 50 μM of melatonin. B, Scatchard plot of the experimental data with the best least-squares regression line. Each experiment used pooled homogenates from 60 pituitaries. Means ± SEM (n = 3). One representative experiment out of 2. C and D, Sections. C, Images were produced on x-ray films by 20-μm serial tissue sections through pituitaries incubated with 100 pM of 2-[^{125}I]iodomelatonin. D, Adjacent sections incubated in the presence of 100 pM of 2-[^{125}I]iodomelatonin and of cold 1 μM melatonin in excess.

sociation constant was 403 ± 136 pM (Fig. 1B). Nonspecific binding increased linearly with increasing ^{125}I-Mel concentrations. Tissue sections incubated in the presence of 100 pM ^{125}I-Mel displayed a radioautographic labeling (Fig. 1C), which was not detected when an excess (1 μM) cold melatonin was added to the incubation medium (Fig. 1D).

Melatonin modulates cAMP levels in cultured trout pituitaries

In the absence of forskolin, no difference was found between organs cultured in the presence or absence of melatonin (Fig. 2). When forskolin (10^{-5} M) was added to the culture medium, cAMP levels displayed an up to 100-fold increase after a 30-min incubation. Under these conditions, melatonin reduced dramatically cAMP levels; the effects were dose-dependent (Fig. 2). Half inhibition was obtained in the presence of 10^{-9} M melatonin, and maximal inhibition (>70% inhibition) was reached with a concentration of 10^{-7} M.

Immunochemical detection of GH and PRL in blots and pituitary sections

In Western blots from pituitary extracts as well as from culture medium deposits, the anti-rtGH recognized a single band of proteins at approximately 24 kDa (Fig. 3). In the same extracts, the anti-rtPRL recognized a single band of proteins at approximately 25 kDa (Fig. 3). In the dot blots, the intensity of the spots increased with increasing amounts of recombinant GH protein layered (from 0.5 to 100 ng; not shown). The data fitted a sigmoid equation, and could be linearized on a logit/log scale for quantification purposes. When measured in parallel, the estimated basal levels of GH release were between 15 and 30 ng/pituitary·3 h. Because recombinant GH protein was available in little amounts, standards could not be systematically added to each blot. However, we were

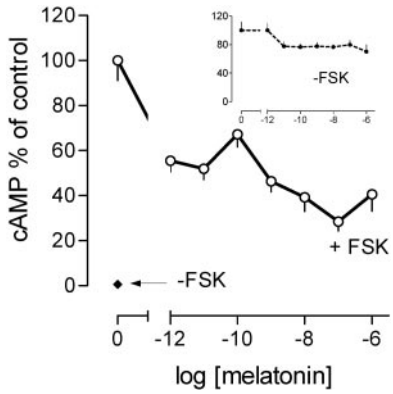

FIG. 2. Effects of melatonin on cAMP accumulation in cultured pituitary glands. Forskolin (FSK; 10^{-5} M) induced an increase in cAMP accumulation. Melatonin reduced this effect in a dose-dependent manner; $P < 0.0001$ (one-way ANOVA); mean ± SEM (n = 6) from one representative experiment which was duplicated. No significant effect is detected in the absence of forskolin (*inset*). For each curve, data were normalized to their respective control (= no melatonin added); absolute values were 0.21 ± 0.02 pmol/pituitary in the absence of FSK, and 18.68 ± 1 pmol/pituitary in the presence of FSK. The *arrow* shows the value from pituitaries cultured in the absence of any drug, normalized to the value obtained in the presence of FSK.

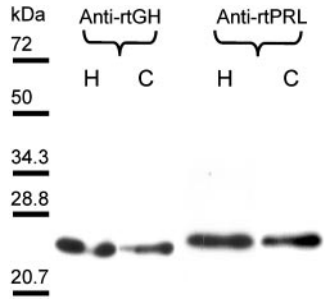

FIG. 3. Western blot analysis of proteins from pituitary glands. Homogenates from pituitary glands (H) and culture medium (C) were obtained after 3 h of culture. A single band of proteins, at approximately 24 kDa, is detected in both extracts with the anti-rtGH antibody. A band at 25 kDa is detected in the same extracts with the anti-rtPRL antibody. Position of the molecular mass markers is indicated on the *left* of the blot.

mainly interested in relative variations, and all the results were normalized to the optical density measured in the controls. The intra assay coefficient of variation was less than 11% for a given sample (n = 5) and less than 25% among samples (n = 6–12). As far as PRL is concerned, recombinant protein was not available. However, there was a linear relationship between the amount of culture medium layered and the density of the immunopositive reactions with an antibody dilution of 1/120,000 (not shown).

In tissue sections, the PRL-labeled areas are distributed in the rostral pars distalis (Fig. 4A), whereas the GH positive cells are localized mostly in the proximal pars distalis (Fig. 4E). A three-dimensional (3-D) reconstruction of pituitary gland, made from serial immunostained sections, indicated there is little overlap between the two areas (Fig. 4, D and H). Only a few clusters of cells in the proximal pars distalis (PPD) of some pituitaries were also PRL immunoreactive. The PRL-positive cells were organized in follicles (Fig. 4B), and the GH-positive cells appeared isolated, or in clusters, or in polylayer cords (Fig. 4, E–G). Whereas the former had an elongated shape (Fig. 4C), the latter were round-shaped (Fig. 4G). In either case, no labeling was seen in the nucleus, and control sections displayed no immunopositive labeling (not shown).

Melatonin modulates GH secretion in cultured trout pituitaries

In a preliminary experiment, the pituitary glands were cultured under the same conditions as those used for the measurement of cAMP. This was dictated by the observation that GH secretion is a cAMP-dependent process. Under these conditions, *i.e.* a 30-min incubation, we observed that forskolin induced an increase in GH secretion, compared with controls (Fig. 5A). This stimulatory effect was no more observed in the presence of picomolar concentrations of melatonin because GH release was seen to resume levels observed in the controls (Fig. 5A). The inhibitory effect was reversed in the presence of nanomolar concentrations of melatonin (10^{-9} and 10^{-8} M). The effects became inhibitory again at higher nonphysiological concentrations of melatonin (Fig. 5A). Physiological concentrations vary between the nanomolar (nighttime) and the picomolar (daytime) range. For

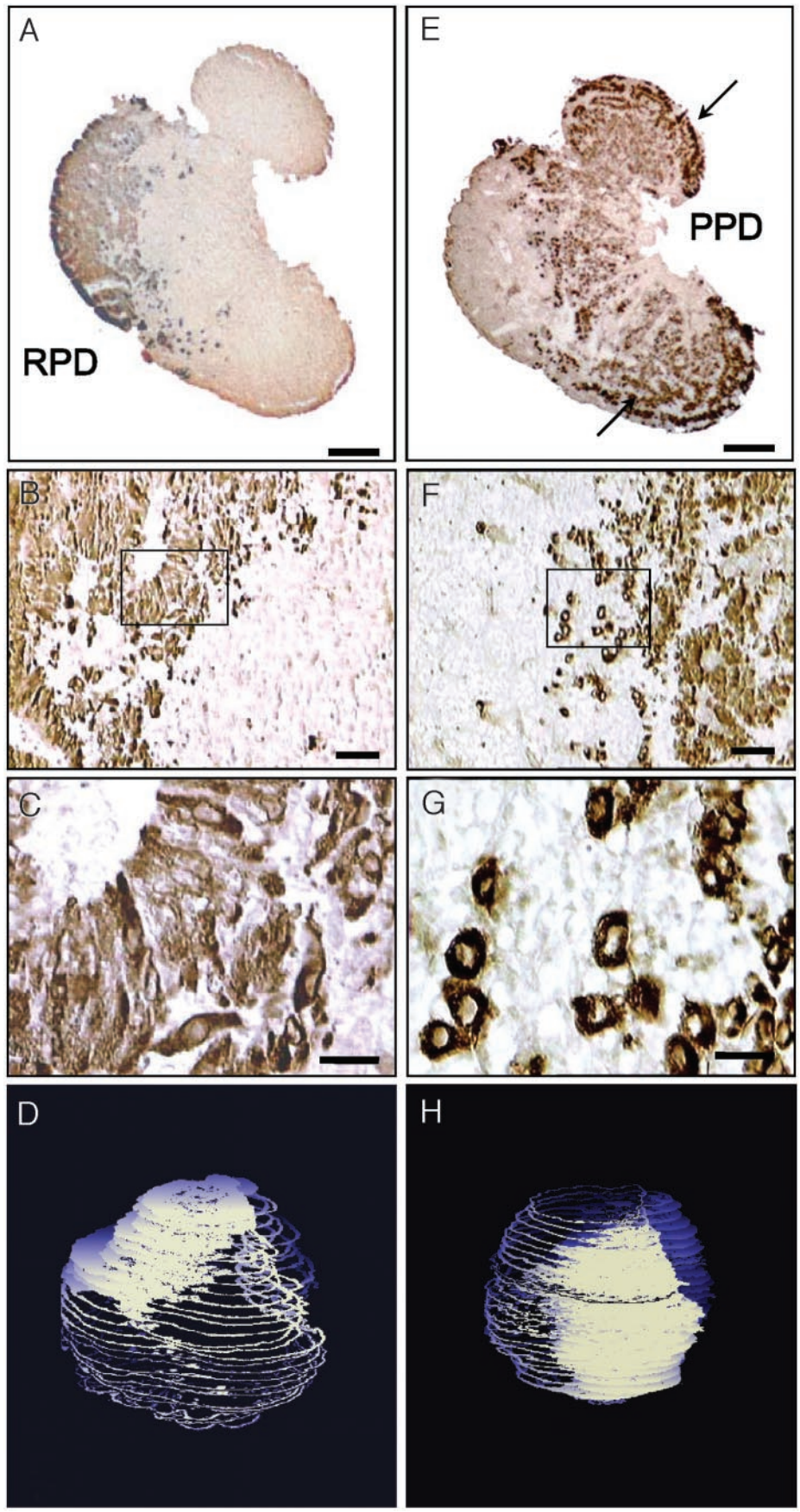

FIG. 4. PRL and GH immunopositive cells in cultured pituitary glands. Images were obtained from 5 μm horizontal sections through rainbow trout pituitaries cultured for 3 h. A–D, PRL. A PRL immunopositive cells are located in the rostral pars distalis (RPD). B, Cells are organized in follicles. C, High magnification of the square in B; PRL cells appear elongated in shape; the cytoplasm is labeled, not the nucleus. D, Lateral view of a 3-D reconstitution of a trout pituitary gland showing the distribution the PRL immunopositive cells (*white areas*). E–H, GH. E, GH immunopositive cells are located in the PPD; *arrows* show monolayer cords. F, At a higher magnification cells also appear in clusters or isolated. G, Higher magnification of the square in B; cells are organized in clusters and appear round-shaped; the cytoplasms, but not the nuclei are labeled. D, Lateral view of a 3-D reconstitution of a trout pituitary shows the distribution GH immunopositive cells (white areas). *Bars*, 200 μm (A and E); 50 μm (B and F); and 20 μm (C and G).

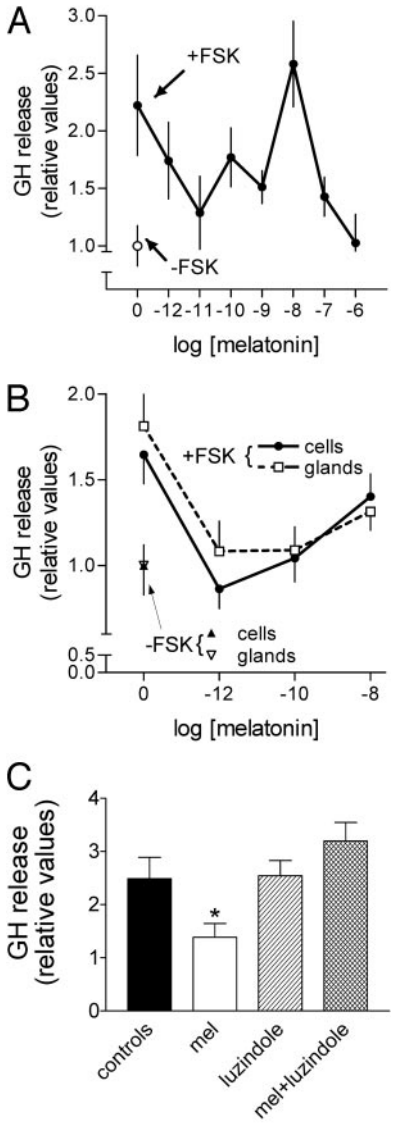

FIG. 5. Modulation of GH release by forskolin (FSK) and melatonin. A, The pituitaries were cultured for 30 min in the absence or presence of forskolin, and of different concentrations of melatonin as indicated. Mean ± SEM (n = 9) $P < 0.01$ (one-way ANOVA). Similar results were obtained in a duplicated experiment. B, Pituitary glands and cells were cultured for 3 h in the presence of forskolin and melatonin at the concentrations indicated. Mean ± SEM; one-way ANOVA indicated $P < 0.03$ (glands; n = 9) and $P < 0.001$ (cells; n = 12). Similar results were obtained in four other experiments. The cells were layered on poly-lysine-coated wells; similar results were obtained with cells layered on collagen coated wells or on polycarbonate Transwells. C, Under pharmacological conditions as in B, the inhibitory effect of melatonin (mel), detected at the concentration of 10^{-12} M, was antagonized in the presence of 10^{-7} M luzindole. Mean ± SEM (n = 6); $P < 0.05$ (Student's *t* test). Similar results were obtained in a duplicated experiment.

this reason three representative concentrations of melatonin were investigated in the following experiments, namely 10^{-12}, 10^{-10}, and 10^{-8} M.

The forskolin induced increase in GH secretion was still observed after 3 h of incubation in the culture media of pituitary glands and cells (Fig. 5, B and C). It usually resulted in a 1.8- to a 2.5-fold increase in GH release (*vs.* untreated glands), and could occasionally reach a 6-fold increase (data from seven independent experiments) (Fig. 5, B and C). Under these conditions, a biphasic response to different concentrations of melatonin was observed. Pituitary glands and cells responded similarly (Fig. 5B), independent of the substrate used to attach the cells (not shown). After 6 or 12 h, the stimulatory effect of forskolin appeared dramatically reduced and the effects of melatonin were not seen anymore (not shown). The inhibitory effect of melatonin, observed at the concentration of 10^{-12} M, was no more observed in the presence of 10^{-7} M luzindole, a melatonin antagonist, which had no effect by its own (Fig. 5C).

Morphometric analysis of the pituitaries was performed in glands cultured for 3 h (as those shown in Fig. 5B). In the forskolin (10^{-5} M)-treated glands, the mean R ratio (Σ_{LA}/Σ_{NLA}) was slightly lower than in the controls, but the difference was not statistically significant (Fig. 6A). The same held true in the glands challenged with both forskolin and 10^{-8} M melatonin. However, in the pituitary glands challenged with forskolin and 10^{-10} M melatonin, the mean R ratio was significantly higher than in all the other groups (Fig. 6A). After normalization of the data, an inverse correlation appeared between the variations in GH release and those of the R ratio (Fig. 6B).

In the absence of forskolin, melatonin induced a dose-dependent increase in GH release after a 30-min, 3-h, or 12-h incubation (Fig. 7A). Two-way ANOVA did not reveal significant differences between the three curves. The stimulatory effect of 10^{-8} M melatonin was not seen in the presence of luzindole, after a 3-h incubation (Fig. 7B). By itself, luzindole had a slight but non significant effect on GH release; this was consistently observed.

Melatonin modulates PRL secretion in cultured trout pituitaries

There was a dose-dependent decrease in PRL release in organs challenged for 30 min, 3, 6, or 12 h in the presence of melatonin (Fig. 8A). Two-way ANOVA indicated the curves obtained at different incubation times did not differ significantly. A similar inhibition of PRL release was also observed with pituitary cells cultured for 3 h in the presence of different melatonin concentrations, independent of the substrate used to attach the cells (Fig. 8B). In pituitary glands, luzindole (10^{-7} M) antagonized the melatonin (10^{-8} M) induced inhibition of PRL release after 3 h of culture (Fig. 8C).

Discussion

This study in the trout provides the first evidence that melatonin modulates GH secretion, and that this results from a direct action of melatonin on trout pituitary cells. It also shows, for the first time in a nonmammalian vertebrate, that melatonin modulates PRL secretion.

Melatonin binding sites are present in trout pituitary

A strong indication that the trout pituitary is a target for melatonin was provided by the binding studies. ^{125}I-Mel was able to bind in a saturable manner to membrane preparations from trout pituitary membranes. The dissociation constant

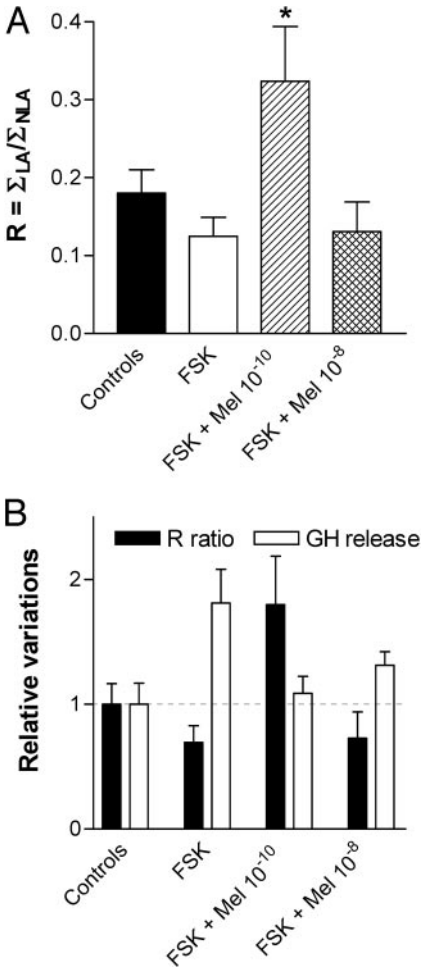

FIG. 6. Morphometric analysis of the GH-immunopositive areas in pituitaries challenged with forskolin (FSK) and melatonin. Five-micrometer-thick pituitary sections were stained for GH immunocytochemistry. For each pituitary, 1) one of every nine serial sections was analyzed to calculate the LA and the NLA; 2) we summed the LAs (Σ_{LA}) as well as the total NLA areas (Σ_{NLA}); and 3) we calculated the ratio $R = \Sigma_{LA}/\Sigma_{NLA}$. A, R value (mean ± SEM, n = 3) as a function of drug treatment. ANOVA indicated the variations were significant ($F = 4.8$, $P < 0.03$); *, statistically different from all the other values. B, An inverse relationship exists between the variations in the R ratio and those of GH release in the medium. Values are from Fig. 6A (*black bars*) and Fig. 5B (glands; *white bars*). They were normalized to their respective control (the 1 value).

was within the range of that found by others in microsomial preparations from trout brain (6). In contrast, we found a much lower number of binding sites (~20-fold less) in the pituitary than in the brain. The results were supported by those obtained with frozen tissue sections showing indeed a faint but specific binding of the radiolabeled melatonin. This is the second demonstration that the fish pituitary is a target for ^{125}I-Mel. Indeed, we have obtained similar results in the pituitary of another Teleost fish, the pike (16). Moreover, cloning and expression experiments indicated two distinct melatonin receptor subtypes are expressed in the pike pituitary (17). It is noteworthy that the binding experiments suggested the presence of only one receptor subtype in the pike (16) and in the trout (this study), whereas cloning studies in the pike (16, 17) and phamacological studies in the trout (see below) suggest two distinct sub-types are present. These apparent discrepancies might result from the fact that 1) the overall amount of binding sites is low, which results in a very low signal-to-noise ratio, and 2) one of the subtypes is expressed in relatively low amounts compared with the other (which is the case in pike; Ref. 17).

Our present and previous results contrast with those obtained by others reporting the absence of melatonin binding sites in the pituitary of nonmammalian vertebrates including birds (23), and two teleost fish, the Atlantic salmon (5) and rainbow trout (6). Also, Mazurais *et al.* (7) could not detect melatonin receptor gene expression in the trout pituitary. The difficulty to evidence melatonin binding sites in the pituitary of the trout and other species might result from technical and/or physiological reasons: low levels of expression, species/strains investigated, age of the animals, possible nycthemeral and/or circannual rhythms of expression. It is possible that the binding sites evidenced here in the trout pituitary, correspond to a yet unknown melatonin receptor subtype. Whatever it may be, these binding sites probably correspond to functional melatonin receptors. Indeed, in the presence of forskolin, the adenyl cyclase activator, melatonin inhibited the accumulation of cAMP in cultured trout pituitaries, a situation similar to the one described in mammals and in the pike (8, 16). The responses reported for the trout were within the order of magnitude of those reported for the other species investigated.

Specificity of the GH and PRL antibodies

Trout pituitaries express two GHs: GH1 and GH2 (19). The anti-rtGH that we used did not distinguish between rtGH1 and rtGH2, and recognized a single band of proteins in extracts from glands and culture medium, at approximately 24 kDa. This corresponds to the estimated molecular weight deduced from the amino acid sequence of the cloned GHs. The characterization and specificity of this antibody has been detailed before (19, 24). In addition, there was a clear-cut linear relationship between the amount of recombinant GH layered on dot blots and the density of the immunopositive reactions under the conditions described in *Materials and Methods* (not shown). In the same extracts, the anti-rtPRL recognized a single band of proteins at a molecular mass of 25 kDa, *i.e.* 1 kDa higher than that reported for GH. A mass of 25 kDa is 1 kDa above the molecular mass deduced from the amino acid sequence of the PRL protein (20). Similar discrepancies have already been reported in other fish species; among other reasons, they might reflect different post-translation processing of the PRL peptide (25).

A further indication that the anti-rtPRL that we used did not cross-react with GH was provided by previous radioimmunological studies (24). Specificity also appeared in the immunocytochemical studies. First, no labeling was seen when the primary antibody was omitted; second, the anti-rtGH and anti-rtPRL antibodies labeled cells located in distinct pituitary gland areas and displaying different organization and shape. A similar regional organization has been described in other fish species (26). A few cells of the PPD were immunoreactive to anti-rtPRL, which is also a feature shared by other fish species (26). In frogs and mammals,

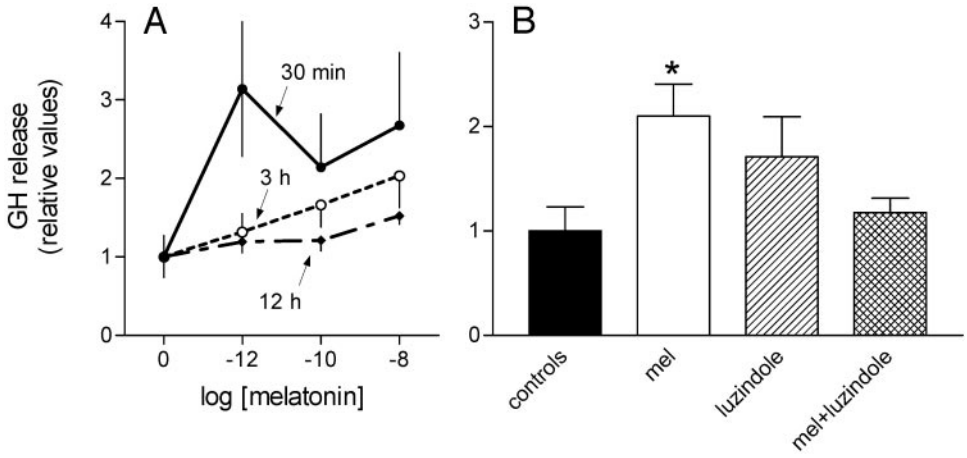

FIG. 7. Effects of melatonin on basal GH release by cultured pituitary glands. A, Glands were cultured in the presence of melatonin, at the indicated concentrations, for 30 min, 3 h and 12 h. This resulted in a dose-dependent increase in GH release. Mean ± SEM (n = 6); $P < 0.05$ for all three curves (one-way ANOVA). Similar results were obtained in two other experiments. B, Under pharmacological conditions as in A, the stimulatory effect of melatonin (mel), detected at the concentration of 10^{-8} M, was antagonized in the presence of 10^{-7} M luzindole. Mean ± SEM (n = 6) $P < 0.05$ (Student's t test). Similar results were obtained in another experiment.

these cells produce both GH and PRL (27). Altogether, our data indicate the immuno(cyto)chemical responses were highly specific for each of the primary antibodies used.

Melatonin modulates GH release by trout somatotropes

GH release is a cAMP-dependent process (18). In good agreement with this, we observed an increase in the amount of GH released in the culture medium under conditions that increased cAMP content (*i.e.* a 30-min incubation in the presence of forskolin). Previous studies had shown that fish somatotropes sustained a continuous release of GH after several days in culture (9, 28). We show here that this nevertheless does not reflect the maximal releasing capacity of the trout somatotropes because forskolin was able to further increase this release. With respect to this, trout somatotropes behave more like eel, goldfish and tilapia somatotropes than like turbot somatotropes; in the latter species, stimulation of GH release was observed provided that the pituitary cells were first challenged with somatostatin to inhibit basal release (28).

A parallel between the variations in cAMP content and GH release was still observed in the presence of picomolar concentrations of melatonin, which induced a clear-cut reduction in GH release. Conversely, at higher (nanomolar range) concentrations the effects of melatonin resulted in a stimulation of GH secretion, in marked contrast with the cAMP levels, which continued to decrease. This indicates a dual modulation of GH release by melatonin; the inhibitory pathway, activated at picomolar concentrations, would involve cAMP, whereas the stimulatory pathway, activated at nanomolar concentrations, would be independent of cAMP. A firm conclusion on the involvement of cAMP will come from studies on the effects of melatonin on adenylate cyclase or protein kinase A activities. Both, the inhibitory and the stimulatory responses to melatonin were suppressed in the presence of luzindole, a nonselective melatonin receptor antagonist, further supporting the view that these processes were mediated through melatonin receptors. Interestingly, in the absence of forskolin, *i.e.* under conditions that do not stimulate cAMP production, melatonin had a stimulatory effect only. Again, this effect was blocked by luzindole. Thus, inhibition of GH release by melatonin requires preactivation of the cAMP pathway. A number of factors are known to activate GH secretion via cAMP, in fish somatotropes, including pituitary adenylate cyclase activating peptide and dopamine (29, 30). Future studies will aim to determine which intracellular pathway(s) mediate the stimulatory response observed in the absence or presence of forskolin. Whereas luzindole alone had no apparent effect in the presence of forskolin, in the absence of forskolin there was a slight but nonsignificant stimulation of GH release. This was consistently observed, and it might reflect a specific property/conformation of the fish melatonin receptors involved (17).

In vertebrates, changes in the activity of pituitary cells can be correlated to changes in their morphology. Thus, variations in the secretory activity of rat somatotropes induced variations in their volume and/or number (31), which implied variations in the overall volume of the GH-producing area (32). Also in fish, *in vivo* administration of melatonin increased the size of the pituitary gonadotropes (2). We hypothesized that a similar situation might hold true for the trout somatotropes. Indeed, we observed significant variations in the ratio $R = \Sigma_{LA}/\Sigma_{NLA}$ as a function of drug treatment. The sum of the areas of the sections analyzed did not vary from one group of pituitaries to another (not shown). Thus, any increase in the R ratio reflected an increase in Σ_{LA} and a concomitant decrease in Σ_{NLA}. The opposite held true when the R ratio decreased. Interestingly, the observed variations in R appeared inversely correlated to the variations of GH release in the culture medium (see Fig. 7B). In other words, the surface of the labeled areas in the pituitaries was high when GH release was inhibited by melatonin. This would suggest that melatonin was indeed acting on the release, but an effect on GH biosynthesis cannot be excluded.

Because the effects of melatonin were investigated after 2 d of culture and were observed with glands and cells as well, we believe the hormone was acting directly on the pituitary cells, and not on some remaining nerve terminals. Furthermore, the kinetics of the cAMP and GH responses (effects were apparent after only 30 min of culture) would suggest that melatonin was acting directly on the GH producing cells, although an action through an intermediary pituitary cell type cannot definitively be ruled out.

A consequence of the effects of melatonin on fish somatotropes might be a modulation of the daily and annual rhythms of circulating GH levels. Although daily variations

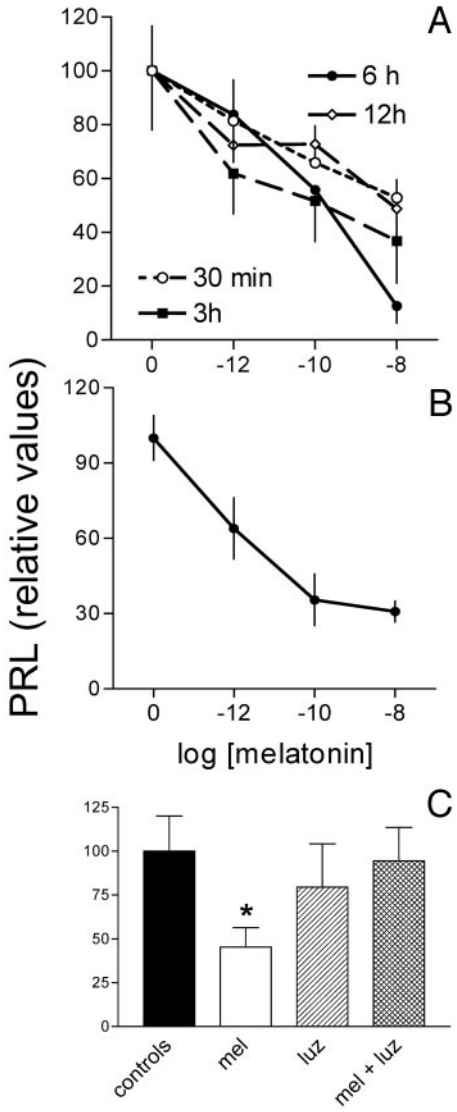

FIG. 8. Effects of melatonin on basal PRL release by cultured pituitary glands and by dissociated pituitary cells. A, Pituitary glands were cultured in the presence of melatonin at the concentrations and durations indicated. Medium was renewed every 3 h. Mean ± SEM (n = 12); $P < 0.05$ (ANOVA) for all the four curves. B, Same as in A, with dissociated pituitary cells cultures on polycarbonate membrane inserts; mean ± SEM (n = 12); $P < 0.0001$ (ANOVA) for all three curves. C, Cultured pituitary glands were challenged for 3 h, either with melatonin (10^{-8} M), or luzindole (10^{-7} M), or both. Mean ± SEM (n = 6); *, $P < 0.05$ (Student's *t* test). Similar results were obtained in two other experiments.

of GH titers have been reported in Teleost (4, 33, 34), contrasting results have been obtained regarding the phase and number of peaks. This might result, from 1) the observation that the control of GH release is a multifactor process, and 2) the experimental conditions (including age, mealtime, photoperiod, species, *etc.*). The trend, however, is toward a nocturnal increase in GH release (above references). Interestingly, we found that the inhibition of GH release was observed at concentrations closer to daytime circulating melatonin levels, whereas the melatonin-induced increase in GH release was observed at concentrations closer to nighttime melatonin levels (35). Thus, a working hypothesis could be that melatonin slows down GH release during day whereas it favors its nocturnal increase.

The daily profile of the melatonin rhythm varies along the year in all vertebrates investigated (1–4). These variations might be involved in the control of the annual variations in GH secretion observed in fish (4). In salmonids and seabream, plasma GH levels increase with increasing photoperiod, *i.e.* from April to June (36). Interestingly, during this period of time, GH plasma levels remain low in salmons placed under continuous illumination (37), a condition that suppresses the nocturnal melatonin surge (1). Conversely, in the same species, decreasing day length in winter season suppressed GH levels, whereas fish kept under long photoperiod during the same period of time did maintain high plasma GH levels (38). Indeed, in this study we observed, for two consecutive years, that trout pituitaries in culture responded neither to forskolin, nor to melatonin during the winter season. The bimodal effects of melatonin on GH release, and the annual variations in the sensitivity to melatonin, might explain why pinealectomy and/or melatonin administration led to opposite growth effects in fish investigated at different times of the year (2, 39).

Melatonin modulates PRL release by trout lactotropes

Recombinant PRL protein was not available. However, an estimation of the relative variations in PRL release was possible because there was a linear relationship between the intensity of the immunoreactions and the amount of culture medium layered on the dot blots, under our experimental conditions. We observed that melatonin inhibited PRL release in a dose-dependent manner, in pituitary glands and cells; the effects were antagonized by luzindole suggesting that melatonin receptors were involved. As for GH, the effects were observed as early as 30 min after drug addition; similarly, we suggest melatonin might have been acting directly on the lactotropes, although this cannot be definitively proven yet.

It has been suggested that an increase in cAMP turnover might play a role in maintaining PRL release under sustained stimulation (40, 41). However, in trout, forskolin (at a concentration similar to the one used in our study) did not modify pituitary PRL content, and increased PRL release by less than 10% over controls (40). In our hands, forskolin had no significant effect on PRL release. It might be that an inhibitory tonus maintained low cAMP levels in trout PRL cells *in vivo*. This can be achieved through dopamine, cortisol, and somatostatin (42, 43). *In vitro*, the absence of such an inhibitory input would result in a maximal accumulation of cAMP, making any stimulation by forskolin ineffective. Future investigations will aim to clarify this point, and to identify which intracellular pathway(s) was (were) activated by melatonin.

Our results are consistent with previous findings showing that 1) pinealectomy affected PRL content in the pituitary (2, 13); 2) daytime melatonin administration reduced by 10-fold pituitary PRL content (the effects depended on the photoperiod (13): and 3) PRL cell activity and PRL plasma levels vary during the 24-h light-dark cycle; higher rates are usually

found at dawn or midday than during nighttime (33, 44, 45). We suggest that nocturnal melatonin production contributes to lower PRL secretion, maybe by a direct action on PRL cells, and that the release of this inhibition during day favors an increase in PRL release. Such a mechanism would also account for the fact that the phase of the PRL rhythm also changes along the annual cycle (44). Indeed, the melatonin-induced inhibition of PRL release was independent of the treatment duration, *i.e. in vivo* the inhibition would be of short duration under long photoperiod, and of long duration under short photoperiod. Our findings are also relevant with regard to the dramatic changes in salinity salmonids have to face during their lives (12, 24). Previous studies had shown that plasma GH levels increase with increasing photoperiod during smoltification in the Atlantic salmon (12). Transfer from fresh water to seawater results in a transient decrease in plasma GH levels, a dramatic and permanent decrease in PRL levels (12), and a concomitant transient increase in cortisol and melatonin levels (46). In rainbow trout, adaptation to salinity is under control by a number of chemical (neurotransmitters, neuropeptides) and physical (osmotic pressure) factors (24, 47). PRL levels are low in the pituitary and high in plasma, as long as fish are maintained in fresh water, whereas the opposite holds true in fish maintained in salt water (12, 24). Our results are consistent with the hypothesis that melatonin contributes to decrease PRL release and plasma titers in the course of transfer from fresh to salt water.

Conclusions

The main finding of this study is the demonstration, for the first time, that melatonin contributes to controlling GH secretion. We also show for the first time in a nonmammalian vertebrate that melatonin inhibits pituitary PRL secretion. These effects are achieved, at least in part, through a direct action on pituitary cells, via melatonin binding sites.

These findings are important because they provide a first clue to understand how melatonin affects GH and PRL release, and related functions in Teleost fish. This is of relevant interest for aquaculture purposes: on the one hand, previous studies have shown an involvement of photoperiod, pineal gland and melatonin in the control of electrolyte balance, body fat, liver, and plasma glycogen, but the results of these *in vivo* investigations were often confusing because apparently contradictory (2); on the other hand, fish GH and PRL are two closely related hormones involved in the control of growth, osmoregulation, immune function, glucose, and lipid metabolism (14, 24, 48–50). Our findings are also important because they provide a first clue to understand how the perception of the photoperiodic information by the neuroendocrine axis has evolved from Teleost fish to mammals. In the latter, melatonin also inhibits PRL release (51); however, via a yet unidentified indirect pathway. Indeed, melatonin receptors are expressed in the pars tuberalis, not in the pars distalis where the lactotropes are (52). It has been hypothesized that the pituitary cells expressing these melatonin receptors might correspond to partially differentiated cells in mammals, and that differentiation leads to the loss of the response to melatonin (52).

Future studies in Teleost fish will aim at determining 1) the nature of the melatonin binding sites and intracellular pathways activated by melatonin; 2) if melatonin was acting directly or indirectly (via some yet unidentified set of pituitary cells) on the GH and PRL producing cells; and 3) how the melatonin signal interacts, along the daily an annual cycles, with the hypothalamic factors known to control GH and PRL productions.

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We dedicate this work to the memory of Niall Bromage who left us this year. Niall's contribution to the study of fish endocrine functions, and their regulation by environmental cues, will remain forever.

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