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Day–night variations in plasma melatonin and arginine vasotocin concentrations in chronically cannulated flounder (*Platichthys flesus*)

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Abstract

Chronically catheterised, free swimming flounder (*Platichthys flesus*) have been used in experiments examining the day–night variations in circulating levels of melatonin (Mel) and arginine vasotocin (AVT). Under normal photoperiod (16 h light/8 h dark) serial blood samples taken from individual fish demonstrated a Mel rhythm with daytime levels at 09.00 and 15.00 h (238 ± 14 and 179 ± 12 fmol·ml^{-1}, respectively) lower than those at 23.00 h (1920 ± 128 fmol·ml^{-1}). Maintenance of fish in 24-h light abolished the light/dark Mel rhythm and circulating levels were comparable to those measured during the day in fish under normal photoperiod illumination. In fish maintained under 24 h dark, although a daily rhythm was still apparent, at the time when it would be normally dark, plasma Mel concentration was reduced and at times when it would be normally light, levels were higher than in fish maintained under normal light/dark illumination. Plasma AVT concentrations were higher in fish during the day (4.4 ± 0.8 fmol·ml^{-1}) than those at night (1.5 ± 0.4 fmol·ml^{-1}), the opposite to that seen with Mel. During acute study infusion of AVT resulted in reduced levels of plasma Mel, although this did not achieve statistical significance. Infusion of Mel did not alter circulating AVT concentration.

Keywords: Arginine vasotocin; Day–night rhythm; Fish; Flounder; Hormone; Melatonin; Neurohormone; Pineal; *Platichthys flesus*; Teleost

1. Introduction

Melatonin (Mel), an indole hormone, is synthesised and secreted primarily by the pineal gland in all vertebrate classes. In all species examined, synthesis and release of this hormone is low during the light period and rises during the dark period (Binkley, 1988), so that Mel is considered to serve as a 'hormone of the night'. Melatonin is not stored in the pineal, so that its plasma level reflects the synthesis capacity of the gland. The hormone has been implicated in a wide spectrum of physiological and behavioural events (Binkley, 1988). The pineal organ of fish appears to be a component of the circadian system. The pineals of most fish species examined: goldfish *Carassius auratus*, white sucker *Catostomus commersoni*,

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pike *Esox lucius*, zebrafish *Danio rerio*, gilthead sea bream *Sparus aurata* contain cellular circadian oscillators within the pineal organ (Iigo et al., 1991; Zachmann et al., 1992a; Bolliet et al., 1996; Cahill, 1996; Molina-Borja et al., 1996). By contrast, the pineal of trout (*Oncorhynchus mykiss* and *Salmo trutta*) and common dentex (*Dentex dentex*) does not appear to contain a circadian oscillator, with melatonin synthesis being directly controlled by illumination (Gern and Greenhouse, 1988; Max and Menaker, 1992; Pavlidis et al., 1999). In addition to the light/dark cycle, chemical signals i.e. catecholamines, adenosine and atrial natriuretic peptide, participate in the control of Mel production (Falcon et al., 1990, 1991). Analysis of the correlation between plasma melatonin and arginine vasotocin levels in rainbow trout suggests an interaction also between these hormones (Kulczykowska, 1999).

Arginine vasotocin (AVT) is a nonapeptide synthesised in the hypothalamic neurosecretory neurones of teleost fish, with axons projecting to the neurohypophysis where the hormone is stored and released. AVT plays a predominant role in osmoregulation and cardiovascular activity (Acher, 1993) interacting with other hormone systems in the regulation of these processes. There is a growing body of evidence that AVT may act as a neurotransmitter and/or neuromodulator in the central nervous system of fish (Goossens et al., 1977; Van den Dungen et al., 1982) and may modulate reproductive physiology and related social behaviours (Foran and Bass, 1999; Goodson and Bass, 2000). There are also some, but scant, data suggesting daily fluctuations in AVT synthesis capacity (Gilchriest et al., 1998) and plasma concentration in fish (Kulczykowska, 1999).

Regulation of an animal's physiological and behavioural response to environmental change is a complex process often involving a number of endocrine factors. It is hypothesised that Mel and AVT interact in controlling the physiological adaptation of fish to daily and seasonal environmental changes (light, temperature, salinity) (Kulczykowska, 1995). Moreover, the established AVT/AVP-melatonin interrelationship reported in mammals (Binkley, 1988; Castillo-Romero et al., 1993; Yasin et al., 1993) provides further support for the current study of interaction between AVT and melatonin systems in fish.

The purpose of this study was first to investigate the character of plasma Mel rhythm in a chronically cannulated, unanaesthetized, free swimming flounder. This model has been subsequently utilised to examine any relationship between melatonin and arginine vasotocin in this benthic, euryhaline marine species.

2. Materials and methods

2.1. Experimental animals

Flounder (*Platichthys flesus*) (300–550 g) of mixed sex were kept in seawater holding tanks at the University of Manchester at 7–10°C under the natural photoperiod. The fish were not fed while in captivity.

2.2. Cannulation of flounder

The fish were anaesthetized in a seawater solution of MS-222 [0.1 $g \cdot l^{-1}$ 3-aminobenzoic acid ethyl ester methanesulfonate (Sigma Chemical Co., Dorset, UK)]. An incision was made close to the tail and the dorsal aorta exposed. A polyethylene cannula (I.D. 0.58 mm, O.D. 0.96 mm, Portex Ltd., Kent, UK) containing 0.9% NaCl and 50 U/ml heparine, was implanted into the dorsal aorta for later serial blood sampling. The wound was then sutured and a minimum period of 48 h elapsed after surgery before fish were used in one of the three experimental procedures described below.

2.3. Experimental protocols

1. Chronically cannulated fish were maintained in seawater experimental tanks. Fish were assigned to one of three experimental groups and exposed to one of the following lighting regimes; continuous darkness (DD; $n = 6$), continuous light (LL; $n = 8$) or were maintained under the natural photoperiod of 16 h light/8 h dark (LD; $n = 8$). Animals were adapted for at least 2 days to the experimental lighting regime before experimentation. Serial blood samples (2.5–3 ml) were collected from the dorsal aortic catheter at 1500, 2300 and 0900 over a 2-day period in each experimental group. After blood centrifugation the separated red blood cells were resuspended in a volume of 0.9% NaCl equal to

the sample volume and returned to the fish. Plasma was rapidly frozen prior to assay for melatonin.

2. Chronically cannulated flounder were maintained in seawater experimental tanks under artificial illumination (lights on: 08.00–20.00) for a period of 2 days before experimentation. Blood samples (2.5–3 ml) were collected at 09.00 and 21.00 h, the times indicated from study 1 that represent predicted low and high levels of melatonin secretion, respectively. After blood centrifugation red blood cells were resuspended in a volume of 0.9% NaCl equal to the sample volume and returned to the fish. Plasma was rapidly frozen prior to assay for melatonin. A parallel series of animals were treated in this way to provide sufficient plasma for measurement of arginine vasotocin at the same time points.
3. To determine the acute effect of AVT on the circulating levels of Mel and the effect of Mel on the circulating levels of AVT, hormone was infused in fish through the arterial cannulae using the following protocol. Fish were held in conditions as for study 2, but 1 h before lights off (19:00) fish were given a bolus injection of 100 $\mu l \cdot 100$ g body wt.$^{-1}$, followed by a 2-h infusion at 4 $\mu l \cdot min^{-1}$ of hormone preparations (56 μM Mel or 3 nM or 10 nM AVT). After 2 h a blood sample was taken, plasma separated and frozen for hormone assay and red blood cells resuspended in saline and returned to the fish. Each fish also received control vehicle (0.9% NaCl) injection and infusion and underwent the same blood sampling protocol on the day before or day after hormone administration.

2.4. Plasma analysis

Blood was centrifuged at $13\,000 \times g$ for 5 min and stored at -70°C prior to plasma analysis. Plasma Mel was measured by HPLC (Kulczykowska and Iuvone, 1998), and plasma AVT by RIA (Warne et al., 1994). Osmolality was determined by vapour pressure measurement using a Wescor Vapour Pressure Osmometer (Wescor Inc., Logan, USA). Plasma sodium, potassium and calcium were measured by an EasyLyte ion analyser (Medica Co., Bedford, USA).

Melatonin was extracted from plasma (2 ml) by solid phase extraction (SPE) using C_{18} Bakerbond cartidges (J.T. Baker, Phillipsburg, NJ, USA; pore size 60 Å, particle diameter 40 μm). The sample was eluted with methanol. The separation and detection were performed with the Beckman modular HPLC system (Beckman Instruments, San Ramon, CA, USA), connected to a Shimadzu spectrofluorometric detector RF-551. Chromatographic isocratic separations were carried out on an Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm particle diameter, 80 Å pore size) connected to a precolumn (45×4.6 mm I.D.) filled with the same material, both obtained from Beckman Instruments (San Ramon, CA, USA). The mobile phase was 60% HPLC-grade methanol (J.T. Baker, Deventer, The Netherlands). Excitation and emission wavelengths were set at 286 and 352 nm, respectively. The detection limit was 3 pg per ml of plasma. The inter- and intra-assay coefficients of variation were 14% ($n = 9$) and 10% ($n = 9$), respectively (see Kulczykowska and Iuvone, 1998 for details). Synthetic melatonin (mol wt. 232.3) for calibration curve was obtained from Sigma Chemical (St. Louis, MO, USA).

Arginine vasotocin was extracted from 1 ml plasma samples using SepPak C_{18} cartridges (Millipore Ltd. UK). Antiserum raised against AVP, which shows 100% cross-reactivity with AVT and AVP was used at a dilution of 1:500 000. The radiolabelled ligand employed in the RIA was ^{125}I AVP (Amersham). Intra-assay variation was 8.3% ($n = 9$) and inter-assay variation was 11.9% ($n = 13$). The minimum detection limit of the assay was 0.24 fmol of AVT per assay tube (see Warne et al., 1994 for details).

2.5. Statistical analysis

Values are presented as means $\pm$ standard error of the mean (S.E.M.). For multiple comparisons, the analysis of variance (ANOVA) followed by Student's *t*-test was used. Significant differences between means for paired sample studies were identified using Student's paired *t*-test. Significance was taken as $P < 0.05$.

3. Results

Plasma melatonin concentrations measured in fish exposed to LD (16-h light/8-h dark), DD (24-h dark) or LL (24-h light) lighting regimes

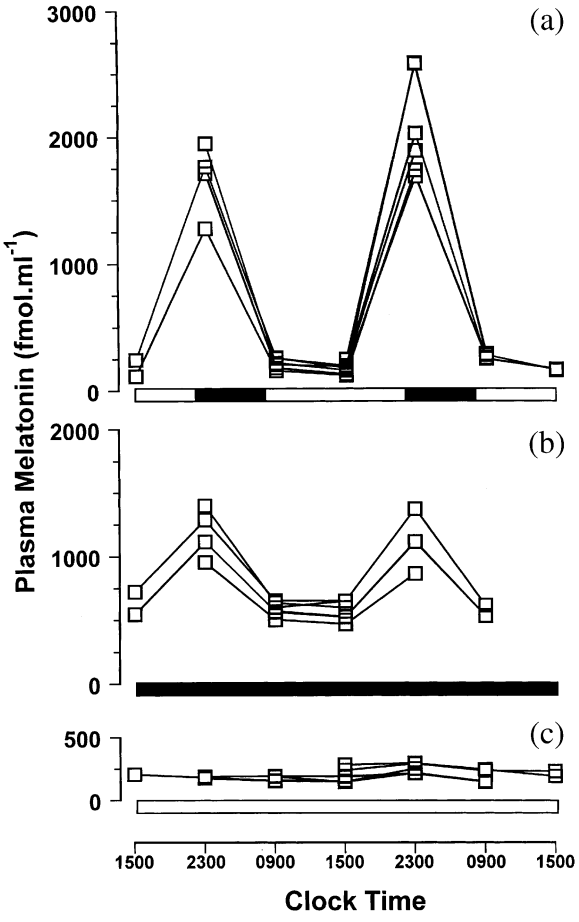

Fig. 1. Plasma melatonin concentration in serial samples from flounder maintained under different lighting regimes. Panel A shows data from individual fish maintained in LD (16 h light: 8 h dark; $n = 6$); panel B from fish in DD (24 h dark; $n = 8$) and panel C from fish in LL (24 h light; $n = 7$). Dark bars represent the period of darkness. Four serial blood samples were taken from each fish with the start of sampling occurring at one of the first four time points of the study.

throughout the two day sampling period are presented in Fig. 1. In the LD group plasma Mel was significantly higher at 23.00 h (dark) (1920 ± 128 fmol·ml^{-1}; $P > 0.001$), than at 09.00 or 15.00 h (light) (238 ± 14 and 179 ± 12 fmol·ml^{-1}, respectively). Similarly, in the DD group, plasma Mel concentrations were higher at 23.00 (1159 ± 77 fmol·ml^{-1}) than at 09.00 or 15.00 (583 ± 18 and 587 ± 30 fmol·ml^{-1}, respectively; $P < 0.01$), although the night time Mel peak in the DD group was lower than that seen in the LD group ($P < 0.001$). Melatonin concentrations in the LL group were immutably low at 09.00, 15.00 and 23.00 h (186 ± 11, 199 ± 12 and 227 ± 15 fmol·ml^{-1}, respectively). Plasma concentrations at 09.00 and 15.00 in the DD group were higher ($P < 0.01$) than values measured in fish at these time points in the LD or LL groups. Plasma Mel concentrations in the LL group at 23.00 h were lower than those in LD and DD groups at this point ($P < 0.001$).

A similar pattern of fluctuation in plasma melatonin also occurred in fish in Experiment II kept under artificial LD (12-h light/12-h dark) illumination and sampled at 09.00 (light) and 21.00 (dark). The results are summarised in Fig. 2. Plasma Mel concentration measured at night was significantly higher than that measured during the day (1240 ± 55 vs. 176 ± 16 fmol·ml^{-1}; $P < 0.001$). Mean plasma arginine vasotocin concentrations were significantly higher in the light than in the dark (4.4 ± 0.8 vs. 1.5 ± 0.4 fmol·ml^{-1}; $P < 0.001$), reflecting an opposite pattern to that seen for melatonin.

Effects of AVT infusion on plasma Mel and

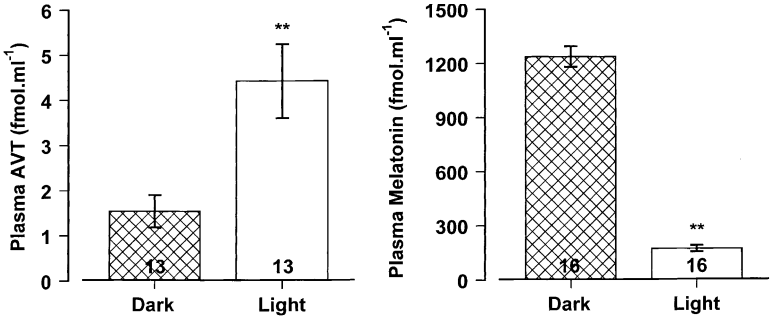

Fig. 2. Plasma AVT and Mel concentrations in samples taken in dark and light from fish maintained 12 h light/12 h dark (21.00 to 09.00). Samples taken 1 h after change in illumination. Number of fish is given in bars. ** $P < 0.01$, for comparison of light and dark measures (Student t paired test).

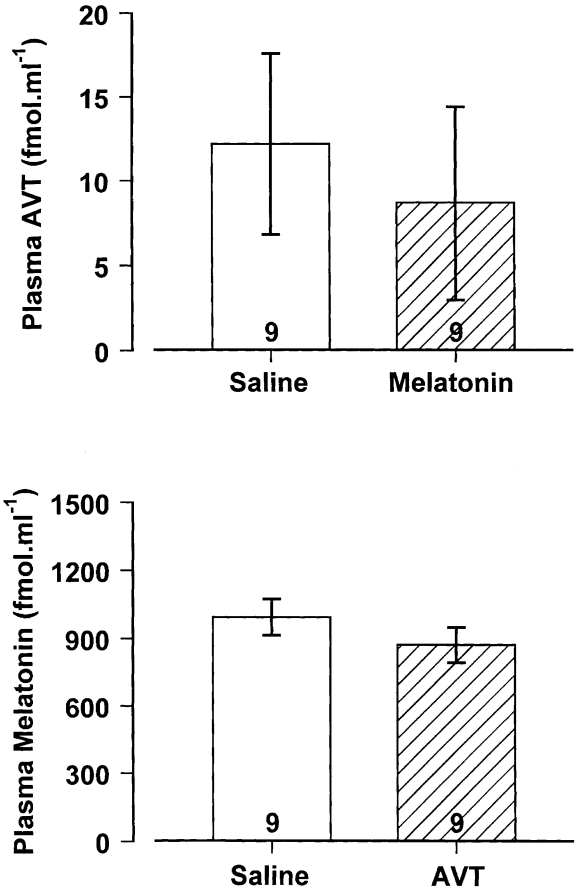

Fig. 3. Plasma AVT concentration following Mel or vehicle (saline) infusion (upper panel) and plasma Mel concentration following AVT or vehicle (saline) infusion (lower panel). Number of fish is given in bars.

Mel infusion on plasma AVT are shown in Fig. 3. The infusion of Mel did not result in any significant or consistent pattern of change in plasma AVT concentration by comparison with levels in saline vehicle infused controls. AVT infusion did result in a lower plasma Mel concentration by comparison with saline-infused controls, but this again did not achieve statistical significance. However, plasma Mel concentration in AVT treated fish was 91% of that in the control group with six of the nine fish showing a fall in a plasma Mel concentration following AVT treatment.

4. Discussion

This is the first serial study in individual free swimming, benthic fish of daily changes in plasma melatonin levels. The melatonin concentrations measured in cannulated fish were similar to those measured in blood samples collected by needle puncture (1138 ± 37 vs. 1146 ± 31 fmol·ml^{-1}). Thus, the chronically cannulated fish offer a useful model for future studies.

Plasma melatonin concentrations measured in flounder were within the range previously reported in other fish: carp, rainbow trout, goldfish, brook trout, Atlantic salmon, common dentex and cod (Gern et al., 1978; Kezuka et al., 1988, 1992; Zachmann et al., 1992b; Randall et al., 1995; Kulczykowska, 1998, 1999; Pavlidis et al., 1999). The very significantly higher nocturnal plasma level of melatonin in flounder kept under the natural photoperiod is consistent with the pattern established for other species. The absence of any melatonin rhythm observed in fish kept under continuous light also reflects the inhibition or suppression of melatonin synthesis by light. The low residual level of circulating melatonin in the light may be due to incomplete inhibition of synthesis or secretion by the pineal or to melatonin synthesis outside the photosensitive pineal organ and retina (Bubenik and Pang, 1997). Although DD conditions reduced the magnitude of the melatonin oscillations seen under LD, an attenuated rhythm was still evident. The flattening of the rhythm in flounder kept under DD conditions is known to occur in some species of fish (Kezuka et al., 1992). Maintenance of the plasma melatonin rhythm after two and three days of keeping flounder in continuous darkness contrasts with the lack of melatonin rhythm observed in rainbow trout and common dentex (Kulczykowska, 1999; Pavlidis et al., 1999). This study suggests the presence of circadian oscillators within the pineal organ of flounder. The pineals of most teleost fish species examined do appear to contain cellular circadian oscillators; the exception thus far being the trout pineal organ (Gern and Greenhouse, 1988; Max and Menaker, 1992).

In addition, the present study demonstrates for the first time differences in day and night concentrations of plasma AVT in flounder. So far, there has been a little information regarding the daily changes in AVT level in fish. These findings agree with the previous observation in rainbow trout (Kulczykowska, 1999) and are comparable with the day/night variations observed for AVP in mammals (Fiske and Greep, 1959; Greenley et al., 1982). The study of a diurnal rhythm of gene transcription in the neurones secreting AVT in

rainbow trout revealed a significant diurnal change in mRNA abundance in the parvocellular neurones. The AVT transcripts increased progressively during the morning reaching the peak in the afternoon and then declined during the night. However, no significant diurnal rhythm was observed in magnocellular neurones (Gilchriest et al., 1998). These results together show that the diurnal fluctuations in plasma AVT level must be taken into consideration in planning experimental study of the AVT system.

Reciprocal day–night variation in the circulating levels of Mel and AVT suggests there may be some interaction between these two hormone systems in flounder. Although infusion of Mel did not result in any consistent change in plasma AVT concentration our data do suggest that AVT infusion may have an effect on plasma Mel concentration, with six of nine fish showing reduced melatonin. Further study is required to confirm this in the flounder, but studies in other fish and in higher vertebrates support this notion. The negative correlation between plasma AVT and Mel shown earlier in rainbow trout (Kulczykowska, 1999) and the considerable data from mammals suggest the existence of a functional relationship between AVT/AVP and melatonin secretion (see Section 1). In the rat pineal, there is a diurnal rhythm in vasotocin content (Calb et al., 1977) and AVT decreased norepinephrine stimulated Mel synthesis (Binkley, 1988). Similarly, AVP inhibited norepinephrine-induced stimulation of Mel release in the bovine pineal gland (Olcese et al., 1993), while vasopressin injected early in the dark phase to rats inhibited the nocturnal Mel synthesis (Schröder et al., 1988).

Data from mammals suggest that nonapeptides may contribute to the circadian system (Uhl and Reppert, 1986; Yamase et al., 1991). The daily rhythmicity in activity in the AVP neurones is unique to the SCN (suprachiasmatic nucleus), considered as the 'biological clock' of the brain. It may suggest that AVP is involved in the generation of the circadian rhythm in mammals (De Kloet et al., 1990). It is generally presumed that in teleosts the preoptic area is the seat of the clock homologous with the mammalian SCN (Ekström and Vanecek, 1992). The neurones in the preoptic area are immunoreactive for AVT (Goossens et al., 1977; Van den Dungen et al., 1982). Furthermore, high levels of Mel binding have been demonstrated there (Martinoli et al., 1991; Ekström and Vanecek, 1992). Drawing analogies with AVT/AVP-melatonin interactions in mammals, the present data may support such an interaction between AVT and Mel as part of the circadian system of fish. Further studies are required to confirm and reveal complex detail of these hormones relationships in fish.

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