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Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

In teleost fish exposed to stressors AVT has been implicated in activation of the hypothalamo-pituitary-interrenal axis. Mel has been shown to counteract several behavioural and endocrine consequences of stress in mammals. These studies were undertaken to investigate the effects of either disturbance or osmotic stress, applied either separately or simultaneously, on plasma AVT, IT and melatonin in rainbow trout (*Oncorhynchus mykiss*). Hormones were determined in plasma by high-performance liquid chromatography preceded by solid-phase extraction. The results showed that both forms of stress caused significant increase in plasma AVT concentration, although more pronounced elevation was observed in physically disturbed fish. Conversely, neither osmotic nor disturbance stress affected plasma IT concentration. The apparent difference in response to stress by the two close related neurohypophysial nonapeptides suggests independent mechanisms controlling their synthesis and/or release and supports the idea that only AVT plays a role in physiological response to stress. Plasma Mel level was depressed in fish subjected to disturbance stress and to both stresses applied simultaneously, an effect possibly associated with the elevation of plasma AVT concentration. Results are discussed in relation to physiological interactions between hormones.

Abbreviations: AANAT – arylalkylamine N-acetyltransferase; ACTH – corticotropin; AVT – arginine vasotocin; AVP – arginine vasopressin; BW – brackish Baltic water; CRH – corticotropin-releasing hormone; FW – freshwater; HPLC – high-performance liquid chromatography; IT – isotocin; Mel – melatonin; SPE – solid phase extraction; SW – seawater.

Introduction

The primary response to stressors in fish involves two major neuroendocrine pathways, the hypothalamus-autonomous nervous system-catecholamine producing chromaffin cells and hypothalamus-pituitary-corticosteroid producing interrenal cells (Pickering 1981).

In teleost fish exposed to different forms of stress AVT has been implicated in activation of the hypothalamo-pituitary-interrenal axis (Fryer and Leung 1982; Gilchriest et al. 2000). As in mammals, hypothalamic hormones corticotropin-releasing hormone (CRH) and AVT (homologue of mammalian vasopressin) trigger the release of corticotropin (ACTH) from fish anterior pituitary gland (Baker et al. 1996). Much less is known about the IT response to stressors.

The pituitary hormones and hypophysiotropic functions of the hypothalamo- pituitary axis have been relatively well preserved throughout vertebrate evolution. Stress response of teleosts has many similarities to that of other vertebrates. However, there is a major difference in organization of pituitary gland in teleosts, including direct innervation of the pars distalis and the lack of a well-distinguished median eminence and portal system (Peter 1986). Therefore, knowledge from well-studied mammalian species may be of limited use for comparison with fish.

Arginine vasotocin and isotocin are synthesised in both magnocellular and parvocellular neurones in hypothalamic region of fish brain and are axonally transported to the terminals in neurohypophysis, from which they are released into the central circulation. In addition, neurones project to the ventral hypothalamus and influence actions of pituitary regulating neurones (Van den Dungen et al. 1982). A number of findings have shown that AVT is one of the key hormones regulating hydromineral balance in fish (Balment et al. 1993; Acher 1995). There are a few studies in teleosts reporting variations in plasma AVT level with respect to salinity and presenting changes in pituitary AVT content during osmotic stress (Harding et al. 1997; Kulczykowska 1997). Increased AVT level was also proposed for use as an early warning indicator of sublethal acid stress in the brook trout (Hontela et al. 1991). Interestingly, in numerous studies it has been demonstrated that both neurohypophysial nonapeptides play a role in neurotransmission and neuromodulation processes in the central nervous system influencing, among other things, reproductive physiology and related social behaviour (Foran and Bass 1999).

The pineal hormone melatonin (N-acetyl-5-methoxytryptamine), well-known major synchroniser of endocrine rhythms in vertebrates, has been shown to counteract several behavioural and endocrine consequences of stress in mammals with most data suggesting an inhibitory role for melatonin (Vaughan et al. 1972). In rats, melatonin attenuates the adrenocortical response to stress (Konachieva et al. 1997). Melatonin is also known to modulate brain activity in mammals and may interact with another compound of pineal origin; it has been shown that melatonin and vasotocin inhibit spontaneous neuronal activity in rat caudate-putamen neurones (Castillo-Romero et al. 1993). However, there are no data on any stress-melatonin links in fish available at the moment.

These studies were undertaken to investigate the effects of either disturbance or osmotic stress, applied either separately or simultaneously, on plasma AVT, IT and melatonin in rainbow trout.

Materials and methods

Animals

Male rainbow trout (*Oncorhynchus mykiss*) (350–480 g) were obtained from a hatchery (Institute of Inland Fisheries in Rutki, Poland). Fish were progeny of single parent spawning. In January, animals were kept in freshwater tanks at 10–14 °C on a commercial trout diet. Fish were maintained under natural photoperiod (the darkness occurred between 15:00 and 8:00).

Experiments

All stress experiments started at 21:00 h. Previous examination of diurnal changes in plasma hormone concentrations in rainbow trout provided information on the best time to study stress effects in that fish (Kulczykowska 1999). Fish were subjected to one of two forms of acute stresses.

I. Fish adapted to freshwater were subjected to a rapid change of tank water (freshwater to brackish Baltic water, BW: 169–173 mOsm kg^{-1}). The control animals were not disturbed in freshwater tank. All blood samples (5–8 ml) were taken at time of sacrifice at 23:00 in control and BW fish.

II. Freshwater-adapted fish were subjected to 5 min enforced displacement in freshwater. The control animals were not disturbed in freshwater tank. Blood samples (5–8 ml) were taken at time of sacrifice at 23:00 in control and disturbed fish.

III. Fish subjected to a rapid change of tank water were immediately after additionally exposed to 5 min disturbance stress.

Blood samples for AVT, IT and Mel were collected from the dorsal aorta of decapitated, unanesthetized fish. Plasma was separated by centrifugation at $1000 \times g$ for 5 min and stored at -70 °C prior to analysis. Plasma osmolality was measured using a vapor pressure osmometer (Wescor Inc., Logan, USA). Plasma sodium, potassium and calcium were measured by an EasyLyte ion analyser (Medica Co., Bedford, USA).

Plasma AVT, IT and melatonin analysis

AVT and IT were extracted from plasma by solid phase extraction (SPE) using C_{18} Bakerbond cartridges (J.T. Baker, Phillipsburg, New Jersey, USA; pore size 60 Å, particle diameter 40 μm). HPLC was performed with a Beckman modular system (Beckman Instruments, San Ramon, California, USA) with UV detector. Chromatographic 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, California, USA). The eluate

was monitored at 215 nm. The inter- and intra-assay coefficients of variation were 17% (n = 15) and 10% (n = 15) for AVT and 15% (n = 15) and 12% (n = 15) for IT, respectively. The method has been described in detail (Kulczykowska 1995a).

Mel was extracted from plasma by SPE using the same cartridge described above. The separation and detection were performed with the Beckman modular system, mentioned above, connected to a Shimadzu spectrofluorometric detector RF-551. Chromatographic separations were carried out on the same Ultrasphere C_{18} column. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. The inter- and intra-assay coefficients of variation were 14% (n = 9) and 10% (n = 9), respectively. The method has been described in detail (Kulczykowska and Iuvone 1998).

Hormones

[Arg^8]vasotocin (mol wt 1050.2, purity 97%), IT (mol wt 966.1, purity 97%) and Mel (mol wt 232.3, purity 99%) were obtained from Sigma Chemical (St. Louis, Missouri, USA). AVT (1 mg ml^{-1}) and IT (1 mg ml^{-1}) were dissolved in HPLC-grade water and stored in stock solutions at −70 °C. Working standards and peptide solutions for injection were prepared directly before use in HPLC-grade water and 0.9% NaCl, respectively. The stock solution of Mel (1 mg ml^{-1}) was prepared by dissolving in HPLC-grade methanol (J.T. Baker, Deventer, the Netherlands). Working standards and solutions for injection were prepared in HPLC-grade water and 0.9% NaCl, respectively, immediately before use. It is strongly recommended that Mel solutions be kept in darkness at −20 °C until use (but not longer than 2 h) to avoid decomposition during storage.

Chemicals

HPLC-grade acetonitrile, water, methanol and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Deventer, the Netherlands). Glacial acetic acid and hydrochloric acid were purchased from E. Merck (Darmstadt, Germany).

Statistics

Values are presented as means ± standard error of the mean (SEM). For multiple comparisons, the analysis of variance (ANOVA) followed by Student's *t*-test was used. Significant differences between two means were identified using Student's paired *t*-test. Significance was taken as $p < 0.05$.

Results

Plasma osmolality, sodium, potassium and calcium in control and experimental fish are shown in Table 1. Plasma osmolality and ions were elevated in BW fish by comparison with FW controls while displacement stress had no effect on plasma composition in either FW or BW fish.

Plasma levels of AVT, IT and Mel in control and experimental fish are presented in Figures 1a, b, c, respectively. Both forms of stress caused significant increase in plasma AVT concentration, although more pronounced elevation was observed in physically disturbed fish. The highest AVT levels were shown in fish exposed to both osmotic and displacement stresses simultaneously (Figure 1a). Neither osmotic nor disturbance stress affected plasma IT concentration (Figure 1b). Melatonin value measured in fish subjected to osmotic stress did not differ from the control. However, plasma Mel concentration was significantly lower than control in fish exposed to displacement stress or to both osmotic and displacement stresses simultaneously (Figure 1c).

Plasma AVT, IT and melatonin concentrations agreed with values presented in rainbow trout previously (Kulczykowska 1999). Plasma AVT concentrations correlated positively with corresponding plasma osmolality and Na^+ values (correlation coefficient of $r = 0.54$, $p < 0.01$ and $r = 0.67$, $p < 0.01$, respectively), similarly to those shown in rainbow trout earlier (Kulczykowska 1997).

Discussion

The objective of the present study was to gain information on the effect of two forms of stress, i.e., disturbance and osmotic stress on plasma AVT, IT and Mel in rainbow trout. It was taken into consideration, that the mechanisms controlling the response to stresses of different nature, employed in this study, are probably different and partly independent.

Herein, the results show increased plasma AVT levels in fish exposed either to osmotic or to displacement stress. Interestingly, fish subjected to both forms of stress simultaneously, responded with an especially pronounced rise of plasma AVT (Figure 1a).

Table 1. Plasma osmolality and ion concentrations in experimental rainbow trout at 23:00 h

	Osmolality mOsm kgH_2O^{-1}	**Na^+** mmol l^{-1}	**K^+** mmol l^{-1}	**Ca^{2+}** mmol l^{-1}
FW control n = 7	315.5 ± 4.2	132.6 ± 1.7	2.51 ± 0.14	1.00 ± 0.02
BW n = 9	339.8 ± 5.3^b	153.0 ± 2.8^a	3.04 ± 0.15^b	1.19 ± 0.03^c
FW displaced n = 10	313.4 ± 3.5	134.5 ± 1.8	2.68 ± 0.16	1.08 ± 0.02
BW displaced n = 8	335.7 ± 4.7^b	152.8 ± 1.6^a	2.99 ± 0.14^b	1.23 ± 0.03^b

Data are given as means ± SEM. Means followed by superscript letters differ significantly from respective fresh water control values: a, $p < 0.001$; b, $p < 0.01$; c, $p < 0.05$.

A function of vasotocin in stress axis in fish seems to be beyond any doubt. In the rainbow trout, corticotropin-releasing hormone and arginine vasotocin synergize to stimulate corticotropin release from the pituitary (Baker et al. 1996). The last increases the secretion of corticosteroid, cortisol, which in fish possesses both glucocorticoid and mineralocorticoid actions. It is also shown that the acute confinement stress is accompanied by the increased biosynthesis of AVT in a group of parvocellular neurones of the preoptic nucleus in rainbow trout (Gilchriest et al. 2000). Hence, the higher plasma AVT concentrations observed in this study were expected. The possible contribution of the second source of plasma AVT in fish, the urophysis, which could participate in response to stress has been also considered, but, in fact, that would have a relatively insignificant impact on the level of circulating AVT (Harding et al. 1997).

As far back as in 1962, Carlson and Holmes demonstrated a considerable decline in the pituitary '*antidiuretic material*', being identified as '*possible vasotocin*', in rainbow trout after transfer to seawater, which would indicate the intense release of that hormone into circulation. In the same study, an increase in pituitary and hypothalamic neurosecretory material, in fish after handling, would suggest a simultaneous increase in both the synthesis and storage of this hormone. Although these early observations were not coupled with plasma hormone measurements, they strongly implied that '*possible vasotocin*' was involved in response to both osmotic and handling stress in fish. The transitory elevated plasma AVT levels in fish exposed to rapid osmotic challenge were reported in rainbow trout (Kulczykowska 1997) and in flounder (Harding et al. 1997). Moreover, Hyodo and Urano (1991) showed that in rainbow trout the magnocellular neurones respond to osmotic challenge by changing expression of AVT precursor genes.

Despite the ability of both forms of stress to enhance plasma AVT concentration, no significant change in plasma IT concentrations was apparent (Figure 1b). Nevertheless, a rise in plasma IT in the rainbow trout transferred from FW to BW has been reported (Kulczykowska 1997), but not observed earlier than 24 h after transfer. Hyodo and Urano (1991) demonstrated reduced synthesis of proIT in SW trout on day 1 after transfer from FW. These data together, suggest that IT plays a role in trout osmoregulation, but seems not to be involved in a quick response to osmotic challenge or disturbance stress. Furthermore, it is reasonable to assume that the vasotocin/isotocin secretion from the different hypothalamic neurones is differentially regulated.

The results of this study showed that both forms of stress caused significant increase in plasma AVT concentration being ineffective to influence plasma IT. On the contrary, in rats, AVP (mammalian counterpart of AVT) responded only to osmotic stress, other than oxytocin (mammalian counterpart of IT), which was affected during both osmotic and non-osmotic stimulation (Jezova et al. 1995).

The present study provided the first experimental evidence that the disturbance stress may affect plasma melatonin in rainbow trout (Figure 1c). Although experimental and clinical studies in mammals have examined the possible physiological association between stress axis and pineal gland the results remain a matter of controversy (Lynch and Deng 1986). The observations in rat indicate that the chronic melatonin treatment influences the adrenocortical secretory re-

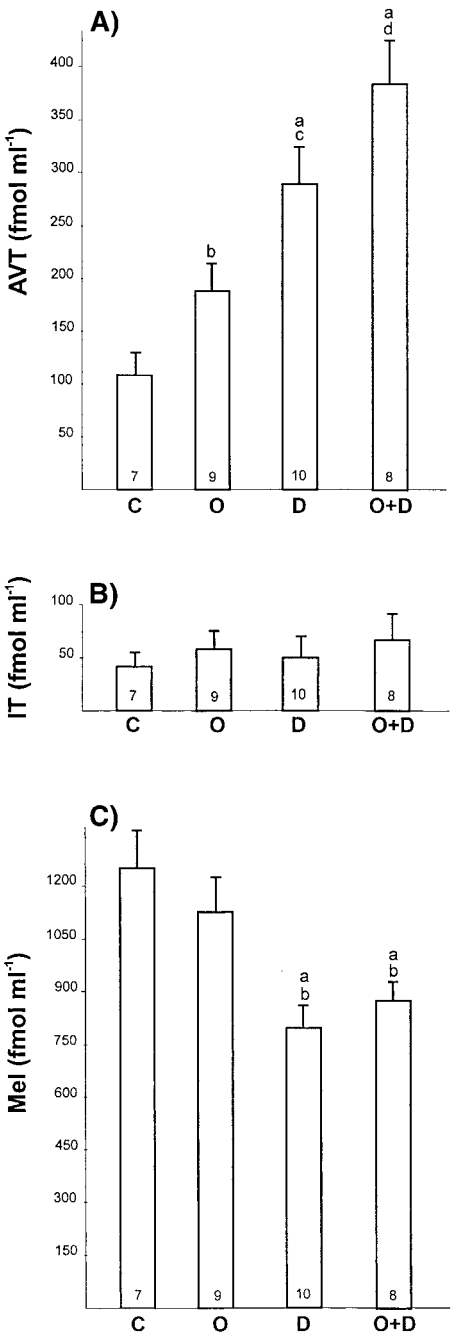

Figure 1. (a) Effect of osmotic (O), disturbance (D) and both stresses (O+D) on plasma arginine vasotocin (AVT) in rainbow trout. C, control value. Values are means ± SEM; n is given in the bars. a, $p < 0.001$ vs. C; b, $p < 0.01$ vs. C; c, $p < 0.01$ vs. O; d, $p < 0.001$ vs.O. (b) Effect of osmotic (O), disturbance (D) and both stresses (O+D) on plasma isotocin (IT) in rainbow trout. C, control value. Values are means ± SEM; n is given in the bars. (c) Effect of osmotic (O), disturbance (D) and both stresses (O+D) on plasma melatonin (Mel) in rainbow trout. C, control value. Values are means ± SEM; n is given in the bars. a, $p < 0.001$ vs. C; b, $p < 0.01$ vs. O.

sponse to stress affecting the biosynthesis and release of CRH, ACTH and AVP (Konakchieva et al. 1997). In general, Mel is assumed to have a stress-protective effect, but it remains unclear which element of stress-axis may be affected by this hormone (Vaughan et al. 1972). In Mel-treated rats, acute stress diminished the release of AVP from hypothalamic organ explants, having no effect on residual content of the hormone in hypothalamus (Konakchieva et al. 1997). However, the physiological links between pineal Mel and hypothalamo-pituitary nonapeptides are still poorly understood at present. The contradictory observations are related to stimulatory or inhibitory effects of vasopressin on the melatonin synthesis/release in mammals (Schröder et al. 1988; Simonneaux et al. 1996).

A possible interrelation between vasotocin and melatonin in fish remains a matter of question. The relationship between AVT and melatonin in fish was considered earlier (Kulczykowska 1995b, 1998), but data are still rather scarce. The lower plasma Mel concentration observed in the present study indicates that melatonin synthesis/release is inhibited in the stress condition, i.e., when the plasma AVT is elevated. This effect might be associated with impairment of melatonin synthesis/release by circulating AVT, or by AVT in vasotocinergic nerve fibers projecting to the pineal organ. That is in agreement with data presented by Schröder et al. (1988) in rats. It is relevant to note, that AVT is effective in inactivating pineal AANAT (arylalkylamine N-acetyltransferase), the rate-limiting enzyme in melatonin-production pathway, especially when the enzyme has been activated by norepinephrine treatment (Sartin et al. 1978). It has been suggested that AVT may act as a modulator of pineal AANAT activity. This could be the case here. Indeed, the fastest primary response to stress in fish is related to the immediate release of epinephrine and norepinephrine into the circulation, similar to that in other vertebrates. In fish the regulatory role of catecholamines in Mel synthesis is not clear yet. It was shown that norepinephrine, at low concentration, had a stimulatory effect on AANAT activity, which was apparently reversed at higher concentration (Falcon et al. 1991). In fact, the release of catecholamines in stress is rapid and short lasting and their effects on melatonin synthesis two hours after stimuli, is rather difficult to predict. The same refers to likely catecholamines-AVT relationships in fish subjected to stress.

In conclusion, this work gives a new insight into hormonal responses to stresses of different nature in

fish and point to the potential role of interrelations between hormones engaged in stress.

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