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Arginine vasotocin (AVT) and isotocin (IT) in fish brain: Diurnal and seasonal variations

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Abstract

An HPLC assay with solid-phase extraction and fluorescence derivatization was developed for measurement of arginine vasotocin (AVT) and isotocin (IT) in the neural tissues of fish. The efficiency and usefulness of the method have been verified in experiments by examination of peptides concentrations in brains of three fish species. The day–night changes in neuropeptides levels have been studied in brains of adult sea bream (*Sparus aurata*) and juvenile Atlantic salmon (*Salmo salar*). Seasonal fluctuations have been investigated in brains of three-spined sticklebacks (*Gasterosteus aculeatus*). The AVT and IT biosynthesis in brain seems to be controlled independently and probably each neuropeptide plays a different role in a circadian time-keeping system and an endocrine calendar in fish.

Keywords: Arginine vasotocin; Derivatization; Fish; Hormone; Hypothalamus; HPLC; Isotocin; Neurohormone; Neurohypophysis; Rhythms

1. Introduction

Arginine vasotocin (AVT) and isotocin (IT) are fish neuropeptides synthesized in the separate hypothalamic parvo- and magnocellular neurons of the NPO (neurosecretory preoptic nucleus) (Goossens et al., 1977; Van den Dungen et al., 1982; Duarte et al., 2001). Single AVT and IT neurons project toward both neurohypophysis and extrahypothalamic regions (Saito et al., 2004a). The peptides are closely related to mammalian vasopressin and oxytocin and have been identified in the hypothalamo-neurohypophysial system of teleosts by Acher et al. (1961, 1962) in the early sixties.

AVT and IT act as neurotransmitters and/or neuromodulators in the central nervous system of fish and both are known to play a role in modulation of reproductive processes and numerous related social behaviours (Godwin et al., 2000; Goodson and Bass, 2000). Many reports have also implicated AVT in cardiovascular activity, fluid management and interactions with other endocrine systems in teleostean fish (Fryer and Leung, 1982; Acher, 1993; Conklin et al., 1997). However, our understanding of the physiological role of AVT and especially of IT in fish is still fragmentary and needs elucidation. A shortage of methods sensitive enough to measure the peptides in a range of their physiological concentrations is a reason for that unsatisfactory state of our knowledge. A lot of difficulties have to be overcome to establish a sensitive assay for the simultaneous measurement of both hormones AVT and IT in one sample (Gozdowska and Kulczykowska, 2004). Our chromatographic assay with derivatization and fluorescent detection preceded by solid-phase extraction gives this unique opportunity.

This study was performed to verify the relevance of the developed HPLC method in experiments and to provide new data on diurnal and seasonal variations of the AVT and IT concentration in fish brain.

Data from mammals strongly suggest that neurohypophysial nonapeptide vasopressin (AVP), analogue of AVT, contributes to the circadian and seasonal time-keeping system (Hofman, 2004). Whether there is a case of AVT in fish is a matter of question, although the hypothetical role of this peptide in the circadian system in teleosts has been considered (Kulczykowska, 1995) and the marked diurnal changes in plasma AVT have been shown in rainbow trout and flounder (Kulczykowska and Stolarski, 1996; Kulczykowska et al., 2001).

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Three alternative experiments on three fish species were conducted. Diurnal variations of AVT and IT concentrations in brain tissue were studied in sea bream (*Sparus aurata*) and in juvenile Atlantic salmon (*Salmo salar*). Changes in the brain neuropeptides concentration throughout the year were investigated in three-spined sticklebacks (*Gasterosteus aculeatus*).

2. Material and methods

2.1. Analytical methods

Frozen brains were weighed quickly and sonicated separately (Microson™ XL 2000) in 1 mL distilled water, then extracted with acetic acid (final 0.25%) and placed in a boiling water bath for 3.5 min according to the procedure described by Pierson et al. (1995). The extracts were cooled on ice before centrifugation (20,000 ×*g*, 30 min, 4 °C). To clean the samples and derivatize peptides, the supernatants were decanted and then loaded into previously equilibrated (2 mL methanol, 2 mL water) spe speedisks (Baker Bond, C18, 20 mg). Columns were washed successively with 0.5 mL water, 0.5 mL acetic acid (4%) and 1 mL water. Then the derivatization procedure different from that previously described (Gozdowska and Kulczykowska, 2004) was applied. The derivatization was performed using 10 μL NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole: 30 mg/mL acetonitrile) in 90 μL borate buffer (pH 9.5) for 20 min at room temperature. After washing by-products with 0.5 mL water and 0.5 mL methanol (10%), derivatized peptides were eluted with mixture of ethanol: 6 N hydrochloric acid (2000:1; 0.5 mL) and directly injected to HPLC system (Beckman modular HPLC system with a Shimadzu spectrofluorometric detector RF-551). Reversed-phase HPLC analysis was performed according to a previously published method (Gozdowska and Kulczykowska, 2004). Briefly, chromatographic separation of peptides was carried out on an Ultrasphere ODS column (250×4.6 mm, 5 μm) using linear gradient system: 48–80% B (0.1% TFA in acetonitrile–water (3:1)) in A (0.1% TFA in water) for 20 min. The column temperature was 22 °C and flow rate 1 mL/min. Fluorescence detection was performed at 470 nm with emission at 530 nm. Derivatization of determined peptides is the crucial step in HPLC-FL analysis. Applying the NBD-F as a derivatizing reagent resulted in high sensitivity of vasotocin and isotocin measurement in fish plasma and allowed detection of these peptides at low physiological concentrations (Gozdowska and Kulczykowska, 2004).

2.2. Animals and experimental protocols

Sea bream (*S. aurata*) (300–500 g) were kept in seawater tanks at the University of Algarve (Portugal) at 19 °C under artificial light. Fish were assigned to one of two experimental groups and exposed to one of the following lighting regimes: continuous darkness (DD) or continuous light (LL). Animals were adapted for at least one week to the experimental lighting regime before experimentation. Brains were removed from decapitated fish from each group at 11:00 and 23:00 h and then stored at −70 °C.

Juvenile Atlantic salmon (approx. 40 g) from wild broodstock of the Dale anadromous strain (Southwest Norway) were kept in freshwater tanks at 8 °C under artificial light with simulated-natural photoperiod (16L:8D) at Bergen University for at least 2 weeks before experimentation. Brains for AVT and IT measurements were removed from decapitated salmon at 12:00 and 24:00 h and stored at −70 °C.

Adult three-spined sticklebacks (*G. aculeatus*) of both sexes used in this study were caught in the Vistula river (Northern Poland) in May (16L:8D), July (16L:8D) and December (8L:16D). Animals were dissected directly after catching. The brains were removed after decapitation, immediately frozen and stored at −70 °C.

2.3. Statistical analysis

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

3. Results

3.1. Analytical step

The concentration of arginine vasotocin and isotocin in the brain tissue was measured using HPLC-FL method (Gozdowska and Kulczykowska, 2004) with significantly changed procedure of extraction and derivatization. The most important improvement was the speedisks which were applied instead of conventional spe cartridges. Moreover, the concentration of derivatizing agent was increased 15-fold. All modifications resulted in a shorter run time of analysis and more effective separation of free peptides in the neural tissue. Each sample was analysed in triplicate. Reproducibility of analysis expressed as RSD (relative standard deviation) for AVT and IT were 4.3% and 1.9%, respectively, and compared favorably with those achieved in our previous method (5.5% and 9.0%, respectively) (Gozdowska and Kulczykowska, 2004). Linearity of standard curve was $r^2=0.995$ and $r^2=0.996$ for AVT and IT, respectively. The detection limit was determined to be 10 fmol/mL.

A typical chromatogram of the AVT and IT in fish brain is shown in Fig. 1.

3.2. Diurnal changes

AVT and IT concentrations measured at 11:00 and at 23:00 h in brains of sea bream exposed to continuous darkness (DD) or continuous light (LL) are presented in Table 1. The AVT level in fish kept in DD displayed no day–night variation; the same applied to the IT in fish adapted to both lighting regimes. In LL adapted fish, noticeably higher concentrations of AVT were