Available online at www.sciencedirect.com
ScienceDirect
General and Comparative Endocrinology 153 (2007) 221–227
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Effect of acute restraint on hypothalamic pro-vasotocin mRNA expression in flounder, *Platichthys flesus*

H. Bond *, J.M. Warne, R.J. Balment

Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK

Received 15 September 2006; revised 16 March 2007; accepted 19 March 2007
Available online 5 May 2007

Abstract

Arginine vasotocin (AVT) stimulates release of adenocorticotrophin hormone (ACTH) in trout. However, AVT's role in fish hypothalamic–pituitary–interrenal-axis (HPIA) is not fully understood. Here, we examined distribution of AVT and glucocorticoid receptor (GR) in the magnocellular preoptic nucleus (PM) and the AVT/cortisol response to acute restraint in flounder.

The GR/AVT distribution in the PM was determined using double immunohistochemistry (IHC). Flounder were confined in nets, immersed in water for 30 m, with plasma and tissue samples taken prior to, 3, 24 and 48 h post-confinement. Plasma osmolality, Na^+, Cl^- and cortisol were taken as indicators of HPIA activation. Plasma AVT was measured proVT mRNA expression in the PM was detected using in situ hybridisation (ISH) with a S35 labelled oligoprobe for homologous flounder proVT. Double IHC showed the presence of GR in AVT synthesising neurones of the PM. Plasma Na^+, Cl^-, osmolality and cortisol (1.0 ± 0.9 to 183.6 ± 3.1 mM; $p < 0.001$) increased significantly 3 h post-restraint: recovering to control levels after 48 h. Plasma AVT levels did not change. However, a concomitant increase in proVT mRNA expression in the magnocellular (PMm) and gigantocellular (PMg) neurones of the PM was observed (11.1 ± 1.8 to $55.2 \pm 9.1\%$ 24 h post-restraint; $p < 0.001$) and levels still remained significantly elevated at 48 h ($p < 0.01$). This suggests that PMm and PMg AVT neurones are associated with HPIA activation following acute restraint, including potential cortisol negative feedback. The extended elevation of hypothalamus proAVT mRNA expression following a single acute stressor affords a possible mechanism to moderate sensitivity of the HPIA to subsequent challenges.

Keywords: Arginine vasotocin; Pro-vasotocin; Glucocorticoid receptor; Cortisol; *Platichthys flesus*; Stress

1. Introduction

The hypothalamic–pituitary–adrenal-axis (HPAA) plays a key role in the integration of the stress response in vertebrates. It is widely recognised that arginine vasopressin (AVP), the mammalian homologue of AVT in fish, acts as a stimulus for ACTH release (Antonii, 1986) and its role in the HPAA has long been established. AVP and corticotrophin releasing factor (CRF) are coexpressed in hypothalamic CRF neurones in the rat (Whitnall et al., 1987) and exert synergistic actions on ACTH secretion from the adenohypophysis (Gillies et al., 1982; Rivier and Vale, 1983; Antonii, 1986; Aguilera, 1998), hence increasing the secretion of corticosteroids. The HPAA exhibits remarkable plasticity. Stress can induce facilitation of subsequent activity of the HPAA, which may balance negative feedback effects of glucocorticoid hormones and maintain responsiveness to new stress as previously shown in the rat (Aguilera, 1994; Aubry et al., 1999; Elias et al., 2004). Repeated stress has been shown to induce increases in AVP and CRF mRNA expression (Bartanusz et al., 1993) and more recently an increase in AVP mRNA, but not CRF mRNA expression was reported in response to stress following repeated restraint, providing further evidence of the importance of AVP in modulation of the HPAA axis during chronic or repeated stress in the rat (Ma et al., 1997).

* Corresponding author. Address: Division of Human Development, The University of Manchester, St. Marys Hospital, Hathersage Road, Manchester M13 0JH, UK.
E-mail address: bond_helen@hotmail.com (H. Bond).

0016-6480/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2007.03.014

Although less understood, the hypothalamic–pituitary–interrenal-axis (HPIA) in teleosts is similar, to the HPAA in mammals. However, hypothalamic control is exerted by direct innervations of the pituitary rather than via a pituitary portal blood supply. AVT and CRF synthesising neurones co-exist in the hypothalamic magnocellular preoptic nucleus (PM) of white sucker and eel (Yulis and Lederis, 1987; Olivereau et al., 1988). AVT acts both independently and synergistically with CRF in stimulating ACTH (Fryer et al., 1985; Baker et al., 1996; Pierson et al., 1996) secretion. A direct neuronal connection between AVT neurosecretory cells (NSCs) and corticotrophic cells in the adenohypophysis has been observed in sea bass and other teleosts (Moons et al., 1989; Batten et al., 1999). Confinement stress has previously been shown to stimulate ACTH release in the trout (Sumpter et al., 1986; Balm and Pottinger, 1995; Ruane et al., 1999) and AVT mRNA expression increases in the parvocellular cells (PMp) of the PM, following repeated restraint stress in the trout (Gilchriest et al., 2000). ProVT neurosecretory cells in the PM are separated by size into three different categories: pars gigantocellularis (PMg), 515 μm^2; pars magnocellularis (PMm), 239 μm^2 and pars parvocelluraris (PMp), 170 μm^2, all of which have direct neuronal connection with the pituitary of trout (Saito et al., 2004).

Evidence so far suggests that AVT has an important modulatory role in the teleost HPIA. Here, we wanted to determine the effect of an acute stress in the euryhaline flounder on AVT and to identify any potential link between AVT and the HPIA and associated feedback by secreted cortisol. To do this we examined the distribution of AVT and glucocorticoid receptors (GR) in the PM and then examined plasma AVT and cortisol responses alongside hypothalamic proVT mRNA expression following acute restraint.

2. Materials and methods

2.1. Experimental animals

Flounder were netted from May to December in Morecambe bay estuary close to Flookborough, in Cumbria and transported to the University of Manchester. Flounder of both sex were used and weighed between 300 to 600 g. Fish were kept in holding tanks containing 250–600 L of SW, which was continuously pumped through activated carbon filters and cooled to between 6 and 11 °C. All animals were subject to normal diurnal lighting and were acclimated to a SW environment for at least two weeks before use. All experiments were carried out under Home Office Licence regulation.

2.2. Detection of rainbow trout glucocorticoid receptor (rtGR)

To determine whether antibody raised to rainbow trout glucocorticoid receptor detected flounder GR, flounder and trout were humanely killed, the liver removed and homogenized in buffer containing 12 mM Hepes (pH 7.6), 300 mM mannitol, 2 μg/ml pepstatin, 1 μg/ml leupeptin and 100 μM AEBSF. The homogenate was centrifuged at 2500*g* for 15 m, the post-nuclear supernatant (PNS) was then removed, pellet discarded and centrifuged at 100,000*g* for 30 min to give a particulate protein pellet. The supernatant/cytosolic fraction was then stored at −80 °C. Protein–SDS complexes were prepared by adding 15 μl sample to 7.5 μl reducing buffer (8 M urea, 5% SDS, 0.04% bromophenol blue, 455 mM dithiothreitol in 50 mM Tris–HCl, pH 6.9). Twenty micrograms protein samples were subjected to SDS–PAGE on a 7% gel. The presence of GR in flounder was checked by Western blot analysis using guinea pig rtGR antibody (1:5000 dilution; kindly donated by Dr. Olivier Kah, Endocrinologie Moleculaire de la reproduction, UPRES-A 6026, France) with rainbow trout liver as a positive control and rtGR preimmune serum with trout liver was used as a negative control.

2.3. Experimental series

Flounder ($n = 18$) weighing between 300 and 600 g were confined in groups of 3 to nets (50 × 50 × 50 cm) and fully immersed in water for a period of 30 m. Groups of six fish were sampled immediately prior to net restraint (controls) and 3, 24 and 48 h post-confinement.

2.4. Blood and tissue sampling

Blood samples (1–5 ml) were taken from the caudal artery via direct needle puncture using a heparinised (50 i.u./ml, Sigma Chemical Co. Ltd, UK) syringe, within 30 s following removal from the tank. Blood was centrifuged at 13,000*g* (microcentrifuge, Hawksley and Sons, Sussex, UK) to separate the plasma. Aliquots of plasma for AVT and Cortisol analysis were snap frozen in dry ice and stored at −80 °C until assay. The remaining plasma was held on ice and analysed immediately for osmolality and ions. Following blood sampling the brain was carefully removed, placed in fixative (4% paraformaldehyde (Sigma, UK) (1 brain to 20 volumes of solution)) and kept at 4 °C for 24 h. The tissue was then dehydrated (automated Citadel 2000 processing unit, Shandon, UK) and set in wax (Klane embedding centre, Staffordshire, UK). 10μm transverse sections of the PM were taken and mounted on gelatin coated slides until further analysis. The PM is part of the hypothalamus found immediately posterior to the optic bulb, continuing until dorsal to the saccus vasiculosus (see Fig. 1). Haematoxylin and eosin staining allowed visual identification and confirmation of the PM.

2.5. Osmolality and ion analysis of plasma

Plasma sodium was measured using a Corning 480-flame photometer (Corning, Essex), plasma Cl^- was measured by electrometric titration using a Corning 925 chloride analyser (Corning, Essex), osmolality was determined by freezing point depression using a Camlab Osmometer (Camlab, Cambridge), calibrated with Roebling standard (298 mOsmol/kg H_2O).

2.6. Radioimmunoassay for AVT

Plasma and pituitary AVT were determined using the radioimmunoassay described by Warne et al. (1994). In brief, AVT was extracted from plasma (0.5–1.0 ml) using reverse phase chromatography (Sep-Pak C18 cartridges, Waters, UK), and from pituitary glands homogenised in 0.4% acetic acid, using 0.5 M acetic acid/0.5% chlorobutanol. The antibody was raised in rabbit against AVP and used at a dilution of 1:500,000. Iodinated AVP (Amersham, Bucks, UK) was the radioligand used and an AVT (Sigma) standard range of 0.1–100 fmol per assay tube was established. The extraction efficiency was 85.2 ± 6.1 % ($n = 12$) for plasma and 83 ± 8.2% ($n = 12$) for pituitary, but no correction has been made for this. The intra- and inter-assay variability were 10.1% ($n = 6$) and 11.2% ($n = 7$) respectively and the assay sensitivity was 0.25 fmol AVT/assay tube.

2.7. Radioimmunoassay for cortisol

Plasma cortisol was determined using the radioimmunoassay as described by Kelsall and Balment (1998) (antibody kindly donated by Dr. B. Baker, University of Bath).