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Cortisol reduces cell proliferation in the telencephalon of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The fish brain grows throughout life, and new cells are added continuously in all major brain areas. As in mammals, the rate of adult brain cell proliferation in fish can be regulated by external factors including environmental complexity and interaction with conspecifics. We have recently demonstrated that the stress experienced by subordinate rainbow trout in social hierarchies leads to a marked suppression of brain cell proliferation in the telencephalon, and that this is accompanied by an increase in plasma levels of cortisol. Corticosteroid hormones are known to suppress adult neurogenesis in mammals, and to investigate whether this is also the case in fish, rainbow trout were fed feed containing either a low or a high dose of cortisol for 6 days. Compared to control animals receiving regular feed, both cortisol treated groups had significantly elevated cortisol levels 24 h after the last feeding, with the high group having levels comparable to those previously reported in socially stressed fish. To quantify cell proliferation, immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed to identify actively cycling cells. The density of PCNA-positive nuclei in the telencephalon was reduced by about 50% in both cortisol treated groups. The effect of cortisol on brain cell proliferation did not reflect a general down regulation of growth, as only the high cortisol group had reduced growth rate, and there was no correlation between brain cell proliferation and growth rate in any group. These results indicate that the reduced proliferative activity seen in brains of socially stressed fish is mediated by cortisol, and that there is a similar suppressive effect of cortisol on brain cell proliferation in the teleost forebrain as in the mammalian hippocampus.

1. Introduction

Teleost fishes of the family Salmonidae, in particular species of the genera *Oncorhynchus* and *Salmo*, have been frequently studied for their tendency to form clear dominance based social hierarchies both in the wild and under experimental conditions [1–8]. Territoriality is expressed at several life stages in salmonids, and subordinate individuals often show a range of behavioural and physiological indicators of chronic stress, including anorexia, altered locomotor patterns and reduced aggression, as well as increased plasma cortisol levels, brain serotonergic activity, and standard metabolic rate [1,6,7,9–12]. We have recently demonstrated that socially subordinate rainbow trout (*Oncorhynchus mykiss*) have reduced brain cell proliferation in the forebrain compared to socially isolated non-stressed individuals [13]. This effect is similar to that seen in contemporary models of psychosocial stress in mammals, where social subordination leads to reduced neurogenesis in the dentate gyrus of the hippocampus [14–16]. A reduced rate of neurogenesis is in turn believed to affect mood and cognition, and is believed to be involved in the pathophysiology of depressive disorders in man (for reviews see Refs. [17–20]).

In comparison to mammals, fish have a much higher prevalence [21–25], and rate of adult brain cell proliferation [24,26], and for this reason fish have been promoted as a potentially important model to understand the evolution and function of adult neurogenesis [27]. The neuroanatomy of adult brain cell proliferation and its role in regeneration after injury is well-studied in fish (for review see Ref. [28]), but it has only recently become evident that the rate of cell proliferation in the fish brain can be regulated in response to external factors. Environmental enrichment [29], pheromone exposure [30] and social communication [31,32] have all been shown to affect the rate of brain cell proliferation in parts of the teleost brain. Yet, very little is known about the mechanisms involved in this regulation.

In our previous study, socially subordinate individuals, which had reduced telencephalic brain cell proliferation also showed significantly elevated plasma levels of cortisol [13]. Cortisol is the main corticosteroid hormone in fish and in addition to its homeostatic function in osmoregulation and energy metabolism (For reviews see Refs. [33,34]) it is typically elevated during acute and chronic stress [34–36]. Cortisol is involved in mediating several of the behavioural

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and physiological effects typically seen in subordinate fish, and treatment with cortisol has been shown to reduce appetite, growth and physical condition in group reared fish [37–39]. Cortisol treatment also affects aggression and locomotor activity during social interaction [40,41], and chronic cortisol treatment leads to increased serotonergic activity in the telencephalon [42]. Cortisol also affects cell addition in the fish brain [31], and is known to reduce adult neurogenesis in the rodent hippocampus [43,44]. Cortisol is thus a likely candidate for mediating the suppressive effect of social stress on telencephalic brain cell proliferation in rainbow trout.

Consequently the aim of the current study was to investigate the effect of cortisol treatment on brain cell proliferation in the rainbow trout. This aim was approached by giving cortisol containing feed to rainbow trout and assessing cell proliferation in the telencephalon by measuring the density of proliferating cell nuclear antigen (PCNA) positive nuclei. This approach allowed for investigation of the effects of cortisol without influence of additional stress associated with injecting the fish with cortisol and a marker of cell proliferation like 5-bromo-3′-deoxyuridine (BrdU).

2. Materials and methods

2.1. Experimental animals and procedure

Juvenile rainbow trout were obtained from a commercial breeder (Valdres Ørretoppdrett, Valdres, Norway) and were transported to the University of Oslo aquarium facility where a group of approximately 250 individuals was maintained in a 750 l holding tank continuously supplied with dechlorinated Oslo tap water (100 l h^{-1}) at 4–6 °C. Lighting followed a 12 h light/12 h darkness cycle, and the fish were fed daily 1% of their body weight with pelleted trout food (3.0 mm, Skretting, Stavanger, Norway) between 12.00 and 16.00. After 6 weeks of group holding, 32 fish weighing from 91.9 g to 225.3 g (156.3 g ± 6.2 g) were isolated in 50 l compartments separated by opaque PVC walls in 250 l glass observation aquaria (c.f. [7,11]). Aquaria were continuously aerated and supplied with dechlorinated Oslo tap water (4.7–5.4 °C). The fish were allowed to acclimate to the experimental set-up for 14 days, during which they were fed pelleted trout feed (1% of body weight) once daily between 12.00 and 16.00 by dropping pellets one by one into the aquarium. After 5 min any remaining food was removed. After acclimation, the fish were anesthetised lightly in a bath of 0.06 mg l^{-1} eugenol (Sigma Aldrich) and weighed. Starting the following day the daily food ration was replaced by control (n = 8), low (n = 12) and high (n = 12) cortisol containing feed. Cortisol was incorporated into the diet by immersing feed pellets in 96% ethanol containing dissolved cortisol (hydrocortisone, Sigma) corresponding to 75 mg cortisol kg^{-1} pellets (low cortisol feed) and 600 mg kg^{-1} pellets (high cortisol feed). Control feed was immersed in pure 96% ethanol. The ethanol was evaporated over night in room temperature to render the cortisol incorporated in the food [45]. The concentrations used were based on the results from Øverli et al. [40], where a concentration of 600 mg kg^{-1} pellets and a daily ration of 1% of the body weight caused plasma cortisol levels similar to those observed in highly stressed rainbow trout (>100 ng ml^{-1}). To address possible dose dependent effects, a low concentration (75 mg kg^{-1} pellets), that was found in a pilot study to produce a low, but significant increase in plasma cortisol levels, was also used. Experimental feed was administered as during acclimation once daily between 12.00 and 16.00 for six days and the exact daily food intake for each fish was recorded by counting the number of feed pellets eaten by each fish.

2.2. Sampling and tissue preparation

After six days of cortisol treatment, 24 h after the last feeding, the fish were anesthetised in a bath of 0.1% Ethyl 3-aminobenzoate methanesulfonate (Sigma), and blood samples were collected from the caudal vein whereupon the fish were killed by decapitation. Blood was centrifuged for 3 min at 4 °C before the plasma was frozen in liquid N_2. Brains were dissected out and fixed in 4% paraformalaldehyde in PBS (phosphate buffered saline, 0.1 mM, pH 7.4) for 24 h, and then transferred to 15% sucrose for 24 h and finally 20% sucrose for 24 h. The brains were embedded in Tissue Tek O.C.T-medium (Sakura) and frozen in isopentane cooled to its freezing point (−160 °C) in liquid N_2. The brains were stored at −80 °C until being sectioned at 25 μm in a cryostat (Microm HM 560) and mounted on SuperFrostPlus (Thermo Scientific) slides.

2.3. PCNA immunohistochemistry

The slides were thawed, washed in PBS (5 × 5 min), and post-fixed in paraformaldehyde (4%, 10 min). Epitope retrieval was performed using citric acid buffer (10 mM, pH 6.0, 85 °C, 60 min). Slides were washed in PBS and unspecific binding blocked with 6% skim milk powder (Acumedic) and 0.03% Triton X-100 (Sigma) in PBS. Sections were treated with primary antibody for 24 h at 4 °C (1:50, Rabbit Anti-PCNA, Dako Cytomation in PBS with 0.6% skim milk powder and 0.03% Triton X-100) and washed 3 × 5 min with PBS. Endogenous peroxidase activity was blocked with 3% H_2O_2 (Sigma, 15 min) and washed 3 × 5 min with PBS. Slides were incubated with secondary antibody for 30 min (EnVision+® System Labelled Polymer-HRP, Anti-Rabbit, Dako) and washed 3 × 5 min with PBS. Finally peroxidase activity was visualized with 3,3'-diaminobenzidine (DAB, Applichem, 15 min), washed 2 × 5 min with dH_2O and coverslipped using permanent mounting medium.

2.4. Quantification of PCNA-positive nuclei

Sections were analyzed and photographed using a Zeiss Axioplan Imaging microscope with an Axiocam HR camera (2600 × 2060 pixels resolution) and Axiovision 3.1 software. All images from each section were stitched together in Adobe Photoshop CS3. Every fourth 25 μm section throughout the telencephalon was analyzed, giving a mean of 22 sections per fish. The number of stained nuclei per investigated brain volume was calculated for each fish based on the total number of stained nuclei in all sections and the investigated volume (calculated from the area of each section as determined using Adobe Photoshop CS3 and the thickness of 25 μm). Areas from the olfactory bulbs and the preoptic areas were excluded from analysis when these were present in the photographed sections.

2.5. Radioimmunoassay quantification of plasma cortisol

Plasma cortisol levels were determined using a radioimmunoassay based on the assay by Pottinger and Carrick [46]. Steroids were extracted from plasma with ethyl acetate (Merck, 1:5 plasma:ethyl acetate), and the extract was vortexed for 30 s followed by centrifugation at 14.000 rpm for 2 min. Supernatants (20–200 μl, depending on the expected stress level of the fish, to ensure that the cortisol levels were within the range of the standard curve) were transferred to 1.5 ml eppendorf tubes. A zero sample and a nonspecific binding (blank) control were made with pure ethyl acetate, and a dilution series standard curve of cortisol (hydrocortisone, Sigma) in ethyl acetate of concentrations from 8 pg/μl to 0.125 pg/μl was produced. All samples, standards and controls were run in duplicate. Identical aliquots (50 μl) of ethyl acetate with approximately 15.000 cpm of [1,2,6,7-^{3}H] cortisol (Amersham Pharmacia Biotech, 60 Ci $mmol^{-1}$) were added to all tubes and the ethyl acetate was evaporated in an exsiccator coupled to a water jet pump. Antibody (Donkey anti cortisol, AbD Serotec, 1:600) in assay buffer; phosphate buffered saline (PBS tablets, Sigma) containing bovine serum albumin (0.1%, Sigma), was added to each tube except the