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Research paper

Thyrotropic activity of corticotropin-releasing hormone in an altricial bird species, the zebra finch (*Taeniopygia guttata*)

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ABSTRACT

In chicken, corticotropin-releasing hormone (CRH) acts as a thyrotropin (TSH)-releasing factor, mediated by the type 2 CRH receptor (CRHR2) on the thyrotropes of the pituitary gland. It is not known whether CRH also controls TSH release in non-precocial avian species that have a different pattern of thyroidal activity during their life cycle. Therefore, we investigated the TSH-releasing capacity of CRH in an altricial species, the zebra finch (*Taeniopygia guttata*). Cellular localisation of type 1 CRH receptor (*CRHR1*) and *CRHR2* mRNA in the pituitary was determined by *in situ* hybridisation, combined with immunohistochemical staining of pituitary thyrotropes. In addition, isolated pituitary glands were stimulated with CRH to determine the effect on TSH release. Lastly, the mRNA levels of hormones and receptors involved in the control of thyroidal and adrenal function were measured by qPCR in zebra finch chicks between hatching and fledging, and in adults. Most of the hypophyseal *CRHR2* mRNA co-localised with thyrotropes, whereas *CRHR1* mRNA was found inbetween thyrotropes. Pituitary glands stimulated *in vitro* with CRH showed increased secretion of TSH-like activity. Pituitary *CRHR2* mRNA expression decreased while pituitary *TSHB* mRNA and brain *CRH* mRNA levels increased towards fledging, similar as seen in chicken hatching. These results suggest that CRHR2 expressed on thyrotropes is likely mediating CRH-induced TSH release in altricial avian species like it does in precocial species, and that the increased thyroid hormone levels towards fledging in altricial birds are the result of increased hypothalamic stimulation, in which the thyrotropic activity of CRH may initially play a role.

1. Introduction

Pronounced increases in circulating thyroid hormones and corticosteroids occur during particular developmental periods in vertebrate life cycles, such as smoltification in salmonids, amphibian metamorphosis, and hatching in precocial birds (e.g., Björnsson et al., 2011; Brown and Cai, 2007; De Groef et al., 2013; Wada, 2008). There is evidence – particularly in amphibians – that the high thyroid hormone and corticosteroid levels during these life stage transitions are caused by increased hypophysiotropic stimulation (Watanabe et al., 2016). In these non-mammalian species, corticotropin-releasing hormone (CRH), which is best known for its role in the hypothalamo–pituitary–adrenal/interrenal (HPA/I) axis by stimulating adrenocorticotropic hormone (ACTH) release from the pituitary gland, also controls the hypothalamo–pituitary–thyroidal (HPT) axis by inducing the secretion of thyrotropin (TSH) (De Groef et al., 2006b). Further studies in chicken and amphibians have shown that CRH stimulates the release of TSH by binding to the type 2 CRH receptor (CRHR2) expressed by pituitary thyrotropes (TSH-producing cells), while it releases ACTH by binding to the type 1 CRH receptor (CRHR1) on pituitary corticotropes (De Groef et al., 2003; Okada et al., 2007). Owing to its dual hypophysiotropic function, CRH is speculated to be an important regulator of both the HPA/I and HPT axes in the period leading up to life stage transitions (Watanabe et al., 2016).

However, bird species differ in their degree of development at hatching as well as in the timing of thyroid hormone and corticosteroid peak secretion (De Groef et al., 2013; McNabb, 2006; Wada, 2008). Precocial birds, like chickens and ducks, are at one end of a developmental spectrum, characterised by hatchlings that are relatively well developed, born with down and with their eyes open, and mobile, and some are capable of feeding on their own. At the other end of the developmental spectrum, fully altricial birds, like pigeons, passerines and parrots, hatch with their eyes closed and without any down, are relatively immobile, and are dependent on the parents for feeding and thermoregulation (Starck and Ricklefs, 1998). Typically, precocial birds have high levels of

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corticosteroids and thyroid hormones in the period before hatching, peaking at hatching and decreasing after hatching. In contrast, the perihatch levels of circulating thyroid hormones and corticosteroids are very low in altricial birds, increasing gradually some days or weeks post hatch towards fledging (De Groef et al., 2013; McNabb, 2006; Wada, 2008). Little is known about the hypothalamic control of fledging in altricial birds. Potentially, the dual hypophysiotropic role of CRH is responsible for the increased activity of the HPT and HPA axes towards fledging in altricial birds. However, it is not known whether the TSH-releasing activity of CRH found in the precocial chicken, can be extrapolated to other avian species, such as altricial birds.

In this study, we investigated whether CRH is a TSH-releasing factor in altricial birds as it is in precocial chicken. Using zebra finch (*Taeniopygia guttata*) as a model, we investigated the localisation of *CRHR1* and *CRHR2* mRNA expression relative to the thyrotropes in the pituitary gland, and the *in vitro* TSH-releasing capacity of CRH in zebra finch pituitary. We also quantified the changes in expression levels of whole-brain *CRH* and *TRH*, and whole-pituitary *CRHR1*, *CRHR2* and thyrotropin-releasing hormone receptor (*TRHR*) mRNA between hatching and fledging, and in adults. Pituitary TSHβ subunit (*TSHB*) and proopiomelanocortin (*POMC*) mRNA were also measured.

2. Materials and methods

This study was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition, 2004) of the National Health and Medical Research Council. All experiments described in this section were approved by the Animal Ethics Committee of La Trobe University (AEC12-10, AEC14-57). A wildlife research permit (number 10007705) was granted by the Victorian State Department of Environment, Land, Water and Planning.

2.1. Cloning of zebra finch CRHR1 and CRHR2 coding sequences

Total RNA was isolated from adult zebra finch pituitary glands using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. One μg of RNA was reverse transcribed in a volume of 20 μl containing 50 U Multiscribe Reverse Transcriptase (Applied Biosystems, Waltham, MA, USA), 1× M-MuLV reverse transcriptase reaction buffer (New England Biolabs, Ipswitch, MA, USA), 0.5 mM deoxyribonucleoside triphosphate (dNTP) mix (Promega, Madison, WI, USA), 0.5 μg oligo-$(dT)_{15}$ primer (Promega) and 40 U RNase inhibitor (Promega). The reaction was performed in a thermocycler at 25 °C for 10 min, followed by 42 °C for 2 h and 85 °C for 5 min. High-fidelity PCR was performed with 5 μl of the cDNA, amplified in a 50-μl mixture containing 1× PCR buffer (Invitrogen), 0.2 mM dNTP mix, 2 mM $MgSO_4$, 1 U High-Fidelity *Taq* DNA polymerase (Invitrogen) and 0.4 μM of each forward primer and reverse primer (*CRHR2* with forward primer 5′-CAAAGAGGTGATCCAGAAGG-3′ and reverse primer 5′-ACCTGTTCCTTCTTTGAATC-3′; *CRHR1* with forward primer 5′-CGCACGGGCAGCTGAGGATG-3′ and reverse primer 5′-CGCTCA GACCGCCGTGGATT-3′; based on the predicted sequences XM_002196341.2 and XM_002198894.3, respectively). The samples were run in a thermocycler with initial denaturation at 94 °C for 4 min, followed by denaturation (94 °C, 30 s), annealing (47 °C for *CRHR2*, 58.5 °C for *CRHR1*, 30 s), and extension (68 °C, 2 min) for 35 cycles. The PCR product was then analysed on a 1% (w/v) agarose gel containing 0.05% (v/v) SYBR Safe DNA gel stain (Invitrogen). The PCR products were excised, purified and inserted in the pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen). Chemically competent *Escherichia coli* (TOP10) cells were transformed with the vector containing the PCR product as insert, and were grown on a culture plate containing Luria-Bertani (LB) medium with 100 μg/ml ampicillin (Bioline, London, UK) and 20 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (50 mg/ml; Bioline). A white colony was subsequently grown overnight in LB medium containing 100 μg/ml ampicillin, and plasmids were purified using the PureLink Quick Plasmid miniprep Kit (Invitrogen). Plasmids were sequenced by the Australian Genome Research Facility (AGRF; Melbourne, Australia) and the obtained sequences were analysed using the default settings of BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Clustal Omega (http://www.ebi.ac.uk) and the ExPASy Translate tool (http://web.expasy.org/tools/translate).

2.2. Localisation of CRHR1 and CRHR2 mRNA in zebra finch pituitary

2.2.1. Animals, tissue processing and sectioning

Adult male zebra finches ($n = 4$) were euthanised by decapitation. Pituitary glands were removed and kept overnight in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$, 1.8 mM KH_2PO_4, pH 7.4) at 4 °C. Glands were then cryoprotected at 4 °C in the same solution containing 30% (w/v) sucrose and were subsequently stored at −80 °C until sectioning. Tissues were cut into 7-μm cryosections and transferred to Polysine slides (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2. In situ hybridisation

A partial zebra finch *CRHR2* sequence of 800 bp (bp 427–1226) was amplified with forward primer 5′-AGGAGTATCA GATGCCTGCG-3′ and reverse primer 5′-GTCTGTTTGATGCTGTG GAA-3′. A partial zebra finch *CRHR1* sequence of 796 bp (bp 449–1244) was amplified with forward primer 5′-TCCGGTGCCTGAGAAACATC-3′ and reverse primer 5′-GATTGCTT GATGCTGTGGAA-3′. These sequences were then inserted into a pCRII-TOPO vector, and the DIG RNA Labelling Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) was used to transcribe RNA copies labelled with digoxygenin following the manufacturer's guidelines. Sense and antisense riboprobes were transcribed from 1 μg of the linearised and purified plasmid using 2 μl (20 U) of the appropriate RNA polymerase (SP6 or T7).

Pituitary sections were first fixed in 4% (w/v) paraformaldehyde in PBS for 5 min at room temperature and washed in PBS. They were then placed in 200 μl of pre-warmed (37 °C) proteinase K buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5) containing 40 μl of proteinase K solution (Promega) for 15 min and washed in PBS. Slides were again fixed in 4% paraformaldehyde in PBS for 5 min at room temperature and washed in PBS. These were then deproteinised with 0.2 M HCl for 10 min, acetylated with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature and washed in PBS. The sections were treated with 1% (v/v) Triton X-100 in PBS for 30 min, then washed in PBS. Prehybridisation was performed in 400 μl of a mixture of 1.8 ml hybridisation buffer (50% (v/v) formamide, 10% (w/v) dextran sulphate, 5x Denhardt's solution, 0.62 M NaCl, 20 mM Na_2-PIPES, 10 mM EDTA, 0.2% (w/v) sodium dodecyl sulfate), 50 μl fish sperm DNA (10 μg/μl; Roche), 100 μl 1 M dithiothreitol and 50 μl total yeast RNA (10 μg/μl; Roche) for 3 h at room temperature. Hybridisation was performed in 200 μl of this solution containing 400 ng/ml of the labelled sense or antisense riboprobe at 65 °C overnight. After hybridisation, slides were washed in 5× saline sodium citrate (SSC) (from 20× SSC concentrate; 3 M NaCl, 0.3 M trisodium citrate dihydrate, pH 7.0) at 72 °C for 45 min, followed by two washes in 0.2× SSC for 5 min at room temperature. After three washes in PBS with 0.1% Triton X-100 for 5 min, slides were incubated in 500 μl of a blocking solution

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