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RESEARCH ARTICLE

How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish

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ABSTRACT

Despite the use of fish models to study human mental disorders and dysfunctions, knowledge of regional telencephalic responses in non-mammalian vertebrates expressing alternative stress coping styles is poor. As perception of salient stimuli associated with stress coping in mammals is mainly under forebrain limbic control, we tested region-specific forebrain neural (i.e. mRNA abundance and monoamine neurochemistry) and endocrine responses under basal and acute stress conditions for previously characterised proactive and reactive Atlantic salmon. Reactive fish showed a higher degree of the neurogenesis marker proliferating cell nuclear antigen (*pcna*) and dopamine activity under basal conditions in the proposed hippocampus homologue (Dl) and higher post-stress plasma cortisol levels. Proactive fish displayed higher post-stress serotonergic signalling (i.e. higher serotonergic activity and expression of the 5-HT_{1A} receptor) in the proposed amygdala homologue (Dm), increased expression of the neuroplasticity marker brain-derived neurotropic factor (*bdnf*) in both Dl and the lateral septum homologue (Vv), as well as increased expression of the corticotropin releasing factor 1 (crf_1) receptor in the Dl, in line with active coping neuro-profiles reported in the mammalian literature. We present novel evidence of proposed functional equivalences in the fish forebrain with mammalian limbic structures.

KEY WORDS: Atlantic salmon, Limbic areas, Neural plasticity, BDNF, Serotonin

INTRODUCTION

Many studies have reported consistent and correlated behavioural and physiological traits in vertebrates, including the correlation between dominant behaviour and lower stress reactivity. Notably, individuals perceive and react differently to their environment, and this affects their robustness to challenges such as stress and diseases (Dingemanse et al., 2010; Koolhaas, 2008; Koolhaas et al., 1999; Seiffge-Krenke, 2011; Øverli et al., 2007). In this context, animals have to balance attention, inhibition of active behaviour and cognitive flexibility in relation to internal and external feedback in an ever-changing environment (Bari and Robbins, 2013). Coping styles have been defined as a set of individual behavioural and physiological responses to stress which are consistent over time, and are commonly used to study individual variations in the stress response of vertebrates, including fish (Koolhaas, 2008; Koolhaas et al., 2007; Øverli et al., 2007). Behaviourally, proactive animals tend to be bolder, more aggressive, dominant and less flexible to changes in routines. Physiologically, proactive individuals are characterised by lower hypothalamic-pituitary–adrenal (HPA) axis reactivity (i.e. lower post-stress cortisol), as well as lower brain serotonergic and higher dopaminergic activity, while reactive individuals exhibit the opposite behavioural and physiological profile (Koolhaas et al., 2007, 2010, 1999). Notably, while differences between coping styles in terms of behaviour, hypothalamic-pituitary–interrenal (HPI) axis reactivity (the fish's HPA equivalent) and monoaminergic activity in multifunctional brain regions, such as the telencephalon, hypothalamus and brainstem, have been reported in fish (Johansen et al., 2012; Schjolden et al., 2006; Silva et al., 2014; Øverli et al., 2001, 2007), a more precise, region-specific, characterisation of telencephalic areas is still lacking. Region-specific studies of functional subdivisions and limbic nuclei are notoriously difficult in fish, as a result of their relatively small size. Yet, by characterisation of conserved neural circuits that regulate adaptive behavioural responses, a neural basis for individual variation can be discerned in teleosts (Maruska et al., 2013). As fish models are becoming increasingly popular for studying central nervous systems diseases (Panula et al., 2006), comprehensive, functional and regional neural studies are needed to allow extrapolation of obtained results to mammalian models.

In contrast to that of mammals, the fish's telencephalon lacks a 6-layered pallium. Instead, teleostean telencephalic pallial areas contain aggregates of neurons (Ito and Yamamoto, 2009), similar to those of birds (Karten, 1991; Shimizu, 2007). Interestingly, the lack of a 6-layered pallium does not imply an absence of so-called 'higher functions', and telencephalic cortical-like functions have been reported in several fish species (Bshary and Brown, 2014; Demski, 1983; Grosenick et al., 2007; Ito et al., 2007; Ocaña et al., 2015). The fish's dorsomedial (Dm) and dorsolateral (Dl) pallium have been characterised as functional homologues of the mammalian amygdala and hippocampus, respectively, and are implicated in stimuli salience, memory and learning (Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011; Vargas et al., 2009). Furthermore, in terms of stimuli salience and emotional coding, the mammalian lateral septum appears to work in conjunction with the amygdala and hippocampus to regulate emotional reactivity and goal-oriented behaviour, respectively

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(Luo et al., 2011; Singewald et al., 2011). The ventral part of the ventral telencephalon (Vv) in fish has been proposed as a putative homologue to the mammalian lateral septum (Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011).

By use of a behavioural paradigm, we characterised contrasting stress coping styles in an individually tagged domestic population of Atlantic salmon (*Salmo salar* L.). Thus, fish that escaped an imposed hypoxia by swimming into an adjacent normoxic tank and those that did not were characterised as proactive and reactive coping styles, respectively. Following a resting period in their home tanks after coping style selection, target regions in the telencephalon were micro-dissected to determine differences in monoamine neurochemistry and gene expression of serotonergic and corticotropin releasing factor (*crf*) systems (both important systems in the regulation of the HPI axis), as well as neural plasticity and proliferation genes, both in control conditions and following an acute stressor. Plasma cortisol levels were assessed as a direct indicator of HPI axis activity, which also gives physiological support to the assessment of proactive and reactive behavioural patterns. *In situ* hybridisation (ISH) analysis was conducted post-stress in order to visualise and identify activated telencephalic areas. We hypothesise that region-specific differences in monoamine neurochemistry and transcript abundance profiles within the telencephalon of proactive and reactive fish will be comparable to those reported for contrasting coping styles in mammals (Koolhaas et al., 2010; Veenema and Neumann, 2007) and believe that our results are important for understanding the association between individual behavioural differences and regulatory monoaminergic and neural plasticity substrates.

MATERIALS AND METHODS

Statement on ethics

This work was approved by the Norwegian Animal Research Authority (NARA), following the Norwegian laws and regulations with respect to experiments and procedures on live animals in Norway.

Animals, facilities and hypoxia-response sorting

The study was conducted at the Aquaculture Research Station in Tromsø (Norway), using 0+ Atlantic salmon (Atlantic QTL-innova IPN). The fish were reared at 10°C, on a continuous light regime and fed *ad libitum* (Skretting Nutra). The fish were individually tagged using internal 12 mm PIT-Tags (HPT12 tags in pre-loaded tray; Biomark, Boise, ID, USA), injected with an MK-25 implant gun. The fish population (n=480, divided over 8 groups) was reared in circular holding tanks (~116 l) with flow-through freshwater. Mean body mass 2 weeks prior to the experiment was 57.1±7.3 g. The experimental setup for the hypoxia sorting consisted of two custom-made circular tanks (~200 l, diameter 65 cm, water depth 60 cm; Cipax AS, Bjørkelangen, Norway), i.e. one low oxygen/hypoxia and one normal oxygen/normoxia tank. The tanks were connected at the surface level by a tube (inner diameter 9 cm). This tube was integrated with a custom-made spool PIT-Tag antenna (Biomark), which was linked to a Biomark FS2001 reader and tag manager software. In this way, we were able to identify fish leaving the hypoxia tank to enter the normoxia tank (i.e. proactive) and those staying (i.e. reactive), independent of the declining oxygen level. Each tank had a separate water inlet and outlet. In the hypoxia tank, the inlet was connected to a N_2 gas exchanger (15 mg N_2 l^{-1}), which deoxygenated the inflowing water. Oxygen levels (mg O_2 l^{-1}) in the tanks were monitored every minute, using an O_2-monitoring system (Loligo Systems, Tjele, Denmark). Control tests, prior to the experiment, demonstrated that the oxygen depletion in the hypoxia tank was homogeneous throughout the water column. Two video cameras were mounted on top of the tanks to observe the fish passing through the tube between the tanks. Each test took approximately 5 h and started at 08:30 h. All tests were conducted in an equal manner. Prior to the test, the tanks were cleaned, the water temperature was adjusted if necessary, and the water flow in each tank was set to 3.5 l min^{-1}. The fish were transferred from their holding tanks to the hypoxia tank as carefully as possible and left undisturbed (behind an opaque curtain) for the duration of the test. The fish were allowed to acclimatise in the system for 2 h prior to the drop in oxygen levels. During the decline in oxygen, water flow in the hypoxia tank was directed through the N_2 gas exchanger, and a sliding door between the hypoxia and normoxia tank was opened, allowing fish to swim freely between the tanks. The experiment was terminated when oxygen levels reached 25% saturation in the hypoxia tank, after which all fish were transferred back to their holding tanks. This test was conducted twice to ensure consistency of the behavioural response.

Sampling protocol

After the sorting experiment, fish were left undisturbed in their holding tanks for a period of 2.5 months, after which they were sampled under basal and acute stress conditions. Reactive and proactive fish were sampled either straight from holding tanks (basal; n=22; 10 reactive, 12 proactive) or after lowering the water level to 5 cm for 30 min (acute stress test; n=28; 17 reactive, 11 proactive). Proactive and reactive fish were collected simultaneously by netting them directly from their holding tanks (each tank contained mixed proactive and reactive fish). Immediately after netting, individuals were killed with an overdose of 1 g l^{-1} MS-222 (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) buffered with 25 mg l^{-1} $NaHCO_3$, which rendered them completely motionless (no opercular movement) within 10 s of immersion. Fish were rapidly weighed, fork length was measured and a blood sample was taken from the caudal vessels with a 1 ml syringe fitted with a 23 gauge needle containing the anticoagulant heparin. Following centrifugation for 5 min at 9289 rcf and 4°C, plasma samples were frozen and stored at −80°C for later analysis. Brain samples were processed in two different ways. (1) Fish were deeply anaesthetised with buffered MS-222 and fixed by vascular perfusion with 4% paraformaldehyde (PF) in 0.1 mol l^{-1} Sørensen's phosphate buffer (PB; 28 mmol l^{-1} NaH_2PO_4, 71 mmol l^{-1} Na_2HPO_4, pH 7.2). The brains were dissected out and post-fixed in fresh 4% PF in Sørensen's PB for 16 h at 4°C. The tissue was washed three times for 20 min in PB, cryopreserved overnight in 25% sucrose in PB at 4°C, embedded in Tissue-Tek O.C.T.-Compound (Sakura Fintek) and stored at −80°C until sectioning for *in situ* hybridisation. (2) Fish were decapitated and whole heads were placed in containers with Tissue-Tek O.C.T. compound and immediately frozen in liquid nitrogen. Frozen brains were then placed in individually labelled tubes and stored at −80°C until sectioning and microdissection for monoamine and gene expression analyses. Right and left lobes were randomised to control for any possible lateralisation differences. As we did not find a lateralisation effect between right and left lobes, the data were pooled (data not shown).

Cortisol radioimmunoassay

Undiluted plasma (in duplicate) was assayed using a radioimmunoassay (RIA) following the procedure described by Gorissen et al. (2012). Intra- and inter-assay variations were 3.5% and 12.5%, respectively, and cross-reactivity of the cortisol

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antibody (antibody xm210; Abcam, Cambridge, UK) was as follows: cortisol 100%, 11-deoxycortisol 0.9%, prednisolone 5.6%, corticosterone 0.6%, 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, testosterone, oestradiol and oestriol all <0.01%.

Brain sectioning and microdissections

Frozen whole heads were sliced in 100 µm serial sections using a Leica CM1950 cryostat (Leica, Wetzlar, Germany), at −18°C. The sliced tissue was thaw-mounted on glass slides, and refrozen at −80°C for microdissection.

The glass slides were placed on a cooling plate set at −14°C. Using a microscope, three areas were microdissected using a modified 23 gauge needle: the Dl as a whole (for the purposes of this study, we did not distinguish between Dl sub-regions), the Dm and the Vv, as depicted in Fig. 1. Brain regions were identified using several salmonid stereotaxic atlases (Carruth et al., 2000; Navas et al., 1995; Northcutt and Davis, 1983). Microdissections for the Vv area were collected until the appearance of the central part of the ventral telencephalon (Navas et al., 1995). On average, 33–42 punches were taken for the Dl, 33–43 for the Dm and 10–12 for the Vv area. Micro-dissected tissue (alternating left and right lobe of the telencephalon) was either injected into 50 µl Trizol® (Invitrogen, Carlsbad, CA, USA) for later analysis of gene expression, or into 50 µl sodium acetate buffer (pH 5) containing an internal standard (3,4-dihydroxybenzilamine hydrobromine; DHBA) for monoamine analysis. All samples were stored at −80°C immediately after extraction.

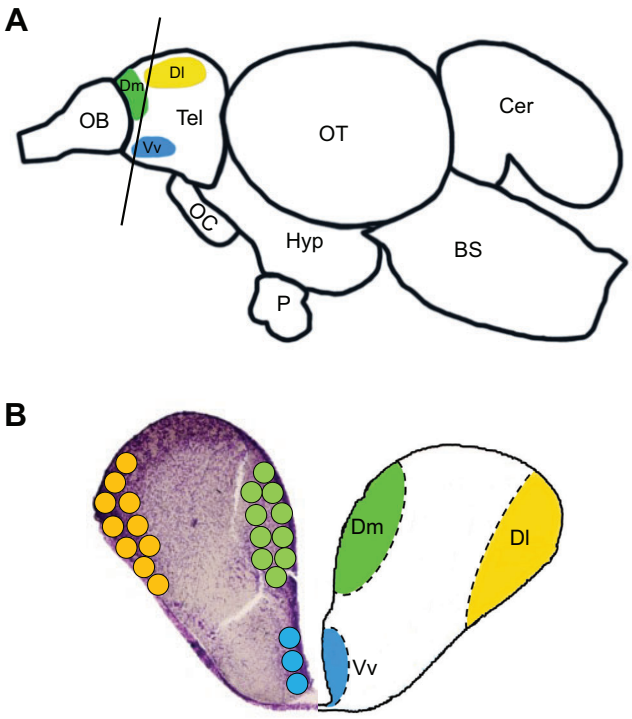

Fig. 1. Atlantic salmon brain. (A) Sagittal view. (B) Telencephalic transverse view. Right, a diagram depicting the location of the dorsolateral pallium (Dl), the dorsomedial pallium (Dm) and the ventral part of the ventral telencephalon (Vv). Left, microdissected areas on a Cresyl Violet Nissl-stained section showing removed tissue sections for each telencephalic subregion. BS, brainstem; Cer, cerebellum; Hyp, hypothalamus; OB, olfactory bulb; OC, optic chiasm; OT, optic tectum; P, pituitary; Tel, telencephalon.

Monoaminergic neurochemistry

Frozen samples were thawed and centrifuged for 10 min at 15,493 rcf and 4°C. The supernatant was used in order to analyse monoamine neurochemistry by high-performance liquid chromatography (HPLC), while the remaining pellet was refrozen at −80°C for later analysis of protein concentration using a Bradford protein assay. Both the HPLC and the protein analysis methodology were performed as described in Vindas et al. (2014a).

Relative transcript abundance

Total RNA from telencephalic microdissected tissue was extracted by thawing frozen samples (immersed in 50 µl Trizol®), which were then vortexed and left for 5 min at room temperature before spinning for 20 min at 13,000 rcf. Ice-cold 70% EtOH was then added to the samples. Next, samples were transferred into an RNAeasy column in 2 ml tubes and the manufacturer's instructions for the RNeasy® Plus Mini kit (Qiagen, Crawley, West Sussex, UK) were followed from this step onwards. RNA concentrations were assessed using a NanoDrop® ND-1000 UV/Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA quality was inferred from RNA integrity numbers (RINs) calculated using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A RIN≥8 was accepted as sufficient RNA quality. First-strand cDNA was synthesised from 0.15 µg DNase I-treated (DNA-free™ Kit, Ambion Applied Biosystems) total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT12–18 primers synthesised by Invitrogen.

Gene sequences were retrieved using NCBI (www.ncbi.nlm.nih.gov/; accession numbers are given in Table S1). Gene-specific primers for Atlantic salmon for the remaining genes of interest were designed using the web-based Primer3 programme (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) and synthesised by Invitrogen. At least four primer pairs were designed, overlapping intron–exon junctions, for each gene, and primer pairs with the lowest Cq-values in PCR and a single peak in the melting curve were chosen and are listed in Table S1. The qPCR products were sequenced to verify that the primers amplified the right cDNA. qPCR was carried out using a Roche LC480 light cycler® (Roche Diagnostics, Penzberg, Germany) as described by Johansen et al. (2011). The reference genes used were *ef1aα*, *S20* and *hprt*1. As *S20* yielded the lowest Cq-values and least variance both between and within plates, this gene was chosen as the internal control for calculation of relative expression (ΔΔCq). All Cq values ≥40 were eliminated as such high numbers imply low efficiency. Furthermore, all Cq values above 35 were rejected based upon comparison between the Cq of the lowest concentration unknown and non-template controls, following procedures described by Bustin et al. (2009).

ISH

ISH for brain-derived neurotropic factor (*bdnf*) and *cfos* transcript abundance (post-stress) was conducted on parallel sections of three Atlantic salmon per coping style. Adjacent transverse 12 µm sections were cut using a Leica CM 1850 cryostat (Leica), collected on SuperFrost Ultra Plus glasses (Menzel Glaser) and dried at 65°C for 10 min. Digoxigenin (DIG)-labelled riboprobes were prepared using a DIG-RNA labelling mix in accordance with the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The ISH probes for *cfos* and *bdnf* were 906 and 485 nucleotides long, respectively, and were cloned using the following primers: *cfos* forward ACTCCGCTTTCAACACCGAC, reverse TGTAGAGAG-GCTCCCAGTCC; *bdnf* forward TCACAGACACGTTTGAGC-

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AGGTGA, reverse ATGCCTCTTGTCTATTCCACGGCA. The quality and quantity of the synthesised riboprobes were assessed by agarose gel electrophoresis. Pre-treatment and treatment of samples for ISH was conducted as specified by Ebbesson et al. (2011).

Statistical analyses

Two-way analysis of variance (ANOVA) was used to compare cortisol levels, monoaminergic neurochemistry and gene expression data, with coping style (reactive versus proactive) and treatment (basal conditions versus acute stress) as independent variables. Models were assessed by their capacity to explain the variability and interaction effects and were accepted or rejected according to total model 'lack of fit' probabilities (provided by the ANOVA model). In addition, when an interaction effect between stress and coping style was found, planned contrast effect tests were conducted in order to ascertain differences between groups. A corrected α=0.01 was used to establish significance for this four-way multiple comparison. Before final acceptance of the model, diagnostic residual plots were examined to ensure that no systematic patterns occurred in the errors (e.g. fitted values versus observed values and Q–Q plots). When necessary, values were either log transformed (concentrations) or arcsine transformed (ratios). Rejection criteria for Cq values resulted in several values being omitted, in particular for genes with a low transcript abundance. Therefore, these samples were not taken into consideration in the statistical analysis. The '*n*' values (after rejection criteria) for each gene of interest are given in Table S2. An overview of average Cq values and efficiencies for all target genes can be found in Table S3.

RESULTS

Hypoxia-response sorting

There was a clear difference in the individual reaction to an increasingly hypoxic environment. Approximately 45% of the fish remained in the hypoxic tank during the entire test period (i.e. reactive), whereas ∼55% left the hypoxic conditions after some time and swam into the neighbouring normoxic tank (i.e. proactive). After the onset of the oxygen decline in the hypoxia tank, we observed a linear reduction in oxygen levels between 0.05 and 0.10 mg O_2 min^{-1}. Most of the salmon remained inactive in the hypoxia tank until approximately 60 min after oxygen decline (at approximately 40% O_2 saturation); from that moment onwards, there was a steady flow of proactive fish migrating towards the normoxic tank. Notably, while some fish crossed back and forth between tanks during the first 60 min of O_2 decline, movement was exclusively unidirectional towards the normoxic tank thereafter. On average, proactive fish left the hypoxic tank after 69 min (at approximately 30.4% O_2 saturation), while reactive fish remained inactive in the hypoxia tank throughout the experiment (the end point of the experiment was set at 25% O_2 saturation, which was reached at approximately 80 min).

Plasma cortisol levels

Cortisol basal and post-stress values were 5±1 and 150±24 ng ml^{-1} for reactive and 6±2 ng ml^{-1} and 96±17 ng ml^{-1} (means±s.e.m.) for proactive fish, respectively. As predicted, both groups reacted with a significant increase in cortisol levels to acute stress (P<0.005). However, reactive individuals had significantly higher cortisol (P<0.001) than proactive fish, after the acute stressor. ANOVA statistics: coping style: $F_{3,108}$=14, P<0.001, stress: $F_{3,108}$=143, P<0.001, interaction (coping style×stress): $F_{3,108}$=9.36, P=0.002.

Monoamine neurochemistry

Regarding serotonin (5-hydroxytryptamine, 5-HT) and its main catabolite 5-hydroxiindoleacetic acid (5-HIAA), we found that only proactive fish displayed higher 5-HIAA concentrations after stress in the Dm (P<0.001; Fig. 2), with a tendency for 5-HIAA levels to be higher in proactive than in reactive fish post-stress (P=0.03; corrected $\alpha \leq 0.01$). Fish from both coping styles responded with an

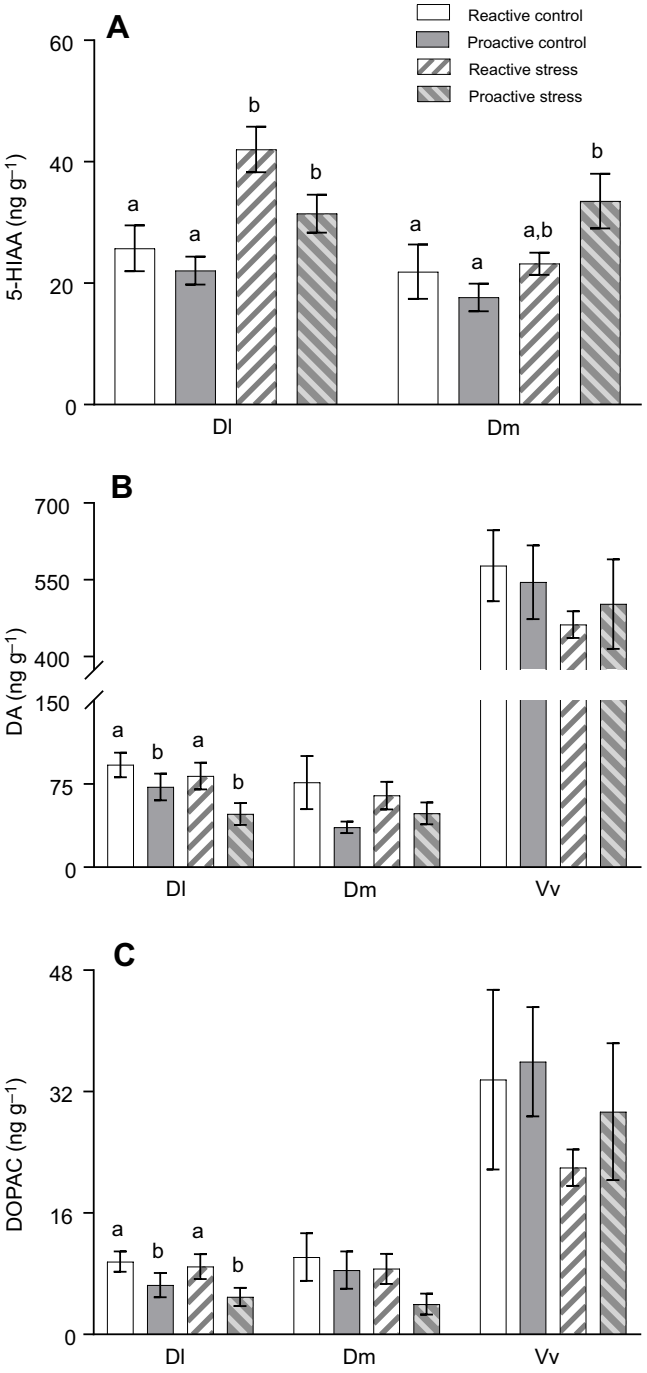

Fig. 2. Effect of coping style (proactive versus reactive) and stress (basal versus acute) on monoamine neurochemistry. 5-Hydroxiindoleacetic acid (5-HIAA; A), dopamine (DA; B) and dihydroxyphenylacetic acid (DOPAC; C) neurochemistry in the Dl, Dm and Vv of Atlantic salmon. Lowercase letters indicate significant ANOVA differences within coping style and/or stress groups in each telencephalic subregion (i.e. not between subregions). Data are presented as means±s.e.m.

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increase in 5-HIAA levels after stress in the Dl (Fig. 2). No significant changes in Dl or Dm 5-HT levels were found. Surprisingly, 5-HT and 5-HIAA levels in the Vv were below the level of detection.

Reactive fish had overall higher concentrations of both dopamine (DA; P=0.02) and its main catabolite 3,4-dihydroxyphenylacetic acid (DOPAC; P=0.007) compared with proactive individuals in the Dl. No other statistically significant differences were evident in the Dm or Vv (Fig. 2). Interestingly, DA and DOPAC concentrations in the Vv were 9- and 7-fold higher than those in the Dl and Dm, respectively. This suggests that DA signalling in this area may be particularly important, but perhaps not under the conditions of our study.

Relative transcript abundance

We analysed two paralogues for both the *5-HT$_{1A}$* receptor (*5-HT$_{1A\alpha}$* and *5-HT$_{1A\beta}$*) and the 5-HT transporter (*5-HTTA* and *5-HTTB*). Region-specific analysis showed an overall higher transcript abundance (i.e. at both basal and acute stress conditions) of both *5-HT$_{1A\alpha}$* and *5-HT$_{1A\beta}$* in the Dm ($P\leq$0.02) and *5-HT$_{1A\beta}$* in the Vv ($P\leq$0.04) in proactive compared with reactive fish (Fig. 3A,B). Both 5-HT transporter paralogues had a low transcript abundance in the microdissected telencephalic areas. In fact, 5-*HTTB* was below detection levels and 5-*HTTA* was mainly expressed in the Dm (Table S2).

The relative mRNA abundance of the neural plasticity marker *bdnf* was significantly increased in response to stress in both the Dl (P=0.002) and Vv (P=0.005) of proactive individuals only (Fig. 3D). The neural proliferation marker proliferating cell nuclear antigen (*pcna*) was higher in the Dl of reactive fish under basal conditions, compared with proactive individuals (P=0.008), and downregulated in the Dl post-stress in reactive fish only (P=0.01, Fig. 3C). There were no statistically significant differences in transcript abundance of the cell differentiation marker *neurod* in any of the studied areas and experimental groups (Table S2).

We also analysed transcript abundance of *crf*, CRF-binding protein (*crfbp*) and CRF receptors 1 (*crf$_1$*) and 2 (*crf$_2$*). We found that relative levels of *crf* mRNA showed no differences in the Dl or Dm (Fig. 4A). There were no effects on *crfbp* expression in the Dl, but there was a tendency for proactive individuals to have higher expression of *crfbp* compared with reactive fish after stress in the Dm (Fig. 4B; P=0.03, corrected $\alpha\leq$0.01). *crf$_1$* expression in the Dl was elevated overall in proactive compared with reactive fish (P=0.02). No differences in *crf$_1$* expression were found in the Dm (Fig. 4C). Expression of *crf$_2$* was not detectable in any of the microdissected areas. In addition, we found little to no expression of any of the studied genes of the CRF system in the Vv.

An overview of coping style and stress-induced differences for all studied variables is given in Table S2. Average Cq values and efficiencies for genes are provided in Table S3. For a full overview of all data used in the statistical analysis, please refer to Table S4.

ISH

ISH analysis of *cfos* and *bdnf* transcript abundance showed clear post-stress activation in the target telencephalic areas, *viz.* Dl, Dm and Vv, in both coping styles (Fig. 5). In addition, we found differences in spatial distribution of *cfos*- and *bdnf*-positive cells between coping styles, which suggests a heterogeneity of activation within target regions. Notably, basal levels of transcript abundance were not detectable, probably due to the fact that as all samples

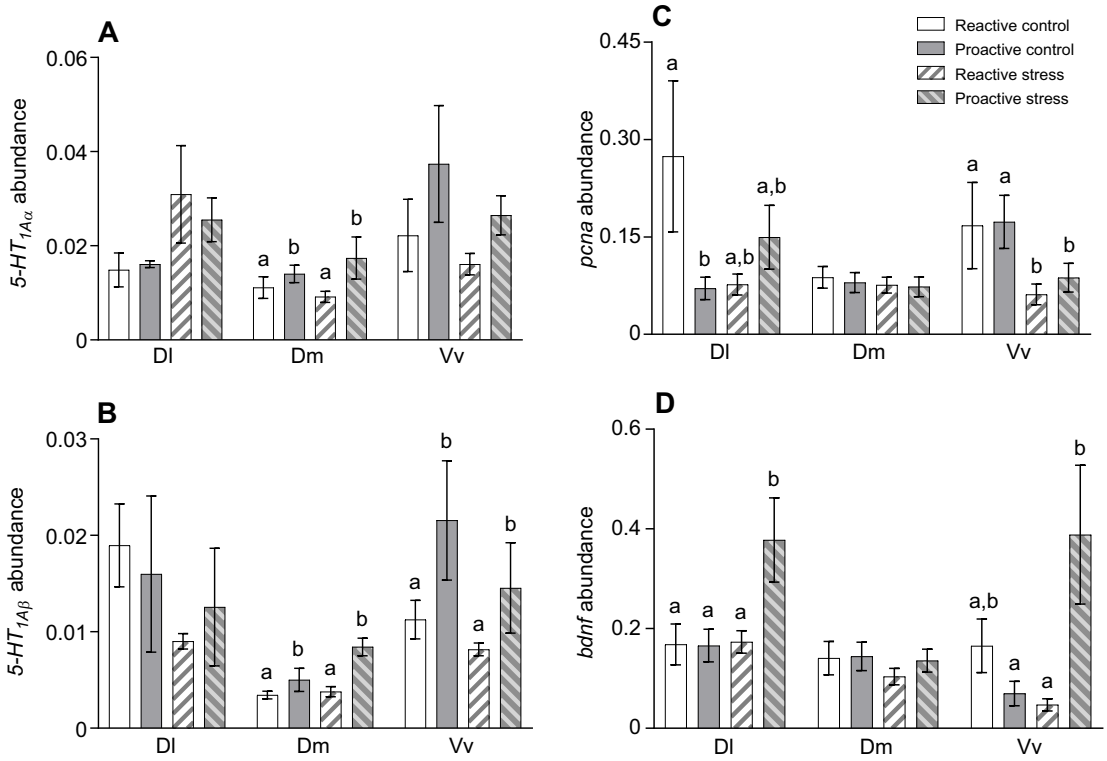

Fig. 3. Effect of coping style (proactive versus reactive) and stress (basal versus acute) on the relative mRNA abundance of serotonin receptors, proliferating cell nuclear antigen (*pcna*) and the brain-derived neurotrophic factor (*bdnf*). Transcript abundance (relative to the *S20* reference gene) of the serotonin receptors *5-HT$_{1A\alpha}$* (A) and *5-HT$_{1A\beta}$* (B), and of *pcna* (C) and *bdnf* (D) in the Dl, Dm and Vv of Atlantic salmon. Lowercase letters indicate significant ANOVA differences within coping style and/or stress groups in each telencephalic subregion (i.e. not between subregions). Data are presented as means±s.e.m.

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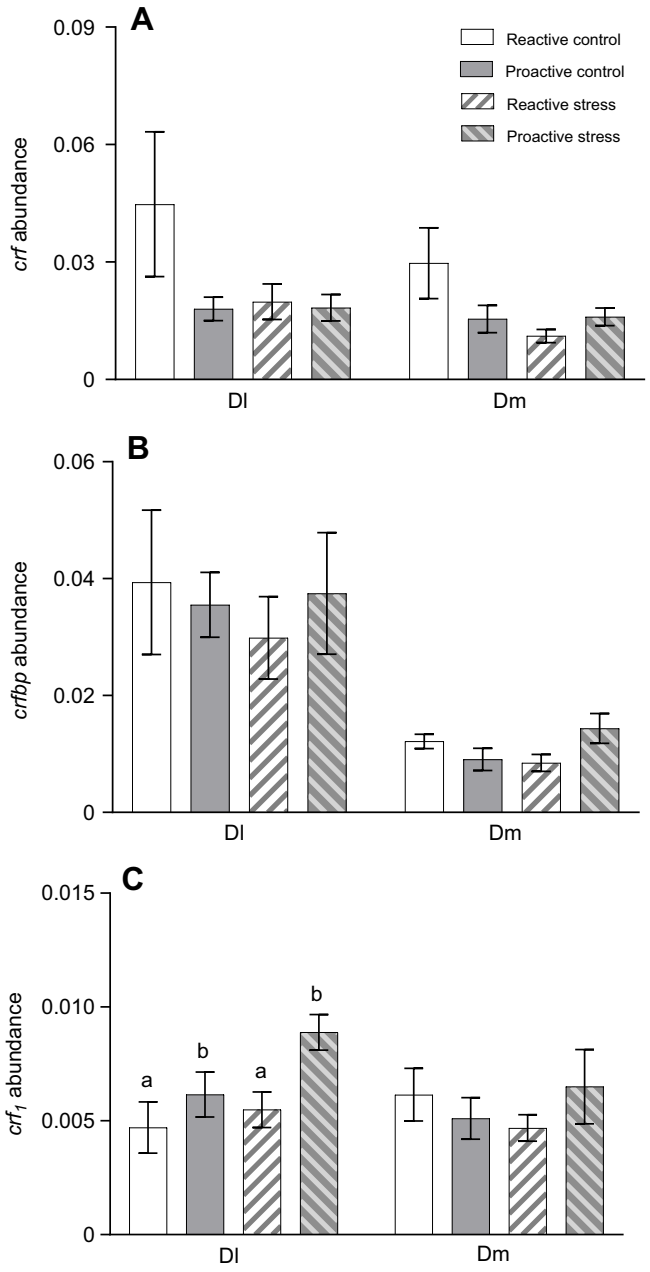

Fig. 4. Effect of coping style (proactive versus reactive) and stress (basal versus acute) on the relative mRNA abundance of corticotropin releasing factor (*crf*), CRF-binding protein (*crfbp*) and CRF receptor 1. Transcript abundance (relative to the *S20* reference gene) of *crf* (A), *crfbp* (B) and crf_1 (C) in the Dl and Dm of Atlantic salmon. Lowercase letters indicate significant ANOVA differences within coping style and/or stress groups in each telencephalic subregion (i.e. not between subregions). Data are presented as means±s.e.m.

were processed together, we had to stop the colouring reaction before any cells were clearly labelled under basal conditions to avoid background staining in post-stress samples.

DISCUSSION

We demonstrate that in response to stress, individual salmon react with a different behavioural output, which is accompanied by specific changes in transcript abundance and monoamine neurochemistry in forebrain areas. We found clear differences between proactive and reactive fish, under both basal and post-stress conditions, with respect to the abundance of signalling molecules in the (cortical-like structures) dorsolateral (Dl) and dorsomedial (Dm) pallium, as well as the subpallial ventral part of the ventral telencephalon (Vv). These signalling molecules include monoamines, downstream genes for the serotonin (5-HT) and corticotropin-releasing factor (CRF) systems, and markers for neural plasticity and cell proliferation. In addition, we found a differential effect of post-stress plasma cortisol concentrations, between coping styles. These results provide evidence that distinct telencephalic neuronal networks in fish are important centres for processing stimuli, which results in distinct and individual behavioural responses; for example, we show how changes in neuronal plasticity and serotonergic signalling in the Dm appear to be characteristic to proactive fish in response to an acute stressor. These results will be fundamental for the advancement of fish animal models, which are increasingly being used in studies on central nervous system (dys)function.

In response to an increasing hypoxic environment, not all individuals showed the same behavioural response. We observed that most of the individuals that, proactively, escaped their immediate hypoxic surroundings into the neighbouring normoxic tank did so once oxygen saturation declined to ∼30%. Once left, they never went back into the hypoxic tank, while others chose a more passive response and remained in their original tank, even at very low oxygen levels (25% O_2 saturation). Notably, the response of fish to hypoxia, as a group-based test for selection of coping styles, has been found to be highly consistent in European seabass (*Dicentrarchus labrax*; Ferrari et al., 2015), as well as Atlantic salmon (Thörnqvist et al., 2015; B.D., T. H. Evensen, Ø.Ø., M.G., L.O.E.E., S. Rey, E.H., unpublished). We found that the fish that stayed exhibited a passive response to hypoxia, accompanied by higher cortisol levels following acute stress compared with the ones that left, which is indicative of reactive and proactive coping styles, respectively (Ruiz-Gomez et al., 2011, 2008; Schjolden et al., 2005; Øverli et al., 2007).

Mechanisms that aid an organism to cope with environmental changes regulate individual differences in motivation, which is only possible through differences in the regulation of the neural network and processing of environmental input (Ebbesson and Braithwaite, 2012; Zupanc and Lamprecht, 2000). It is now generally accepted that a complex structural and functional activation of neural networks (in particular, forebrain cell populations), molecular processes and neurotransmitter systems (e.g. the CRF and the 5-HT system) underlie different coping styles (Koolhaas et al., 2010; Puglisi-Allegra and Andolina, 2015). In our study, proactive fish were characterised by increased serotonergic signalling, particularly in the Dm (the proposed homologue of the amygdala). That is, proactive fish responded to stress with a significant increase in serotonergic activity (measured as changes in the main catabolite of 5-HT, 5-HIAA; Shannon et al., 1986) in the Dm and had an overall higher expression (under both basal and acute stress conditions) of both the $5\text{-}HT_{1A}$ receptor paralogues in the Dm and of $5\text{-}HT_{1A\beta}$ in the Vv (the proposed lateral septum homologue). In agreement with our results, proactive mammals are characterised by higher 5-HT neurotransmission, particularly after acute stress (Koolhaas et al., 2007, 2010, 1999), specifically in the amygdala and lateral septum (Veenema and Neumann, 2007). Notably, regional differences in $5\text{-}HT_{1A}$ transcript abundance are important as these results support the notion that differential 5-HT receptor distribution in neuronal networks (at least partially) determines active and passive coping strategies (Puglisi-Allegra and Andolina, 2015).

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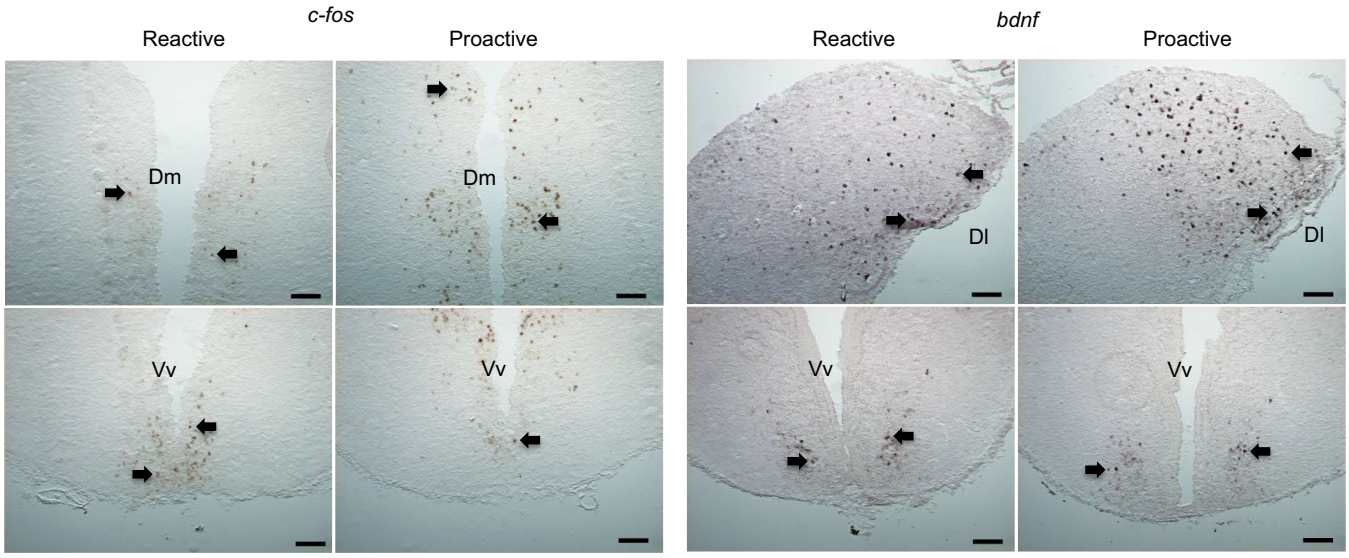

Fig. 5. *In situ* hybridisation of the immediate early gene *c-fos* and *bdnf* after an acute stress challenge in the Dl, Dm and Vv of proactive and reactive fish. Arrows indicate stained cells. The scale bars represent 100 μm.

Proactive fish responded to the stressor with increased *bdnf* mRNA abundance in the Dl, which is the proposed hippocampus homologue (Goodson and Kingsbury, 2013; O'Connell and Hofmann, 2011; Vargas et al., 2009), and the Vv. Synaptic plasticity is promoted by *bdnf*, as are neurogenesis, cell survival and the strengthening of learning and memory (Mattson et al., 2004). Recently, Smith et al. (2014) characterised forebrain *bdnf* expression in mice that displayed differential behavioural responses to social aggression and fear conditioning. They reported that mice that chose to escape an aggressive conspecific showed higher *bdnf* abundance in the amygdala compared with individuals that did not escape. Similarly, we also found increased *bdnf* mRNA abundance after an acute stressor in proactive fish, which had previously chosen to leave an increasingly unfavourable (i.e. hypoxic) environment, although this was in the Dl and Vv and not in the Dm. The increase of *bdnf* in different functional brain areas might be due to the nature of the stressful stimuli utilised in each experiment [in Smith et al.'s (2014), study, the mice were exposed to an aggressive conspecific, while in our experiment, fish were subjected to crowding stress]. In mammals, the hippocampus and lateral septum are associated with memory, learning and goal-oriented behaviour (Jarrard, 1993; Luo et al., 2011; O'Connell and Hofmann, 2012). When we extrapolate these functional roles to the fish's proposed telencephalic equivalents, it is tempting to hypothesise that this increase in *bdnf* may help proactive fish in displaying a greater behavioural reactivity to acute stressors (i.e. active coping), particularly considering that the fish Dl is strongly associated with memory and spatial navigation (Broglio et al., 2015; Vargas et al., 2009). It would therefore be interesting to characterise the learning ability of proactive and reactive individuals in response to different stressful situations to further explore this hypothesis.

Interestingly, reactive fish had higher basal *pcna* transcript abundance in the Dl compared with proactive individuals. In agreement with our results, Johansen and colleagues (2012) report higher telencephalic *pcna* abundance in reactive compared with proactive rainbow trout after short-term confinement (i.e. acute stress). This may be particularly important as reactive fish show greater behavioural flexibility regarding routine formation than proactive individuals (Ruiz-Gomez et al., 2011). Notably, we believe that our results complement the information previously reported by Johansen and colleagues (2012), as we pinpoint a specific telencephalic subregion, the Dl, in which there is higher *pcna* abundance in reactive fish. However, in our experiment, we did not find an increase in *pcna* abundance in response to acute stress in the studied telencephalic subregions; in fact, there was an overall downregulation in *pcna* in response to stress in the Vv. We believe that this illustrates the importance of studying region-specific areas within the brain, as it may allow for a better understanding of the activation of specific neuronal populations in response to stimuli. In our experiment, reactive fish also exhibited higher DA activity in the Dl. DA signalling in limbic areas is associated with increased attention and arousal (Alcaro et al., 2007; Redgrave et al., 1999). Notably, in a previous study, we found that Atlantic salmon experiencing unpredictability of reward were characterised by a potentiated brain dopaminergic system (Vindas et al., 2014b), which suggests that the link between DA signalling and increased attention is also present in salmon. Our current results indicate that, compared with proactive fish, reactive individuals express elevated markers for increased perception and attention in the Dl, which are important for memory and learning (O'Connell and Hofmann, 2012).

The biological effect of CRF is mediated through its receptors (CRF_1 and CRF_2) and binding protein (CRFBP), which regulate the stress response and appetite, and modulate the immune response (Flik et al., 2006; Manuel et al., 2014). We found that crf_1 mRNA levels in the Dl were higher in proactive fish (under both basal and acute stress conditions). In mammals, telencephalic CRF mediates an array of responses, such as anxiety-like behaviour, increased arousal and altered locomotor activity (Owens and Nemeroff, 1991). At present, we cannot say whether this holds in fish, but considering our results and the fact that CRF receptors have been associated with alternative coping styles (Puglisi-Allegra and Andolina, 2015), further investigation should be focused on the role of this system in the regulation of alternative coping styles in fish. Notably, we found that the abundance of *crf* and *crfbp* (as well as the crf_1 and crf_2 receptor) genes was low in the Vv (see Table S3 for Cq values). There is evidence that both *crf* and *crfbp* are expressed in the Vv of zebrafish (Alderman and Bernier, 2007), so either there are notable species-specific differences amongst teleosts or the

conditions studied in our experiment result in downregulation of these genes in the Vv. It is likely that these genes show higher regulation in hypothalamic areas, like the preoptic area, as it is there that the stress axis is activated. Therefore, it would be interesting to study this area under similar conditions in future studies.

Interestingly, our ISH results on *bdnf* and *c-fos* mRNA abundance show differences in spatial distribution of post-stress labelled cells between coping styles, which suggests heterogeneity of activation within target regions. That is, while *c-fos*-labelled cells in the Dm of proactive fish show an even distribution over the whole region, this is not the case in reactive fish, in which labelled cells are found mainly in the upper part of the Dm. Similarly, *c-fos*-labelled cells in the Vv of proactive individuals were found mainly in the upper area of this region, while they were distributed throughout the Vv in reactive fish. Interestingly, while *bdnf*-labelled cells in the Vv of reactive and proactive fish showed similar activation, this appears to be the result of not only the same but also different subpopulations within the neuronal network of the Vv. It has become increasingly clear that telencephalic neuronal populations are highly heterogenic in teleost fishes, where subpopulations within regions, such as the Dl, contain functionally equivalent structures to mammalian nuclei (Broglio et al., 2015). In the present study, the entire Dl, Dm and Vv were sampled; the differential activation within these regions remains to be determined. Further research should be directed towards dissecting these complexes within distinct teleostean telencephalic areas, especially as it is becoming increasingly clear that the brain in early vertebrates is not as simple as it was once thought (Ocaña et al., 2015).

Conclusions

Vertebrate models, such as fish, are increasingly being used to study human mental disorders and dysfunctions (Panula et al., 2006). Notably, knowing the evolutionary history of mammalian forebrain networks, as well as their functional equivalents in fish, is crucial for the advancement and correct interpretation of these translational models. Here, we present original data on the proposed teleostean functional equivalents to the amygdala, hippocampus and lateral septum, in a fish population screened for different coping styles. We found that there are marked differences between reactive and proactive fish, particularly after stress, that find resemblance in mammalians. Proactive fish were characterised by a stress-induced increase in 5-HT signalling in the Dm as well as higher *bdnf* transcript abundance in the Dl and Vv, accompanied by lower post-stress cortisol levels, compared with reactive individuals. Under basal conditions, however, reactive fish showed increased *pcna* mRNA levels and DA activity in the Dl. We hope that these results inspire more functional neuroanatomical research in fish to understand how evolutionarily conserved and complex neural systems regulate perception, attention and stimulus salience to the surrounding environment, and how they are linked to disease vulnerability.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualisation, B.D., L.O.E.E., E.H., Ø.Ø., S.W., G.F., M.A.V.; Methodology, B.D., M.A.V., L.O.E.E.; Validation, B.D., M.A.V., V.T.; Formal Analysis, M.A.V., M.G., E.H., V.T.; Investigation, B.D., M.A.V., L.O.E.E., Ø.Ø., M.G.; Resources, T.O.N., P.-O.T.; Writing – Original draft, M.A.V.; Writing – Review and Editing, M.A.V., M.G., G.F., Ø.Ø., L.O.E.E., E.H.; Supervision, B.D., Ø.Ø., E.H., L.O.E.E., G.F., S.W.; Project Administration, L.O.E.E., B.D.; Funding Acquisition, L.O.E.E., Ø.Ø., E.H., B.D., G.F., S.W.

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Supplementary information

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