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RESEARCH ARTICLE

Severe hypoxia exposure inhibits larval brain development but does not affect the capacity to mount a cortisol stress response in zebrafish

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ABSTRACT

Fish nursery habitats are increasingly hypoxic and the brain is recognized as highly hypoxia sensitive, yet there is a lack of information on the effects of hypoxia on the development and function of the larval fish brain. Here, we tested the hypothesis that by inhibiting brain development, larval exposure to severe hypoxia has persistent functional effects on the cortisol stress response in zebrafish (*Danio rerio*). Exposing 5 days post-fertilization (dpf) larvae to 10% dissolved O_2 (DO) for 16 h only marginally reduced survival, but it decreased forebrain neural proliferation by 55%, and reduced the expression of *neurod1*, *gfap* and *mbpa*, markers of determined neurons, glia and oligodendrocytes, respectively. The 5 dpf hypoxic exposure also elicited transient increases in whole-body cortisol and in *crf*, *uts1* and *hsd20b2* expression, key regulators of the endocrine stress response. Hypoxia exposure at 5 dpf also inhibited the cortisol stress response to hypoxia in 10 dpf larvae and increased hypoxia tolerance. However, 10% DO exposure at 5 dpf for 16 h did not affect the cortisol stress response to a novel stressor in 10 dpf larvae or the cortisol stress response to hypoxia in adult fish. Therefore, while larval exposure to severe hypoxia can inhibit brain development, it also increases hypoxia tolerance. These effects may transiently reduce the impact of hypoxia on the cortisol stress response but not its functional capacity to respond to novel stressors. We conclude that the larval cortisol stress response in zebrafish has a high capacity to cope with severe hypoxia-induced neurogenic impairment.

KEY WORDS: Environmental stressors, Neural proliferation, Stress response, Hypoxia tolerance, Developmental programming, Hypothalamic–pituitary–interrenal axis

INTRODUCTION

Hypoxia is a natural phenomenon in aquatic ecosystems, but its prevalence worldwide is rapidly expanding. It is now considered one of the most significant environmental challenges faced by fish (Pollock et al., 2007; Vagner et al., 2019). Increases in nutrient discharge linked to anthropogenic activities have rapidly increased the spread of hypoxia over the last century in both freshwater and marine habitats (Jenny et al., 2016; Breitburg et al., 2018). Global warming is further exacerbating the spread of hypoxia, increasing both its frequency and magnitude (Breitburg et al., 2018). While fish have evolved a variety of physiological and metabolic adaptations to cope with seasonal or diurnal fluctuations in O_2 levels (Richards, 2009; Nilsson and Randall, 2010; Mandic and Regan, 2018), behavioural avoidance is a common strategy adopted by juvenile and adult fish to reduce the negative effects of hypoxia (Breitburg, 1994; Chapman and McKenzie, 2009; Craig, 2012; Switzer et al., 2015). However, the early developmental stages of various fish species may be unwilling or unable to escape the hypoxic conditions observed in nursery habitats because of food availability, predator avoidance or reduced mobility (Meire et al., 2013; Dubuc et al., 2017). Despite evidence linking hypoxia on nursery grounds to reductions in survival, species richness and adult fisheries production (Hughes et al., 2015), the short- and long-term physiological effects of early hypoxia exposure in fish remain poorly understood (Vagner et al., 2019). In fact, although the brain is widely recognized as the most hypoxia-sensitive organ in vertebrates, to our knowledge the impact of hypoxic conditions on brain development and function in larval fish has yet to be examined.

The complexity of the developing brain makes it particularly sensitive to environmental stressors such as hypoxia. In general, it is known that the effects of hypoxia on the mammalian brain are specific to development stage, brain region and neural cell type (Schmidt-Kastner and Freund, 1991; Nyakas et al., 1996). Depending on its severity, duration and timing, fetal and perinatal hypoxia can stimulate or inhibit neurogenesis and gliogenesis, restrict brain growth, and lead to a loss of all neural cell types (Huang et al., 2004; Romanko et al., 2007; Yang et al., 2007; Spadafora et al., 2010; Baydyuk et al., 2020). Reoxygenation of the neonatal mammalian brain post-ischaemia is also known to impair neurogenesis and lead to irreversible neuronal damage (Torres-Cuevas et al., 2019). In contrast, although severe hypoxia and reoxygenation can cause brain cell death and affect neural cell proliferation in adult fish (Lefevre et al., 2017; Das et al., 2019), it is unknown whether these conditions affect the brain of larval fish. Unlike mammals, fish are characterized by substantial post-embryonic brain growth (Kaslin et al., 2008). Whereas post-embryonic neurogenesis in mammals is almost exclusively restricted to ventricular proliferative zones, neurogenesis is also observed in several non-ventricular proliferative centres in the larval zebrafish forebrain (Mueller and Wullimann, 2002). Therefore, while the larval fish brain is likely impacted by environmental hypoxia, the effects are predicted to differ from those observed in mammals.

An important brain function that may be compromised as a result of developmental hypoxia exposure is the regulation of the endocrine stress response. In rats and sheep, acute and chronic hypoxia exposure during the perinatal period can lead to a variety of cerebral deficits including short- and long-term effects on basal and stress-induced hypothalamic-pituitary–adrenal (HPA) axis function (Mikhailenko et al., 2009; Chintamaneni et al., 2014;

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Newby et al., 2015). Whether these alterations in HPA axis function are consequences of hypoxia-induced damage to stress-sensitive neural circuitries remains to be determined (Fajersztajn and Veras, 2017). Hypoxic insults during the neonatal period can also activate the HPA axis and lead to increases in glucocorticoid levels (Xiong and Zhang, 2013; Ducsay et al., 2018). Therefore, like other developmental stressors, hypoxia can have widespread effects on the HPA via the programming effects of excess glucocorticoids. In fish, although most components of the hypothalamic-pituitary–interrenal axis (HPI; the fish homologue of the HPA axis) and the capacity for *de novo* cortisol synthesis are present prior to hatching, a stress-mediated increase in cortisol does not take place until hatching or during the larval stage (Alderman and Bernier, 2009; Fuzzen et al., 2011; Peterson et al., 2019). While acute hypoxia exposure post-hatching can elicit a cortisol stress response in rainbow trout (*Oncorhynchus mykiss*; Fuzzen et al., 2011) and channel catfish (*Ictalurus punctatus*; Peterson et al., 2019) larvae, the potential of those stressors to interfere with neural development or with the maturation and function of the HPI axis was not determined. Similarly, although acute anoxia (Ivy et al., 2017) and chronic hypoxia (Wilson et al., 2016) exposure during embryonic development may have programming effects on the HPI axis of zebrafish, the impact of acute larval hypoxia exposure on brain development and HPI axis function in this species remains unknown.

Therefore, using zebrafish larvae, we sought to (1) determine whether acute sub-lethal hypoxia exposure affects brain development, (2) identify the neural specificity of this environmental challenge, (3) characterize its effects at different levels of the HPI axis, and (4) establish whether acute larval hypoxia exposure has programming effects on the cortisol stress response. To this end, we first characterized the hypoxia tolerance of 5 days post-fertilization (dpf) larvae, a developmental stage in zebrafish at which the HPI axis responds to a variety of stressors (Alderman and Bernier, 2009; Wilson et al., 2013; Yeh et al., 2013). Next, to determine the effects of acute larval hypoxia exposure on brain development, we used the nucleotide analogue 5-bromo-2-deoxyuridine (BrdU) to label dividing cells in the forebrain of 5 dpf larvae and quantified the gene expression of cell type-specific markers of neurogenesis and differentiation. To assess the capacity of acute larval hypoxia to stimulate a stress response, changes in whole-body cortisol were quantified with the mRNA levels of several key effectors of the HPI axis. Finally, to test the hypothesis that acute hypoxia exposure-induced delays in brain development have persistent functional effects on the HPI axis, we quantified the cortisol stress response of 10 dpf larvae and adults that were previously exposed to normoxia or acute hypoxia at 5 dpf.

MATERIALS AND METHODS

Experimental animals

Adult zebrafish, *Danio rerio* (F. Hamilton 1822), were obtained from AQUAlity Tropical Fish (Mississauga, ON, Canada) and housed at the Hagen Aqualab (University of Guelph, Guelph, ON, Canada) in 4 l tanks (Aquatic Habitats, Apopka, FL, USA). Adult fish were maintained at a density of 4–5 fish l^{-1} on a 14 h:10 h light:dark photoperiod in normoxic conditions at 27°C. Fish were fed twice daily with newly hatched brine shrimp (Aquafauna Bio-Marine Inc., Hawthorne, CA, USA) in the morning and TetraMin fish flakes (United Pet Group, Blacksburg, VA, USA) in the evening. For each experiment, fertilized eggs were collected at 09:00 h and pooled from 4–5 divided chamber breeding baskets each containing 4 adult males and females. Embryos were incubated at 28.5°C in egg water: 60 µg l^{-1} Instant Ocean Sea Salts (United Pet Group, Cincinnati, OH, USA) and 0.1 ppm Methylene Blue as an antifungal. Egg water was changed twice daily. Both eggs and larvae were reared on a 12 h:12 h light:dark photoperiod (lights on at 07:00 h) in normoxic conditions. Larvae were fed twice daily with Hatchfry Encapsulon larval diet (Argent Laboratories, Redmond, WA, USA) commencing at 5 dpf. All procedures were carried out in accordance with the Canadian Council for Animal Care guidelines and approved by the University of Guelph Animal Care Committee.

Experimental protocol

Series 1: effects of hypoxia exposure on survival in 5 dpf larvae

Mesh-bottomed (100 µm) 90 ml wide-mouth cups (ThermoFisher Scientific, Waltham, MA, USA) containing 75 larvae each were placed in 4 l tanks with a microbubble diffuser (Point-Four Systems, Port Moody, BC, Canada) and a custom-built flow-through barrier at the water's surface. The flow through barrier ensured that all cups were submerged and that the larvae were unable to use aquatic surface respiration throughout the experiment. The larvae were counted into the cups 24 h prior to exposure to allow acclimation and the cups were sealed with a meshed lid. Duplicate cups of larvae were exposed to the following dissolved oxygen (DO) levels: 100% (7.7 mg l^{-1}), 20%, 10%, 6.5%, 5%, 3% and 0%. A clear plastic sheet was placed on top of the flow-through barrier in the hypoxic treatments to maintain consistent exposure conditions. Experiments commenced when nitrogen gas (or air for the 100% DO treatment) was bubbled into the tank for 20 min to achieve the target DO level. The O_2 saturation was maintained within ±1% of the target value and measured continuously using an O_2 electrode (Liquid Dissolved Oxygen Field Probe, Hach, Loveland, CO, USA). All experiments commenced at 17:00 h with 4 dpf larvae, were terminated at 09:00 h once they reached 5 dpf, and were performed at 28.5°C. Survival rates were assessed following 16 h of chronic exposure. Larvae were considered dead if their hearts had ceased beating; this was determined by observing the larvae for a 30 s interval through a dissecting microscope.

Series 2: effects of hypoxia exposure on neural proliferation in 5 dpf larvae

A BrdU saturation-label approach was used to quantify the impact of hypoxia exposure on neural proliferation (Mueller and Wullimann, 2002). The saturation-label approach is optimized to reveal nearly all proliferative cells and early post-mitotic cells that may already express neuronal differentiation markers (Mueller and Wullimann, 2002). Briefly, 4 dpf larvae were placed in 24-well plates containing egg water (without Methylene Blue) and 10 mmol l^{-1} BrdU (Sigma-Aldrich, MI, USA), and exposed to 100%, 20% or 10% DO levels for 16 h. These DO levels were selected based on the results of Series 1 above and our aim to use sub-lethal hypoxic conditions. The 16 h exposure duration was chosen to approximate the length of one to two cell cycles in 5 dpf zebrafish larvae (Mueller and Wullimann, 2002, 2005). The uncovered plates were placed in a custom-made air-sealed Plexiglas chamber. O_2 saturation of the wells was monitored with a MI-730 micro-O_2 electrode (Microelectrodes Inc., Bedford, NH, USA) and maintained using a gas-mixing pump (Wosthoff AG, Cortmund, Germany). All experiments were carried out with 4 dpf larvae, commenced at 17:00 h, and were terminated at 09:00 h once the larvae reached 5 dpf. During the 16 h exposure, the chamber was kept in an incubator and maintained at 28.5°C. The larvae were euthanized immediately in ice water and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; NaCl, 137 mmol l^{-1}; Na_2HPO_4, 15.2 mmol l^{-1}; KCl, 2.7 mmol l^{-1};

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KH_2PO_4, 1.5 mmol l^{-1}; pH 7.8) at 4°C overnight. Larvae were rinsed with PBS and cryopreserved in 3 successive overnight incubations in: (1) 30% sucrose, (2) 1:1 30% sucrose:Cryomatrix (Thermo Fisher Scientific), and (3) Cryomatrix.

Series 3: effects of hypoxia exposure in 5 dpf larvae on the expression of neural markers at 5 and 10 dpf

Larvae were exposed to 100%, 20% or 10% DO levels for 16 h to assess the impact of hypoxia exposure on the expression of cell type-specific markers of neurogenesis and differentiation. Specifically, we quantified the gene expression of: neurogenin1 (*neurog1*; Blader et al., 2003) as a marker of pro-neuronal cells, neurogenic differentiation 1 (*neurod1*; Mueller and Wullimann, 2003) as an indicator of post-mitotic determined neurons, and finally glial fibrillary acidic protein (*gfap*; Nielsen and Jørgensen, 2003) and myelin basic protein A (*mbpa*; Brösamle and Halpern, 2002) as markers of radial glia (or astrocytes in mammals) and oligodendrocytes, respectively. All exposures were carried out with 4 dpf larvae, commenced at 17:00 h, were terminated at 09:00 h (at 5 dpf) and were performed at 28.5°C. The larvae were either sampled immediately after exposure or reared for an additional 5 days prior to sampling at 09:00 h (at 10 dpf). Exposures were performed in mesh-bottomed (100 µm) 90 ml wide-mouth cups as described above in Series 1 using pools of 25 larvae (n=7–8 pools at 5 dpf; n=11–13 pools at 10 dpf). Following exposure, cups were immediately placed in ice water to euthanize the larvae. The larvae were then transferred into tubes kept on dry ice (25 larvae per tube), and samples were stored at −80°C until further analysis.

Series 4: effects of hypoxia exposure on the cortisol stress response and the expression of key regulators of the HPI axis in 5 dpf larvae

To assess the impact of hypoxia exposure on whole-body cortisol levels and the expression of corticotropin-releasing factor b (*crfb*), urotensin 1 (*uts1*), CRF binding protein (*crfbp*), melanocortin 2 receptor (*mc2r*), steroidogenic acute regulatory protein (*star*), cytochrome P450 side-chain cleavage (*cyp11a1*), 11β-hydroxylase (*cyp11c1*), 11β-hydroxysteroid dehydrogenase 2 (*hsd11b2*) and 20β-hydroxysteroid dehydrogenase 2 (*hsd20b2*), 4 dpf larvae were exposed to 100%, 20% or 10% DO levels for 2 h ending at 5 dpf (between 07:00 and 09:00 h) or for 16 h ending at 5 dpf (between 17:00 and 09:00 h). Exposures were performed as in Series 1 using pools of 25 larvae (n=7–10 pools). Samples were collected as in Series 3 and stored at −80°C until further analysis.

Series 5: effects of hypoxia exposure in 5 dpf larvae on the cortisol stress response at 10 dpf and in adults

The effects of hypoxia exposure during larval development on the cortisol stress response in later life were assessed in a series of three experiments. In all experiments, 4 dpf larvae were first exposed to either 100% or 10% DO levels for 16 h ending at 5 dpf (between 17:00 and 09:00 h). Exposures were performed as in Series 1 using pools of 25 larvae in mesh-bottom cups. Post-exposure, the larvae were: (1) raised to 10 dpf and re-exposed to either 100% or 10% DO for 2 h (between 07:00 and 09:00 h; n=12–14 pools of 25 larvae), a duration selected based on the results of Series 4; (2) raised to 10 dpf and exposed to 0 or 180 rpm for 20 min (between 08:40 and 09:00 h; n=9–10 pools of 25 larvae), a previously validated vortex stressor (Williams et al., 2017a); or (3) raised to adulthood (4 months post-fertilization) and re-exposed to either 100% or 10% DO for 1 h (n=12–15 individuals; between 09:00 and 10:00 h), conditions selected based on the results of Williams et al. (2017b). After the initial exposure at 5 dpf, cups with larvae were immediately placed in a normoxic water bath maintained at 28.5°C and fed twice daily. In the larvae re-exposed to hypoxia at 10 dpf, cups were returned to 4 l tanks with a microbubble diffuser and flow-through lid barrier 24 h preceding the experiment. In the larvae exposed to the vortex stressor at 10 dpf, pools of 25 larvae were placed in 6-well plates with 5 ml of water and acclimated on an orbital platform shaker (0 rpm) for 2 h prior to increasing the shaker speed to 180 rpm as per Williams et al. (2017a). In the larvae re-exposed to hypoxia as adults, mixed-sex adult fish were acclimated to 4 l tanks for 4 days and fed twice daily. On the day preceding the experiment, a microbubble diffuser and a surface flow-through barrier were placed in each tank. Exposure tanks were transferred to a larger container filled with water to maintain a temperature of 27°C for the duration of the experiment. Survival between 5 dpf and adulthood was closely monitored and did not differ between the normoxia- and hypoxia-exposed larvae. At the end of each exposure, fish were immediately euthanized in ice water, placed on dry ice, and stored at −80°C for later determination of whole-body cortisol.

Series 6: effects of hypoxia exposure in 5 dpf larvae on hypoxia-tolerance of 10 dpf larvae

To test whether larval hypoxia exposure affects hypoxia tolerance in later life, 4 dpf larvae were first exposed to either 100% or 10% DO levels for 16 h, ending at 5 dpf (between 17:00 and 09:00 h). Exposures were performed in mesh-bottomed (100 µm) 90 ml wide-mouth cups as described in Series 1 using pools of 25 larvae. Larvae were then raised at 28.5°C under normoxic conditions and fed twice daily until 10 dpf. Groups of 25 larvae in 90 ml mesh-bottomed cups were then submerged in 4 l tanks containing water with 100% or 5% DO for 4 h (n=8–10). The percentage of larvae that survived within each cup was quantified immediately after exposure. Survival was assessed as in Series 1.

Analytical techniques

Immunohistochemistry and quantification of forebrain cell proliferation

To quantify forebrain cell proliferation, fixed and cryopreserved 5 dpf larvae from the three DO regimes of Series 2 were embedded in Cryomatrix (ThermoFisher Scientific) and stored at −80°C until sectioning. Cross-sections (12 µm) obtained with a cryostat (Leica CM 3050S, Leica Microsystems, Wetzlar, Germany) were thaw mounted on Superfrost Plus glass slides (ThermoFisher Scientific) and stored at −80°C until further analysis. Triton X-100 (TX) was used to reduce non-specific hydrophobic interactions and improve antibody penetration. Slides were rinsed 3×10 min in PBS-TX (0.5%) and tissue was denatured for 20 min in 4 mol l^{-1} HCl. Using an InsituPro VSi robot (Intavis Bioanalytical Instruments, Köln, Germany), slides were permeabilized with 3×10 min washes in PBS-TX, blocked in 10% serum for 30 min, and incubated for 10 h at 4°C in anti-BrdU primary antibody (1:200; Abcam ab6326) diluted in PBS-TX. Following incubation with primary antibody, the slides were rinsed 3×10 min with PBS-TX. Slides were then incubated with the green fluorescently labelled secondary antibody Alexa Fluor 488 (1:200; Molecular Probes A11006) diluted in PBS-TX for 4 h in darkness. Following the final 3×10 min washes in PBS, slides were mounted with coverslips and Vectashield with the blue-fluorescent stain 4′6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlington, ON, Canada) to prevent photobleaching and label cell nuclei, respectively. Control experiments were performed in which the primary antibody was excluded to control for non-specific cross-reactivity of the secondary antibody. Immunolabelled sections were analysed by fluorescence microscopy (Leica DMLA, Leica

Microsystems) and images were captured using Andor iQ software (version 2.6; Andor Bioimaging Division, Belfast, UK).

BrdU- and DAPI-labelled forebrain cells were quantified using ImageJ and the manual Cell Counter plugin (version 1.45; National Institute of Mental Health, USA). All photomicrographs were analysed at 60× magnification. Taking into consideration the known regional heterogeneity of neural proliferation in the larval zebrafish brain (Mueller and Wullimann, 2005), the forebrain was divided into seven proliferative regions. Using the larval zebrafish brain atlas of Mueller and Wullimann (2005) as a guide, sections that were unequivocally determined to be within one of these defined regions were quantified. Forebrain cell proliferation is reported as the ratio of BrdU- to DAPI-labelled cells within each section and was averaged over all sections within a given proliferation region. The number of BrdU- and DAPI-positive nuclei was counted on an average of 2.9±0.4 (mean±s.e.m.) sections per forebrain region (range 2–5) for each larva (n=3–5 larvae per treatment). The number of DAPI-labelled cells did not differ across treatments within a region for all seven forebrain regions quantified.

Cortisol extraction from larval zebrafish

Pooled larvae (25 larvae per sample) in 200 µl of homogenizing buffer (80 mmol l^{-1} Na_2HPO_4; 20 mmol l^{-1} NaH_2PO_4; 100 mmol l^{-1} NaCl; 1 mmol l^{-1} EDTA) were sonicated (Vibracell©, Sonics and Materials Inc., Danbury, CT, USA) 2×8 s on ice. Homogenates were extracted as per Fuzzen et al. (2010). In brief, the 200 µl samples were extracted twice with 800 µl of methanol. The extracts were combined and dried under nitrogen at room temperature. The extract was then reconstituted in 300 µl of acetate buffer (2.35 ml glacial acetic acid, 1.23 g sodium acetate trihydrate, in 1 litre; pH 4.0) and purified using C-18 solid phase extraction columns (Agela Technologies, Wilmington, DE, USA). Cortisol eluted from the column was dried under nitrogen at room temperature and stored at −20°C until analysis. Total protein was assessed using a Bradford assay (BioRad Protein Assay Dye Reagent, BioRad, Hercules, CA, USA) on a fraction of the original homogenate.

Cortisol extraction from adult zebrafish

Zebrafish were thawed and blotted dry, weighed, and placed into individual 16×100 mm glass tubes with 4 ml of homogenizing buffer (see above). Fish were then homogenized for 30 s using a Euro Turrax T 20b homogenizer (IKA Labortechnik, Wilmington, NC, USA). A 200 µl sample of homogenate was then extracted as per the larval cortisol extraction protocol above.

Cortisol radioimmunoassay

Whole-body cortisol concentration was measured in triplicate by radioimmunoassay as described by Bernier et al. (2008). The cortisol antibody (product code # XM210, Abcam, Cambridge, UK) concentration used (1:5000) was adjusted so that 40% of the radiolabelled cortisol was bound in the absence of competitor. The lower detection limit of the assay was 62.5 pg ml^{-1}. The intra- and inter-assay coefficients of variation were 3.2% (n=6) and 5.3% (n=6), respectively. Cortisol values are given as pg cortisol μg^{-1} total protein in the larvae, and as ng cortisol g^{-1} body mass in the adult fish and were corrected for extraction efficiency (85%).

Quantification of mRNA by real-time RT-PCR

As a means of providing an independent assessment of the effects of hypoxia exposure on neuronal proliferation and the stress response, the gene expression levels of several cell type-specific markers of neurogenesis and differentiation (*neurod1*, *neurog1*, *gfap*, *mbpa*) and key effectors of the HPI axis (*crfb*, *crfbp*, *cyp11a1*, *cyp11c1*, *hsd11b2*, *hsd20b2*, *mc2r*, *star*, *uts1*) were quantified. Embryonic samples were homogenized with a 3 ml syringe fitted with a 23G needle. Total RNA was extracted from pools of 25 embryos using Trizol (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. To increase recovery of total RNA, 10 µg of RNA grade glycogen (Roche Diagnostics, Laval, QC, Canada) was added to each sample prior to precipitation of nucleic acid by isopropanol and incubation at −80°C overnight. Total RNA concentration was quantified using a NanoDrop 8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). From each total RNA pool, 1 µg of RNA was treated with Ambion DNAse (ThermoFisher Scientific) and used to synthesize cDNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT. Separate samples were treated identically without the addition of reverse transcriptase or without the presence of RNA to verify the absence of genomic DNA or contaminated reagents. Triplicates of each cDNA, no reverse transcriptase and no template sample were amplified by real-time quantitative PCR (qPCR) using an ABI Prism 7000 system (Applied Biosystems, Foster City, CA, USA) with Quanta Perfecta Supermix (Quanta Biosciences, Beverly, MA, USA) using the primers listed in Table 1. The 20 µl reactions contained 10 µl 2× master mix, 5 µl of 10-fold diluted first strand cDNA template and 2.5 µl of both forward and reverse primers (0.4 µmol l^{-1}). The cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 1 s and 59°C for 30 s. Melting curve analysis was performed after each run to verify the specificity of each PCR product. Only samples with a unimodal dissociation curve and predicted melting temperature were analysed. To account for differences in amplification efficiency, a standard curve was constructed for each target gene using serial dilutions of cDNA. To correct for minor variations in template input and transcriptional efficiency, the input values were normalized to the housekeeping gene elongation factor 1α (*ef1α*). Note that the expression of *ef1α* did not differ between any of the treatments (P>0.05) at any developmental stage.

Statistical analysis

The results are presented as means±s.e.m. All comparisons were done by one- or two-way ANOVA followed by Holm–Šidák *post hoc* test (SigmaPlot 12.5, San Jose, CA, USA) where assumptions of normality and equal variance were met. If these assumptions were not met, the data were $\log_{10}$ transformed prior to the above analysis. Data that still failed equal variance assumptions were tested using a one-way ANOVA followed by the Dunnett T3 *post hoc* test (IBM SPSS 24, New York, NY, USA). Statistical significance was defined as P<0.05.

RESULTS

Series 1: effects of hypoxia exposure on survival in 5 dpf larvae

Exposure of 5 dpf zebrafish larvae to 100% or 20% DO levels for 16 h was associated with 99% survival rate (Fig. 1). At 10% DO, survival decreased to 95%. Below 10% DO, there was a rapid and marked decline in survival such that all hypoxia exposures to DO levels below 6.5% resulted in survival rates of less than 50%.

Series 2: effects of hypoxia exposure on neural proliferation in 5 dpf larvae

Immunohistochemistry against BrdU identified proliferating cells within the forebrain of 5 dpf zebrafish larvae across seven proliferative

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Table 1. Nucleotide sequences of zebrafish primers used for reverse transcription-qPCR

Gene	Accession no.	Sequence (5′–3′)	Efficiency
crfb	NM_001007379	F: GCCGCGCAAAGTTCAAAA R: GCGAGGAGAATCTGTGCGTAA	101%
crfbp	NM_001003459	F: CGAGAGTTACCAGAGGAGTTTGTGTA R: ACCCTCTACGGCCACCATATC	90%
cyp11a1	BC154309	F: GGCAGAGCACCGCAAAA R: CCATCGTCCAGGGATCTTATTG	97%
cyp11c1	NM_001080204	F: CCTGGGCCACACATCGA R: CCGTTCTTGAGGAAGACTCCTTT	92%
ef1α	NM_131263	F: GGGCAAGGGCTCCTTCAA R: CGCTCGGCCTTCACTTTG	101%
gfap	NM_131373	F: GCAGGAGACTGAGGAGTGGT R: CCATTACGATTGGCTGCAT	95%
hsd11b2	NM_212720	F: TGCTGCTGGCTGTACTTCAC R: TGCATCCAACTTCTTTGCTG	88%
hsd20b2	KM_279631	F: GCTGGGCTGTTGTAACTGGT R: TTGGCAAGCTCTTCAGCATA	101%
mbpa	XM_002665561	F: CCTGGATCAAATCAGCAGGT R: TGGGGTCTCTTTCCCTTTCT	90%
mc2r	NM_180971	F: AAACCGAATCGCGTCTATGC R: CAGGCCGCTTTTCCTGTGT	95%
neurod1	NM_130978	F: ATCATAACAAGCTTTCAACACACC R: ATCATGCTTTCCTCGCTGTA	102%
neurog1	NM_131041	F: CCAGCCCACCAATAAGGTTATAC R: CGAAAAGGAGTAGTCACAGCTTGA	97%
star	NM_131663	F: ACCTGTTTTCTGGCTGGGATG R: GGGTCCATTCTCAGCCCTTAC	101%
uts1	NM_001030180	F: CACGCTTCCTCAACCGCTACT R: CAGTCGCGCACTTCTCGAT	98%

crfb, corticotropin-releasing factor b; *crfbp*, CRF binding protein; *cyp11a1*, cytochrome P450 side-chain cleavage; *cyp11c1*, 11β-hydroxylase; *ef1α*, elongation factor 1α; F, forward; *gfap*, glial fibrillary acidic protein; *hsd11b2*, 11β-hydroxysteroid dehydrogenase 2; *hsd20b2*, 20β-hydroxysteroid dehydrogenase 2; *mbpa*, myelin basic protein a; *mc2r*, melanocortin 2 receptor; *neurod1*, neuronal differentiation 1; *neurog1*, neurogenin 1; R, reverse; *star*, steroidogenic acute regulatory protein; *uts1*, urotensin 1.

regions (Fig. 2A). Representative brightfield (Fig. 2B,E), BrdU labelling (Fig. 2C,F) and DAPI nuclear staining (Fig. 2D,G) images from a larval zebrafish exposed to 100% and 10% DO are shown in Fig. 2. DO level significantly affected the ratio of BrdU- to DAPI-positive nuclei and this effect was dependent on forebrain region (DO level: $F_{2,81}$=26.99, P<0.001; region: $F_{6,81}$=25.49, P<0.001; DO level×region: $F_{12,81}$=0.98, P=0.482) (Fig. 2H). Overall, while the ratio of BrdU- to DAPI-positive nuclei did not differ between the 20% DO exposure and the normoxic treatment (P=0.880), the number of proliferating cells was lower in the 10% DO treatment than in either the 20% DO (P<0.001) or the normoxic (P<0.001) treatments.

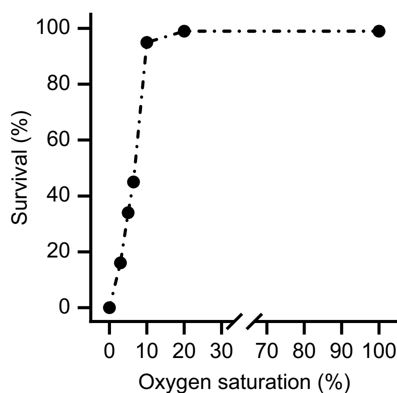

Fig. 1. Effects of dissolved O_2 saturation levels on percentage survival in 5 dpf zebrafish larvae. Duplicate groups of 75 larvae were exposed to each O_2 saturation level for 16 h. Symbols indicate the different O_2 saturation levels tested.

Specifically, the ratio of BrdU- to DAPI-positive nuclei was lower in the 10% DO treatment than in the normoxic treatment in region 1 (P=0.036), and lower than both the normoxic and 20% DO treatments in regions 4 (P=0.014; P=0.008), 5 (P<0.001; P=0.002) and 7 (P=0.025; P=0.006). Note that the effects of DO on the ratio of BrdU- to DAPI-positive nuclei was specifically due to a decrease in the number of BrdU-positive nuclei (DO level: $F_{2,81}$=30.32, P<0.001; region: $F_{6,81}$=2.30, P=0.046; DO level×region: $F_{12,81}$=0.99, P=0.472) and independent of the number of DAPI-positive nuclei (DO level: $F_{2,81}$=0.90, P=0.411; region: $F_{6,81}$=41.60, P<0.001; DO level×region: $F_{12,81}$=0.37, P=0.970) (Fig. S1). Within the normoxic treatment, the ratio of BrdU- to DAPI-positive nuclei was ∼3-fold lower in regions 2–5 relative to region 1 (the olfactory bulb), and ∼7-fold lower in regions 6 and 7 relative to region 1. Average neural proliferation across all forebrain regions differed between treatments ($F_{2,12}$=11.94, P=0.001) (Fig. 2I). While the normoxic and 20% DO treatments did not differ (P=0.282), average neural proliferation in the 10% DO treatment was 55% (P=0.002) and 47% (P=0.008) lower than the normoxic and 20% DO treatments, respectively.

Series 3: effects of hypoxia exposure in 5 dpf larvae on the expression of neural markers at 5 and 10 dpf

Relative to the normoxic treatment, exposing 5 dpf larvae to 20% DO or 10% DO for 16 h did not have any immediate effect on the expression of *neurog1* at 5 dpf (P=0.259) (Fig. 3A) or delayed effect at 10 dpf (P=0.600) (Fig. 3B). In contrast, *neurod1* expression differed across treatments at 5 dpf ($F_{2,22}$=6.437, P=0.007) and 10 dpf ($F_{2,35}$=5.201, P=0.011). While the 10% DO treatment reduced *neurod1* expression by 32% at 5 dpf (P=0.006) and 44% at 10 dpf (P=0.009) relative to the normoxic treatment, the 20% DO treatment

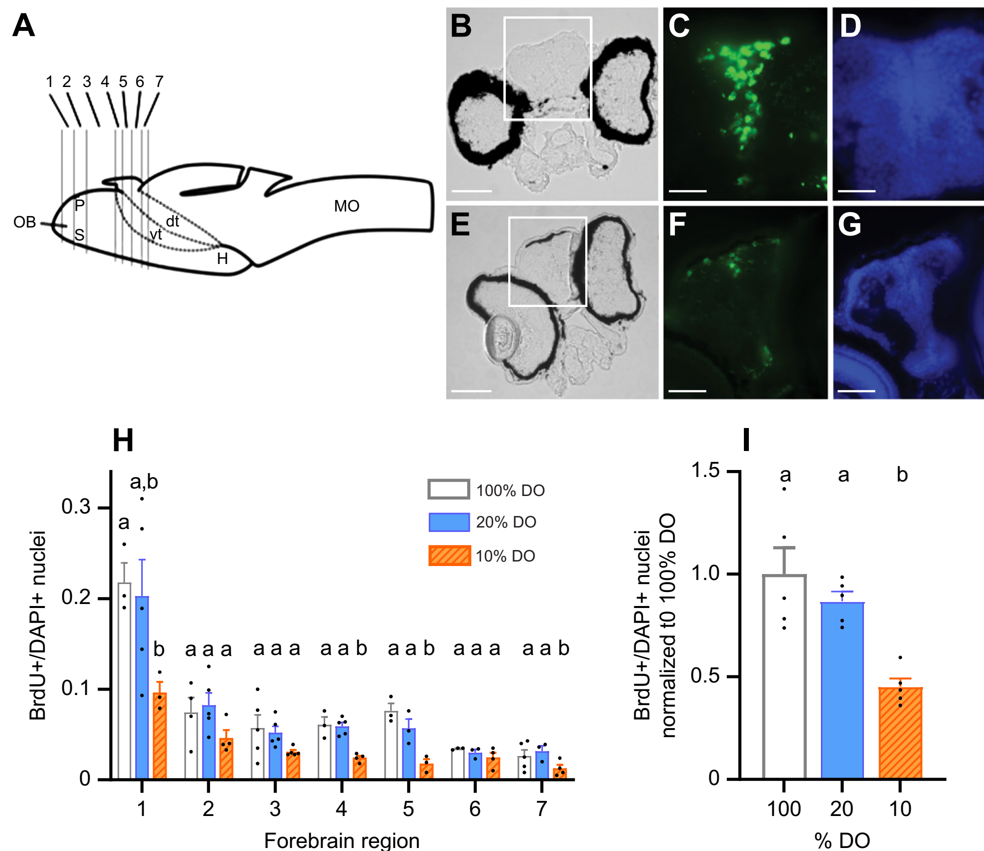

Fig. 2. Effects of hypoxia exposure on forebrain proliferation in 5 dpf zebrafish larvae. Larval zebrafish were exposed for 16 h to 100%, 20% or 10% dissolved O_2 (DO) in the presence of bromodeoxyuridine (BrdU) to label dividing cells. (A) Sagittal view of a 5 dpf zebrafish larval brain showing the seven forebrain regions used for BrdU quantification (adapted from Mueller and Wullimann, 2005). Along the rostral to caudal axis, the seven proliferative regions included were: (1) olfactory bulb (OB); (2) pallium (P) and subpallium (S); (3) pallium, subpallium and anterior commissure; (4) pallium, ventral thalamus (vt) and preoptic region; (5) habenula, dorsal thalamus (dt), ventral thalamus and preoptic region; (6) habenula, dorsal thalamus, ventral thalamus and rostral hypothalamus (H); (7) torus longitudinalis, pretectum, dorsal thalamus, ventral thalamus, posterior tuberculum and rostral hypothalamus. MO, medulla oblongata. (B) Brightfield image representative of a transverse section from region 2 in A from larval zebrafish exposed to 100% DO. (C) BrdU immunohistochemistry and (D) DAPI nuclear staining of the boxed area in B. (E) Brightfield image representative of a transverse section from region 4 in A from larval zebrafish exposed to 10% DO. (F) BrdU immunohistochemistry and (G) DAPI nuclear staining of the boxed area in E. Scale bar: 0.1 mm in B and E, and 0.05 mm in C, D, F and G. (H) Effects of DO saturation levels on the ratio of BrdU- to DAPI-positive nuclei within the seven defined forebrain regions shown in A. The number of BrdU- and DAPI-positive nuclei was counted in an average of 2.9±0.4 sections (mean±s.e.m) per forebrain region for each larva (n=3–5 larvae per treatment). Statistical differences between values were determined by two-way ANOVA followed by a Holm–Šidák test for multiple comparison (P<0.05). Differences between treatments within a given forebrain region are indicated by different lowercase letters. (I) Effects of DO saturation levels on forebrain neural proliferation averaged over all regions assessed in H (n=5). The ratio of BrdU- to DAPI-positive nuclei was normalized to the proliferation level of the 100% DO treatment. DO treatments that do not share a common letter are significantly different from each other as determined by a one-way ANOVA on ranks followed by a Holm–Šidák test for multiple comparisons (P<0.05). Values are means+s.e.m.

had no effect at either developmental stage (P=0.063; P=0.197). Hypoxia exposure did not have any immediate effect on *gfap* expression at 5 dpf (P=0.700) but resulted in a difference between treatments at 10 dpf ($F_{2,35}$=4.210, P=0.024). Specifically, relative to the 100% DO treatment, the 10% DO treatment reduced *gfap* expression by 39% at 10 dpf (P=0.022) and the 20% DO treatment had no effect (P=0.139). Similarly, hypoxia exposure did not have any immediate effect on *mbpa* expression at 5 dpf (P=0.809) but elicited a difference between treatments at 10 dpf ($F_{2,34}$=3.972, P=0.029). The 10% DO treatment reduced *mbpa* expression by 36% at 10 dpf (P=0.025) and the 20% DO treatment had no effect (P=0.211).

Series 4: effects of hypoxia exposure on the cortisol stress response and the expression of key regulators of the HPI axis in 5 dpf larvae

DO level significantly affected whole-body cortisol levels in 5 dpf larvae and this effect was dependent on exposure duration (DO level: $F_{2,54}$=4.616, P=0.015; time: $F_{1,54}$=22.2, P<0.001; DO level×time: $F_{2,54}$=4.405, P=0.017) (Fig. 4A). After the 2 h exposure, whole-body cortisol was 1.8-fold higher in the 10% DO treatment than in the normoxic group (P=0.012), and the 20% DO and normoxic treatments did not differ (P=0.532). After the 16 h exposure, whole-body cortisol was lower in the 20% DO treatment than in the normoxic group (P=0.043) and did not differ between the 10% DO and normoxic (P=0.510) treatments. Hypoxia exposure duration also had a significant effect on *crfb* expression in 5 dpf larvae (DO level: $F_{2,47}$=1.528, P=0.229; time: $F_{1,47}$=6.579, P=0.014; DO level×time: $F_{2,47}$=3.061, P=0.057) (Fig. 4B). While *crfb* expression was 1.3-fold higher in the 10% DO treatment than in the normoxic group (P=0.039) after the 2 h exposure, the two treatments did not differ after the 16 h exposure (P=0.742). In contrast, *crfb* expression in the 20% DO and normoxic treatments did not differ after either the 2 h (P=0.813) or 16 h (P=0.667) exposure. The 10% DO treatment

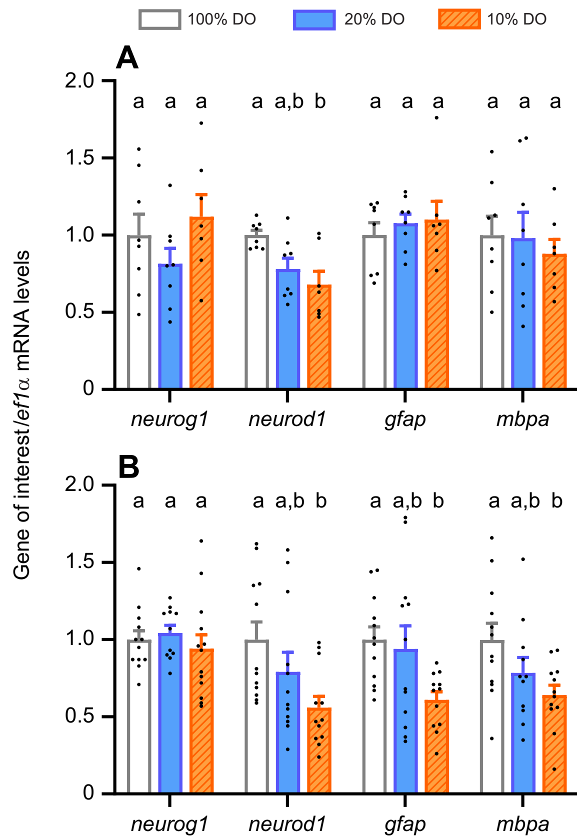

Fig. 3. Effects of exposing 5 dpf zebrafish larvae for 16 h to 100%, 20% or 10% DO on the gene expression of neural markers. The larvae were either sampled immediately after exposure (A: 5 dpf; *n*=7–8) or reared for an additional 5 days prior to sampling (B: 10 dpf; *n*=11–13). Gene expression of neurogenin 1 (*neurog1*), neurogenic differentiation 1 (*neurod1*), glial fibrillary acidic protein (*gfap*) and myelin basic protein a (*mbpa*) was normalized to the expression of elongation factor 1α (*ef1α*) and is expressed relative to the 100% DO treatment. Bars that do not share a common letter are significantly different from each other, as determined by one-way ANOVA followed by a Holm–Šidák test for multiple comparisons (P<0.05). Values are means+s.e.m.

had similar transient stimulatory effects on the expression of *uts1* and *hsd20b2* in 5 dpf larvae. The expression of *uts1* was dependent on hypoxia exposure duration (DO level: $F_{2,47}$=1.355, P=0.269; time: $F_{1,47}$=11.99, P=0.001; DO level×time: $F_{2,47}$=4.637, P=0.015) (Fig. 4C). The mRNA levels of *uts1* were 2.0-fold higher in the 10% DO treatment than in the normoxic group (P=0.013) after the 2 h exposure, but the two treatments did not differ after the 16 h exposure (P=0.655). The *uts1* expression in the 20% DO and normoxic treatments did not differ after either the 2 h (P=0.828) or 16 h (P=0.967) exposure. DO level significantly affected *hsd20b2* expression in 5 dpf larvae and this effect was dependent on exposure duration (DO level: $F_{2,49}$=4.333, P=0.019; time: $F_{1,49}$=12.553, P<0.001; DO level×time: $F_{2,49}$=2.547, P=0.09) (Fig. 4D). The expression of *hsd20b2* was 1.6-fold higher in the 10% DO treatment than in the normoxic group (P=0.004) after the 2 h exposure, and the two treatments did not differ after the 16 h exposure (P=0.878). Exposure to 20% DO did not affect the expression of *hsd20b2* after either the 2 h (P=0.095) or 16 h (P=0.778) exposure. In contrast, neither DO level nor exposure duration had an effect on the mRNA levels of *crfbp*, *mc2r*, *star*, *cyp11a1*, *cyp11c1* and *hsd11b2* in 5 dpf larvae (Fig. S2).

Series 5: effects of hypoxia exposure in 5 dpf larvae on the cortisol stress response at 10 dpf and in adults

The cortisol stress response to hypoxic conditions in 10 dpf larvae was dependent on hypoxia exposure history at 5 dpf (5 dpf: $F_{1,51}$=0.702, P=0.406; 10 dpf: $F_{1,51}$=13.94, P<0.001; 5 dpf×10 dpf: $F_{1,51}$=4.565, P=0.038) (Fig. 5A). Relative to larvae kept in normoxia at both 5 and 10 dpf, the larval zebrafish reared in normoxic conditions at 5 dpf and exposed to 10% DO at 10 dpf responded with a 2.2-fold increase in whole body cortisol (P<0.001). In contrast, while exposure to 10% DO at 5 dpf did not affect basal cortisol levels in 10 dpf larvae (P=0.354), it blunted the cortisol stress response associated with exposure to 10% DO at 10 dpf (P=0.265).

The cortisol stress response to a vortex stressor in 10 dpf larvae did not depend on hypoxia exposure history at 5 dpf (5 dpf: $F_{1,37}$=0.011, P=0.916; 10 dpf: $F_{1,37}$=115.146, P<0.001; 5 dpf×10 dpf: $F_{1,37}$=0.612, P=0.439) (Fig. 5B). Larvae kept in normoxic conditions at 5 dpf, increased whole-body cortisol 2.6-fold in response to vortexing at 10 dpf (P<0.001). Similarly, larvae exposed to 10% DO at 5 dpf, increased whole-body cortisol 3.0-fold in response to vortexing at 10 dpf (P<0.001).

The cortisol stress response to hypoxic conditions in adult zebrafish was independent of the hypoxia exposure history at 5 dpf (5 dpf: $F_{1,53}$=0.652, P=0.423; adult: $F_{1,53}$=50.206, P<0.001; 5 dpf×adult: $F_{1,53}$=0.012, P=0.914) (Fig. 5C). Larvae kept in normoxic conditions at 5 dpf, increased whole-body cortisol 4.7-fold in response to hypoxia as adults (P<0.001). Similarly, larvae exposed to 10% DO at 5 dpf, increased whole-body cortisol 5.2-fold in response to hypoxia as adults (P<0.001).

Series 6: effects of hypoxia exposure in 5 dpf larvae on hypoxia tolerance of 10 dpf larvae

Hypoxia tolerance in 10 dpf larvae was dependent on hypoxia exposure history at 5 dpf (5 dpf: $F_{1,35}$=40.652, P<0.001; 10 dpf: $F_{1,35}$=53.53, P<0.001; 5 dpf×10 dpf: $F_{1,35}$=30.5, P<0.001) (Fig. 6). Whereas exposure to 5% DO decreased survival in 10 dpf larvae that were previously exposed to normoxic conditions at 5 dpf (P<0.001), it did not affect survival in 10 dpf larvae that were previously exposed to hypoxia at 5 dpf (P=0.201).

DISCUSSION

Fish nursery habitats are growing increasingly hypoxic and the brain is recognized as a highly hypoxia-sensitive organ. However, there is a lack of information on the short- and long-term effects of hypoxic conditions on the development and function of the larval fish brain. This study provides original evidence that in larval zebrafish severe acute hypoxic conditions elicit marked region-specific reductions in neural proliferation, as well as cell-specific and time-dependent reductions in the gene expression of markers for neurons and glial cells. We also demonstrate that severe hypoxic conditions are needed to elicit transient increases in whole-body cortisol levels and in the gene expression of select effectors and regulators of the HPI axis. While hypoxia exposure at 5 dpf can abolish the cortisol stress response to hypoxic conditions in 10 dpf larvae, it also increases the hypoxia tolerance of these animals. Moreover, hypoxia exposure at 5 dpf does not affect the cortisol stress response to a novel stressor in 10 dpf larvae or to a hypoxic challenge in adult zebrafish. Therefore, whereas exposure to severe hypoxia at the larval stage can at least transiently inhibit brain development, these conditions do not appear to affect the short- and long-term functional capacity of the HPI axis to respond to stressors.

The relative hypoxia sensitivity of 5 dpf zebrafish larvae observed in this study is in marked contrast to the recognized anoxia tolerance

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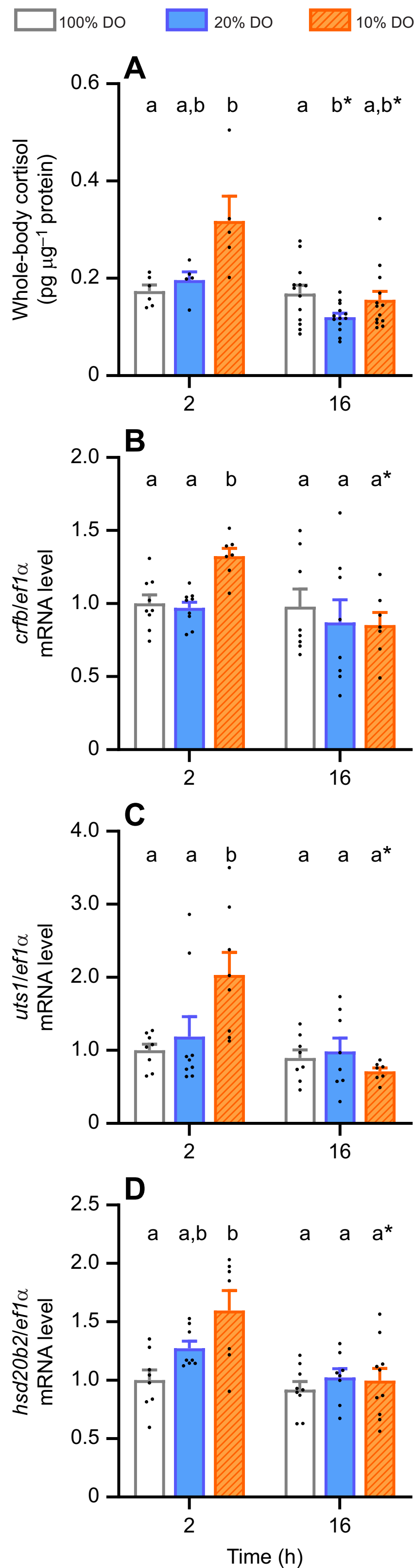

Fig. 4. Effects of exposing 5 dpf zebrafish larvae for 2 or 16 h to 100%, 20% or 10% DO on whole-body cortisol, and on gene expression of key effectors of the hypothalamic-pituitary-interrenal (HPI) axis. (A) Cortisol (*n*=5–12), (B) corticotropin-releasing factor b (*crfb*; *n*=8–9), urotensin 1 (*uts1*; *n*=8–9) and 20β-hydroxysteroid dehydrogenase 2 (*hsd20b2*; *n*=8–9). Gene expression was normalized to the expression of *ef1α* and is expressed relative to the 2 h 100% DO treatment. Statistical differences between values were determined by two-way ANOVA and by Holm–Šidák *post hoc* tests ($P<0.05$). Bars within a given exposure duration that do not share a common letter are different from one another. For a given treatment, a difference between exposure durations is indicated by an asterisk. Values are means+s.e.m.

of zebrafish embryos. While 1 dpf zebrafish embryos can arrest metabolism and enter a state of suspended animation in response to severe O_2 deprivation, the window of anoxic viability closes gradually with development and shuts around hatching, i.e. at ∼2–3 dpf (Padilla and Roth, 2001; Mendelsohn et al., 2008). Post-hatching, our results show that survival in 5 dpf larvae is unaffected by a 16 h exposure to 20% DO, slightly reduced by exposure to 10% DO (95% survival) and significantly impacted by exposure to O_2 levels below 10% DO. Similarly, in a previous study, the survival of 4 dpf zebrafish larvae exposed to 5% O_2 saturation in air (∼24% DO) for 24 h was unaffected, but significantly reduced when 5 dpf larvae were exposed to 2.5% O_2 saturation in air (∼12% DO) for 3.5 h (Long et al., 2015).

Consistent with a heightened sensitivity to reduced O_2 levels post-hatching, our results demonstrate for the first time in larval fish that hypoxic conditions can be associated with a marked reduction in neural proliferation. The BrdU saturation-labelling technique used in this study identified the mitotic cells in the various proliferation zones of the forebrain as well as recent post-mitotic cells which express early neuronal differentiation markers such as *neurod* (Mueller and Wullimann, 2002, 2003). Consistent with previous studies using 5 dpf larval zebrafish kept under normoxic conditions, we observed that the large majority of proliferating cells were located in the periventricular region, that neural proliferation is heterogeneous across the forebrain, and that the olfactory bulbs have the highest ratio of proliferating cells (Wullimann and Puelles, 1999; Mueller and Wullimann, 2002, 2005). In larvae exposed to hypoxia, while the 20% DO treatment had no effect on neural proliferation, exposure to 10% DO elicited a marked overall reduction in forebrain BrdU signal, suggesting that the hypoxia tolerance of proliferating neural cells and recent post-mitotic neurons in 5 dpf zebrafish larvae drops sharply at a DO threshold that is only slightly above the O_2 level that induces lethality. Also, as the critical level of O_2 uptake in 5 dpf zebrafish larvae is ∼30–34 mmHg, i.e. ∼20% DO (Rombough and Drader, 2009; Pan et al., 2019), our results suggest that neural proliferation in larval zebrafish decreases below the partial pressure of O_2 at which metabolic rate first becomes O_2 limited.

Similarly, severe perinatal hypoxia depletes the subventricular zone of neural stem cells in rodents (Levison et al., 2001; Brazel et al., 2004). The region-specific inhibitory effects of the 10% DO treatment observed in this study infer a differential sensitivity of neural proliferative cell populations to hypoxic conditions. In mammals, while cerebral ischaemia does not appear to induce cell death in the main olfactory bulb (Choi et al., 2010), severe asphyxia in full-term human infants results in selective injury to the sensorimotor cortex, basal ganglia and thalamus (Johnston et al., 2002). Although future double-labelling immunohistochemistry studies are needed to specifically identify the nuclei affected by severe hypoxia exposure in larval fish, the thalamus and the subpallium (the zebrafish homologue of the basal ganglia) (Cheng et al., 2014) were among the forebrain regions characterized by reduced neural proliferation in this study. In general, the selective vulnerability of brain regions to hypoxic insults is well established and attributed to a differential activation of *N*-methyl-*D*-aspartate

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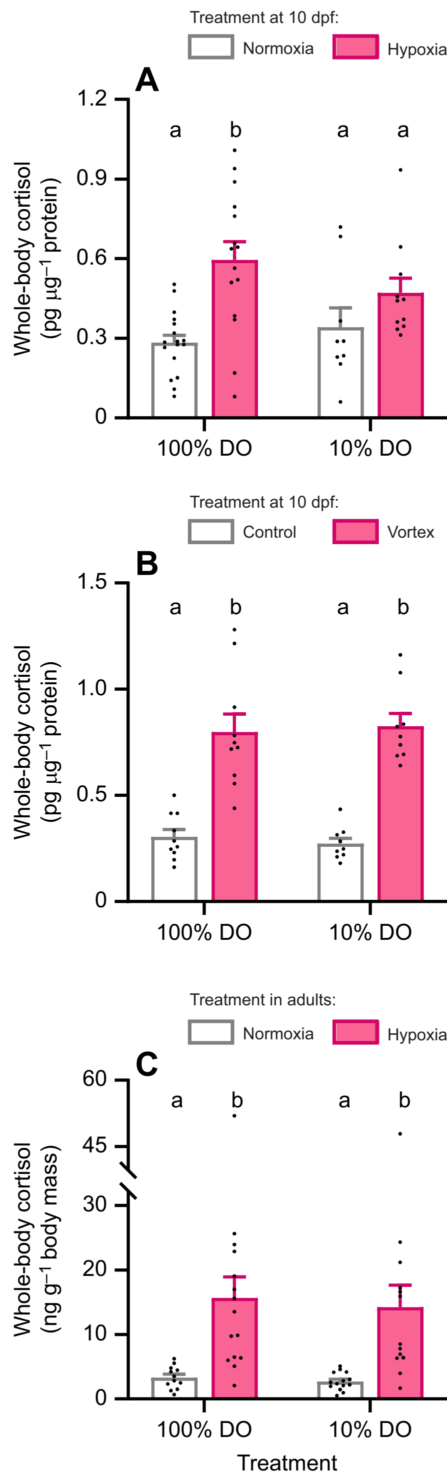

Fig. 5. Effects of exposing 5 dpf zebrafish larvae to hypoxia on the cortisol stress response at 10 dpf and in adults. (A) Effects of 2 h exposure to 100% (normoxia) or 10% (hypoxia) DO on whole-body cortisol in 10 dpf zebrafish larvae previously exposed at 5 dpf to 100% or 10% DO for 16 h (*n*=9–17). (B) Effects of 20 min exposure to no vortexing (control) or continuous vortexing at 180 rpm in an orbital shaker on whole-body cortisol in 10 dpf zebrafish larvae previously exposed at 5 dpf to 100% or 10% DO for 16 h (*n*=9–10). (C) Effects of 1 h exposure to 100% (normoxia) or 10% (hypoxia) DO on whole-body cortisol in adult zebrafish previously exposed at 5 dpf to 100% or 10% DO for 16 h (*n*=12–15). Treatments that do not share a common letter are significantly different from each other as determined by a two-way ANOVA followed by a Holm–Šidák test for multiple comparisons (*P*<0.05). Values are means+s.e.m.

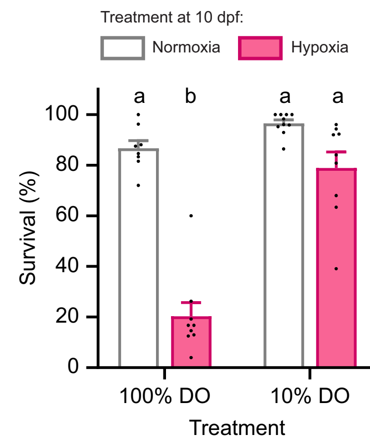

Fig. 6. Effects of 4 h exposure to 100% (normoxia) or 5% (hypoxia) DO on percentage survival in 10 dpf zebrafish larvae previously exposed at 5 dpf to 100% or 10% DO for 16 h. Treatments that do not share a common letter are significantly different from each other as determined by a two-way ANOVA followed by a Holm–Šidák test for multiple comparisons (*P*<0.05). Values are means+s.e.m. (*n*=8-10).

(NMDA) glutamate receptors in immature neurons (Hattori et al., 1989; Nyakas et al., 1996; Johnston et al., 2002; Waters and Machaalani, 2004).

The inhibitory effects of severe hypoxia on the gene expression of neural markers observed in this study also indicate that a lack of O_2 constrains larval brain development in zebrafish and that the effects may be neural cell type specific and time dependent. Despite the marked overall effects of severe hypoxia on forebrain neural proliferation, the 10% DO treatment did not have any immediate or delayed effects on the expression of the proneuronal transcription factor *neurog1*. As *neurog1* is a marker of the least differentiated state assessed, our results suggest that undifferentiated progenitors in zebrafish larvae may have a higher hypoxia tolerance than more differentiated cell types, a finding that is consistent with the properties of mammalian neural stem cells (Horie et al., 2008; Panchision, 2009). In 5 dpf larvae, the fact that *neurod1* was the only cell lineage marker affected by the 10% DO treatment indicates that recently determined neurons are more sensitive than other neuronal cell types to the immediate effects of severe hypoxia. In mammals, the early phase of hypoxia–ischaemic damage is generally attributed to elevations in extracellular glutamate levels, the opening of NMDA glutamate receptors and a sharp increase in injurious intracellular Ca^{2+} (Johnston et al., 2002). As a result of differences in the composition and properties of NMDA receptors, while determined post-mitotic neurons are susceptible to glutamate excitotoxicity, immature neuronal precursors, astrocytes and oligodendrocytes are not (Bickler and Hansen, 1998; Dzamba et al., 2013; He et al., 2013). However, given the forebrain region-specific effects of severe hypoxia on neural proliferation observed in this study, interpretation of whole-larvae neural marker gene expression data should be done with caution as cell-type specific effects could be masked by region-specific responses to the hypoxic stressor. In the 10 dpf larvae previously exposed to hypoxia at 5 dpf, the reductions in *neurod1*, *gfap* and *mbpa* expression suggest that severe hypoxia exposure has similar delayed effects on determined neurons, radial glia and oligodendrocytes, respectively. In rodent models of neonatal hypoxia, the re-oxygenation phase that follows a severe hypoxic insult is associated with a second wave of neural cell damage that affects both neurons and glia. The cause for this delayed wave of neurological impairment is complex and associated

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with several processes including the production of O_2 radicals, the synthesis of nitric oxide and inflammation (Torres-Cuevas et al., 2019). Overall, although further research is needed to verify that the transcriptional changes observed here translate to the protein level and have a functional impact, and to identify the specific mechanisms that contribute to the impairment in neural proliferation and differentiation elicited by severe hypoxia, our results imply that the mechanisms of the immediate and delayed effects of hypoxia on neural development in larval zebrafish may differ.

As previously observed in zebrafish (Wilson et al., 2013), rainbow trout (Fuzzen et al., 2011) and channel catfish (Peterson et al., 2019), we show that exposure to hypoxic conditions in larval fish can elicit an increase in whole-body cortisol levels. Overall, previous results in rainbow trout (Bernier and Craig, 2005; Fuzzen et al., 2011) and zebrafish (Wilson et al., 2013; Ivy et al., 2017; Williams et al., 2017b), and those from the current study, suggest that moderate hypoxia has no impact on whole-body cortisol in larval fish, that severe hypoxic conditions are needed to elicit a relatively modest increase in whole-body cortisol, and that the larval HPI axis is less responsive to hypoxic conditions than the HPI axis of adults. As in larval and adult rainbow trout (Fuzzen et al., 2011; Bernier et al., 2008), we also observed that exposure of larval zebrafish to severe hypoxia is associated with an initial increase in whole-body cortisol levels followed by a return to basal values despite continuous exposure to the hypoxic conditions.

Our analysis of the transcriptional activity of key effectors of the stress response suggests that CRF and UTS1 are involved in the regulation of the HPI axis in response to acute hypoxia exposure. Consistent with the changes in preoptic area *crf* and *uts1* expression elicited by chronic hypoxia exposure in adult rainbow trout (Bernier et al., 2008), the 10% DO treatment in this study was characterized by transient increases in *crf* and *uts1* expression that paralleled the changes in whole-body cortisol levels. CRF and UTS1 are potent stimulators of pituitary adrenocorticotropic hormone (ACTH) secretion in fish, the primary effector of cortisol production from the interrenal cells (Bernier et al., 2009). Although CRF is broadly expressed within the brain and in peripheral tissues, the preoptic area is the principal site of CRF synthesis in developing zebrafish (Chandrasekar et al., 2007; Alderman and Bernier, 2009), suggesting a potential role for CRF in the regulation of the HPI axis in response to severe hypoxic conditions in this study. In contrast, there is very limited *uts1* expression in the hypothalamus and preoptic area of larval zebrafish (Bräutigam et al., 2010). Instead, the principal site of *uts1* expression in zebrafish larvae is the midbrain nucleus of the medial longitudinal fascicle (nmlf; Bräutigam et al., 2010). The mammalian homologue to the nmlf, the non-preganglionic Edinger Westphal (npEW) nucleus, is the prominent site of urocortin 1 (UCN1) synthesis (the mammalian ortholog of fish UTS1; Kozicz, 2007). In rodents, the UCN1-npEW neurons are recruited by a variety of stressors and are implicated in the energy-dependent regulation of the stress response (Xu et al., 2012), an avenue of the stress response to hypoxic conditions which has yet to be explored. Conversely, the lack of hypoxia-induced change in the expression of *mc2r*, the interrenal ACTH receptor, and in the expression of *star*, *cyp11a1* and *cyp11c1*, key genes involved in corticosteroid synthesis (Fuzzen et al., 2010), imply that acute hypoxia exposure in this study did not have any immediate effects on the capacity of ACTH to stimulate cortisol production in larval zebrafish.

Several mechanisms may also be involved in dampening the magnitude and duration of the hypoxia-induced cortisol stress response in larval zebrafish. For example, as an important means of regulating the biological action and excretion of glucocorticoids, the enzymes 11β-HSD2 and 20β-HSD2 are responsible for the conversion of cortisol to the inactive and more hydrophilic steroids cortisone and 20β-dihydrocortisone, respectively (Alderman and Vijayan, 2012; Tokarz et al., 2012, 2013). These enzymes are broadly expressed in larval and adult zebrafish (Alderman and Vijayan, 2012; Tokarz et al., 2012), and previous studies have shown that the expression of *hsd11b2* and *hsd20b2* can increase in response to cortisol treatment and to some stressors (Fuzzen et al., 2010; Alderman and Vijayan, 2012; Tokarz et al., 2013). Here, we provide original evidence that the expression of *hsd20b2* increases transiently in response to severe hypoxic conditions and that the expression of *hsd11b2* remains unchanged. The transient nature of the hypoxia-induced cortisol stress response may also result from the negative feedback effects of cortisol on the HPI axis, a mechanism already functional by 2 dpf in zebrafish (Nesan and Vijayan, 2016). In addition, zebrafish embryos and larvae exposed to severe hypoxia are characterized by increased mRNA levels of the hypoxia-responsive gene leptin (Yu et al., 2012), a pleiotropic cytokine with known inhibitory effects on the HPI axis of fish (Gorissen et al., 2012). Therefore, the regulation of the HPI axis in larval zebrafish under hypoxic conditions is likely to be multifactorial.

An important factor involved in the regulation of neurogenesis is glucocorticoids. As such, given the stimulatory effects of hypoxia on the HPI axis, cortisol may be involved in regulating neural cell proliferation in response to severe hypoxic conditions. However, the relationship between cortisol and neural cell proliferation in fish is rather complex. For example, while the marked and sustained increases in cortisol associated with social subordination in zebrafish and rainbow trout have been shown to suppress forebrain cell proliferation (Sørensen et al., 2011, 2012; Tea et al., 2019), modest rises in plasma cortisol mediate at least a portion of the increase in brain cell addition elicited by long-term social interactions in the brown ghost knifefish (*Apteronotus leptorhynchus*; Dunlap et al., 2006, 2011). Similarly, in adult rainbow trout, the expression of genes involved in neural plasticity and neurogenesis is generally increased by short-term confinement stress and inhibited by long-term social stress (Johansen et al., 2012). In developing zebrafish, while the microinjection of cortisol into the yolk at the one-cell stage increased neurogenesis in the pallium and the preoptic region of 1 dpf embryos (Best et al., 2017), continuous cortisol treatment from 0 to 5 dpf decreased cell proliferation in the forebrain of 5 dpf larvae (Higuchi, 2020). Taken together, these results and similar observations in mammals are evidence that the effects of cortisol on neurogenesis are biphasic: stimulatory when the stress-induced increases in cortisol are mild and/or acute and inhibitory when cortisol levels are high and/or sustained (Sørensen et al., 2013; Saaltink and Vreugdenhil, 2014). Therefore, although this remains to be verified experimentally, given the modest and transient increase in whole-body cortisol levels elicited by the 10% DO treatment, we suggest that the severe hypoxia-induced increase in cortisol levels is unlikely to have contributed to the reduced rate of neural cell proliferation and differentiation of the hypoxic larvae.

In contrast, we show that exposure to 10% DO for 16 h in 5 dpf zebrafish larvae blunts the cortisol stress response elicited by exposure to severe hypoxia at 10 dpf. We suggest that three factors may contribute to this response: (1) the programming effects of cortisol on the HPI axis; (2) the detrimental effects of hypoxia on stress-sensitive neurocircuitry that regulates HPI axis activity; and/or (3) an increase in hypoxia tolerance. The fact that excess glucocorticoids or physical stressors during early development can

affect the cortisol stress response in larval and adult zebrafish (Nesan and Vijayan, 2016; Wilson et al., 2016) and rainbow trout (Auperin and Geslin, 2008) implies that cortisol exposure can have programming effects on the HPI axis. Similarly, the observation in zebrafish that severe hypoxia in 36 h post-fertilization embryos affects the responsiveness of the HPI axis to social stress in later life suggests that a lack of O_2 during early development can affect the regulation of the HPI axis independent of the developmental actions of cortisol (Ivy et al., 2017). In this study, however, while acute larval hypoxia exposure blunted the cortisol stress response to a second hypoxic bout at 10 dpf, it had no effect on basal cortisol levels in later life, on the cortisol stress response associated with a novel vortex stressor at 10 dpf, and on the cortisol stress response elicited by a hypoxic challenge in adult fish. So, despite the capacity of hypoxia to increase whole-body cortisol levels and impair brain development, we did not find any evidence that the severe acute hypoxia treatment used here on larval fish had either programming or detrimental effects on the function of the HPI axis in zebrafish. Instead, we propose that the reduced cortisol response following repeated hypoxia exposure in 10 dpf larvae is due to a reversible increase in hypoxia tolerance.

The marked increase in survivorship in response to lethal hypoxia in larvae that were previously exposed to the 10% DO treatment provides direct evidence that exposure to severe acute hypoxia leads to hypoxic pre-conditioning in zebrafish larvae. Similarly, as evidenced by a decrease in critical O_2 tension (P_{crit}), previous studies have shown that exposing embryos (Robertson et al., 2014) or larvae (Mandic et al., 2020) to hypoxic conditions can increase hypoxia tolerance in zebrafish. Together, these results indicate that in response to developmental hypoxia, fish recruit various biochemical and physiological mechanisms whose actions persist beyond the initial exposure and facilitate an adaptive response upon re-exposure to reduced O_2 levels. However, in this study, the fact that severe hypoxia exposure at 5 dpf had no effect on the hypoxia-induced cortisol stress response in adult fish suggests that the adults derived from the hypoxia- and normoxia-exposed 5 dpf larvae had similar hypoxia tolerance and that the pre-conditioning induced by severe hypoxia is reversible. Recent findings demonstrate that a key regulator of the mechanisms responsible for hypoxic pre-conditioning in zebrafish is the master regulator of hypoxia responsive genes, hypoxia-inducible factor-1 (HIF-1; Robertson et al., 2014; Mandic et al., 2020). In mammals, HIF-1 and its target genes are also known to play an essential role in promoting neural stem cell survival, proliferation and differentiation following hypoxic–ischaemic brain injuries (Qi et al., 2017; Parry and Peeples, 2018). Therefore, beyond its role in hypoxic pre-conditioning, future studies should assess whether HIF-1 contributes to the repair of the larval brain following severe hypoxia exposure.

The acute severe hypoxic conditions used in this study are common in shallow nursery habitats during spring and summer. Detailed longitudinal studies of water quality have shown that shallow estuaries, salt marshes and tidal tributary headwaters are impacted by dynamic temporal and spatial fluctuations in O_2 levels over the diel cycle (Beck et al., 2001; Cheek et al., 2009; Hughes et al., 2011; Campbell and Rice, 2014). In these habitats, hypoxic events range in intensity from moderate to severe (i.e. anoxia) and persist in duration from hours to days. Driven by diel photosynthesis and respiration cycles of phytoplankton and macroalgae, these acute periods of night-time hypoxia can be repeated daily for weeks and interspersed with daytime hyperoxia (Smith and Able, 2003; Tyler and Targett, 2007; Stierhoff et al., 2009; Caffrey et al., 2010). In deeper estuaries, lakes and marine systems, stratification and organic decay after spring algal blooms can lead to large-scale chronic seasonal hypoxia, which typically last weeks to months (Diaz and Rosenberg, 2008). Therefore, beyond the threat of acute severe hypoxia, larval and juvenile fish may be exposed to recurrent diel cycles of hypoxia and hyperoxia, as well as chronic hypoxia. These conditions may have more detrimental effects on brain development and stress-coping abilities than those used in this study and thus should be given future consideration.

In conclusion, our findings do not support the hypothesis that acute severe hypoxia exposure-induced delays in larval brain development have persistent functional effects on the HPI axis of zebrafish. Although severe acute hypoxic conditions did inhibit the cortisol stress response to hypoxia in larvae, we suggest that reversible hypoxic pre-conditioning is responsible for this transient dampening of the cortisol stress response. Similarly, we previously observed that acute anoxia exposure in zebrafish embryos does not affect the cortisol stress response to hypoxia in adults (Ivy et al., 2017). Together, these results demonstrate that developmental exposure to severe acute hypoxia does not have long-term effects on either the hypoxia-responsive neurocircuitry that regulates the HPI axis or the capacity of the HPI axis to mount a cortisol stress response. However, we have also previously observed that embryonic anoxia exposure favours the development of a dominant and aggressive phenotype in adult zebrafish (Ivy et al., 2017), which implies that developmental exposure to hypoxic conditions can have lasting effects on neuronal functions. Therefore, towards a broader understanding of the impact of hypoxic conditions on brain development and function, future studies should assess whether hypoxia exposure in larval fish can differentially affect stress-sensitive neuronal circuitries and lead to cognitive deficits and behavioural disorders.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.V.M., N.J.B.; Methodology: K.V.M., Z.A.Z., N.J.B.; Validation: K.V.M., N.J.B.; Formal analysis: K.V.M., N.J.B.; Investigation: K.V.M., Z.A.Z., N.J.B.; Writing - original draft: K.V.M., N.J.B.; Writing - review & editing: K.V.M., Z.A.Z.; Visualization: K.V.M., N.J.B.; Supervision: N.J.B.; Funding acquisition: N.J.B.

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Data availability
All data used in this paper are available from the Dryad digital repository (Bernier et al., 2022): https://doi.org/10.5061/dryad.gxd2547hv

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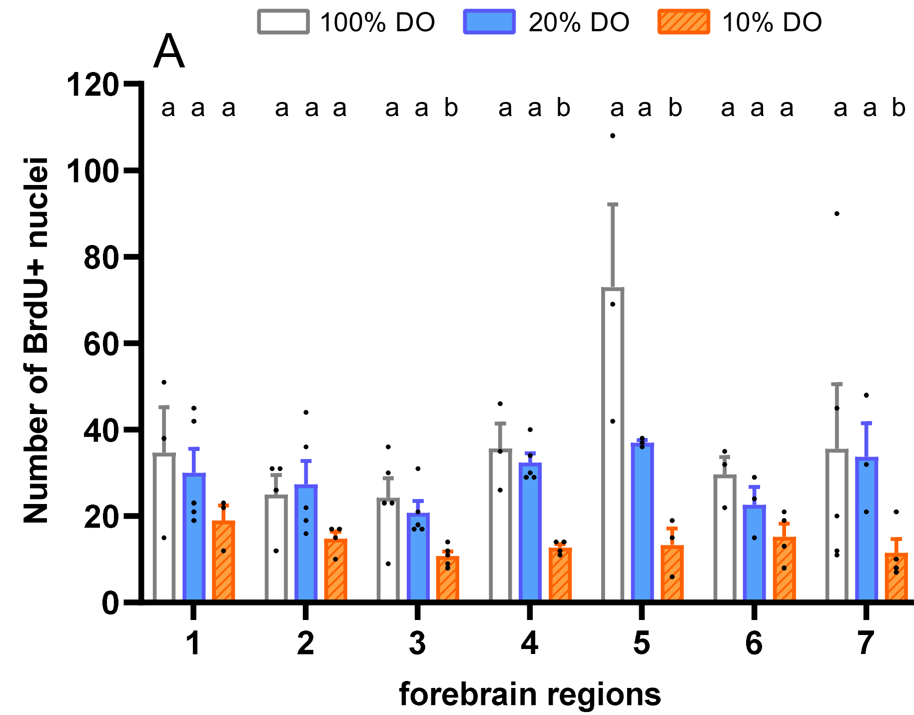
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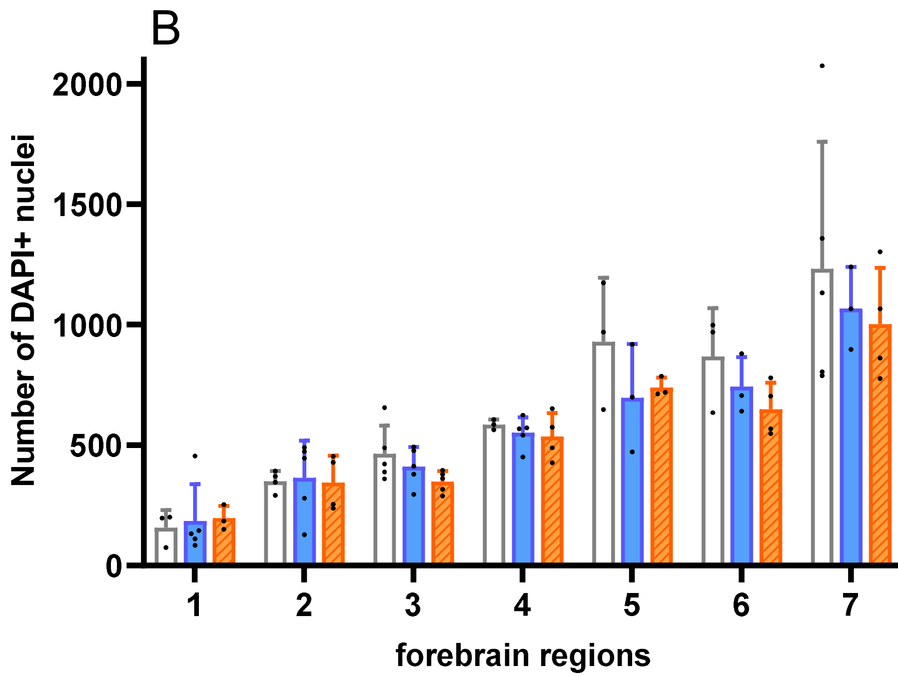
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forebrain regions

Fig. S1. Effects of hypoxia exposure on forebrain proliferation in 5 dpf zebrafish larvae. Larval zebrafish were exposed for 16 h to 100%, 20% or 10% dissolved O_2 (DO) in the presence of bromodeoxyuridine (BrdU) to label dividing cells. (A) Effects of DO saturation levels on the number of BrdU-positive nuclei within seven defined forebrain regions. (B) Effects of DO saturation levels on the number of DAPI-positive nuclei within seven defined forebrain regions. The number of BrdU- and DAPI-positive nuclei were counted on an average of 2.9 ± 0.4 sections (mean ± s.e.m) per forebrain region for each larva (n=3-5 larvae per treatment). Statistical differences between values were determined by two-way ANOVA followed by a Holm-Sidak test for multiple comparison (P<0.05). Differences between treatments within a given forebrain region are indicated by different letters. The seven forebrain regions used for BrdU quantification along the rostral to caudal axis included: 1) olfactory bulb; 2) pallium and subpallium; 3) pallium, subpallium, and anterior commissure; 4) pallium, ventral thalamus, preoptic region; 5) habenula, dorsal thalamus, ventral thalamus, and preoptic region; 6) habenula, dorsal thalamus, ventral thalamus, and rostral hypothalamus; 7) torus longitudinalis, pretectum, dorsal thalamus, ventral thalamus, posterior tuberculum, and rostral hypothalamus. See Figure 2 and Result section for further details. Values are means + s.e.m.

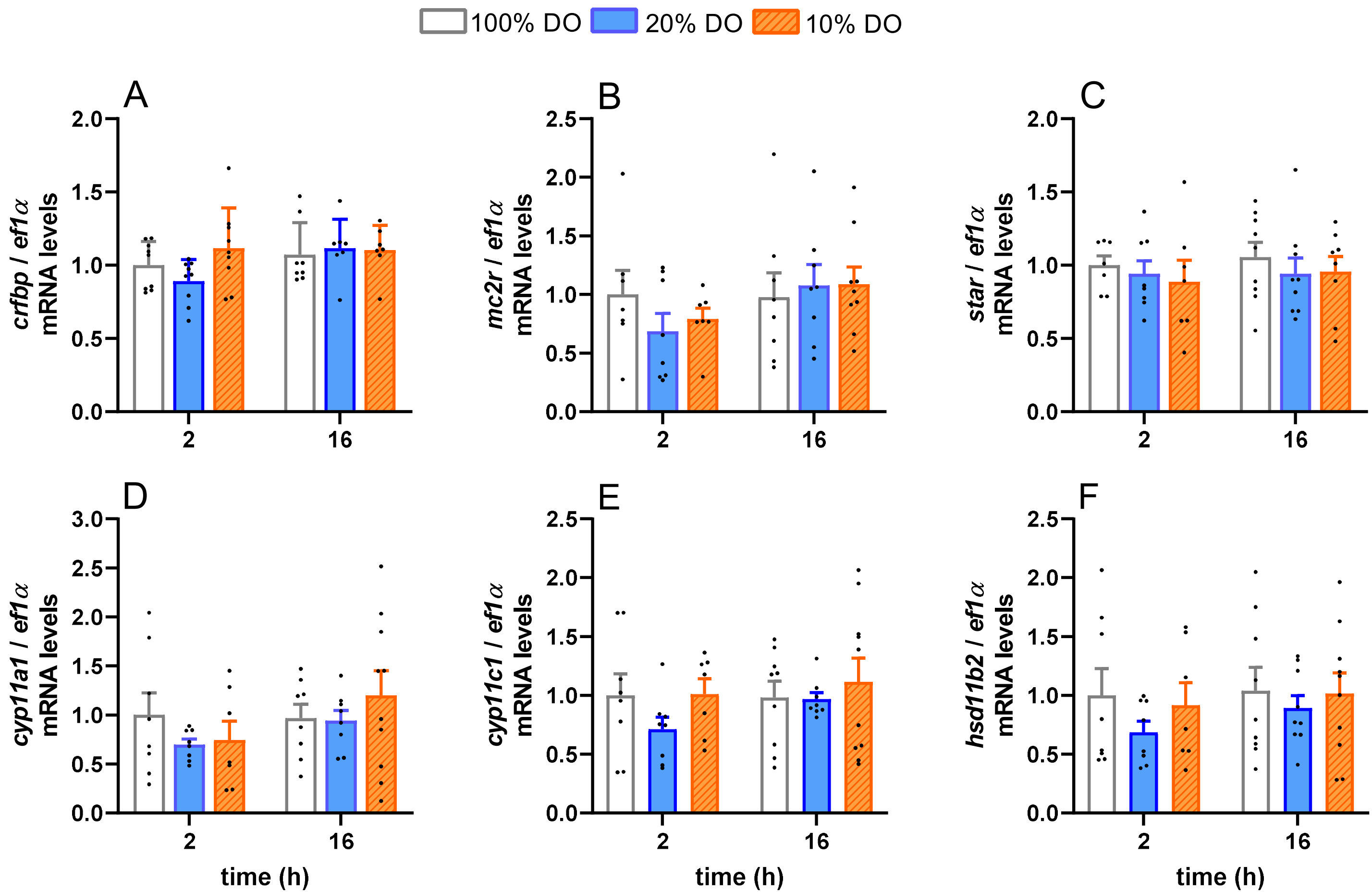

Fig. S2. Effects of exposing 5 dpf zebrafish larvae for a 2 or 16 h period to 100%, 20% or 10% dissolved O_2 (DO) saturation levels on the mRNA levels of corticotropin-releasing factor binding protein (*crfbp*; A), melanocortin 2 receptor (*mc2r*; B), steroidogenic acute regulatory protein (*star*; C), cytochrome P450 side-chain cleavage (*cyp11a1*; D), 11β-hydroxylase (*cyp11c1*; E), and 11β-hydroxysteroid dehydrogenase 2 (*hsd11b2*; F). Gene expression is normalized to the *)⍰1fe(* ⍰1 rotcaf noitagnole fo noisserpxe and is expressed relative to the 2 h 100% DO treatment. A two-way ANOVA was performed to determine the effects of time and O_2 treatment on the mRNA levels of each gene. No statistical difference was observed (P>0.05). Values are means + s.e.m. (n=7-10).