

RESEARCH ARTICLE

Social stress increases plasma cortisol and reduces forebrain cell proliferation in subordinate male zebrafish (*Danio rerio*)

Jonathan Tea¹, Sarah L. Alderman^{2,*} and Kathleen M. Gilmour¹**ABSTRACT**

Many animals, including zebrafish (*Danio rerio*), form social hierarchies through competition for limited resources. Socially subordinate fish may experience chronic stress, leading to prolonged elevation of the glucocorticoid stress hormone cortisol. As elevated cortisol levels can impair neurogenesis, the present study tested the hypothesis that social stress suppresses cell proliferation in the telencephalon of subordinate zebrafish via a cortisol-mediated mechanism. Cell proliferation was assessed using incorporation of the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU). After 48 and 96 h of social interaction, subordinate male zebrafish exhibited elevated plasma cortisol concentrations and significantly lower numbers of BrdU⁺ cells in the dorsal but not ventral regions of the telencephalon compared with dominant or group-housed control male fish. After a 2 week recovery in a familiar group of conspecifics, the number of BrdU⁺ cells that co-labelled with a neuronal marker (NeuN) was modestly reduced in previously subordinate male fish, suggesting that the reduction of cell proliferation during social stress may result in fewer cells recruited into the neuronal population. In contrast to male social hierarchies, subordinate female zebrafish did not experience elevated plasma cortisol, and the number of BrdU⁺ cells in the dorsal telencephalic area was comparable among dominant, subordinate and group-housed control female fish. Treating male zebrafish with metyrapone, a cortisol synthesis inhibitor, blocked the cortisol response to social subordination and attenuated the suppression of brain cell proliferation in the dorsal telencephalic area of subordinate fish. Collectively, these data support a role for cortisol in regulating adult neurogenesis in the telencephalon of male zebrafish during social stress.

KEY WORDS: Behaviour, Glucocorticoid, Neurogenesis, Telencephalon, Metyrapone, BrdU, Fish, Brain

INTRODUCTION

Adult neurogenesis refers to the sequential proliferation, differentiation and incorporation of new neurons in the post-embryonic brain. Although adult neurogenesis is a widespread phenomenon in vertebrates, the fish brain has emerged as an important comparative model for studying the cellular and molecular mechanisms that regulate this process. Teleost fish retain the highest capacity for adult neurogenesis among vertebrates,


having cell proliferation rates that are orders of magnitude above those of mammals (Hinsch and Zupanc, 2007; Zupanc and Horschke, 1995). This high rate of cell addition occurs in dense proliferation zones that extend across the entire rostro-caudal axis of the brain (Ekström et al., 2001; Maruska et al., 2012; Zupanc and Horschke, 1995; Zupanc et al., 2005), with many of the newborn cells differentiating into neurons and boasting long survival times (Zupanc et al., 2005). The number of proliferation zones in the adult brain of other vertebrates is reduced along a phylogenetic continuum, but all vertebrates studied to date exhibit adult neurogenesis in the forebrain (Kaslin et al., 2008). In general, the heightened capacity for adult neurogenesis in teleosts is important for injury repair and for supporting ongoing integration of peripheral inputs associated with indeterminate growth (Zupanc, 2006). But niche-specific changes in neurogenesis are known to occur under a variety of experimental contexts (Lindsey and Tropepe, 2014; Lindsey et al., 2014) suggesting that, on a finer scale, the ability to change the relative complement of cell types in select brain regions may serve discrete functions (Dunlap, 2016; Sørensen et al., 2013).

The forebrain of fish includes the paired lobes of the telencephalon, which are grossly divided into dorsal and ventral subregions, homologous to the pallial and subpallial regions of the mammalian cerebrum, respectively. Accordingly, the telencephalon is a major integrative centre of the brain that processes diverse sensory inputs relayed from the thalamus to govern behaviour, learning and memory. It is also the site of abundant cell proliferation, which dominates along the ventricular zones (Grandel et al., 2006; Lindsey et al., 2014; Maruska et al., 2012; Sørensen et al., 2007; von Krogh et al., 2010; Zupanc and Horschke, 1995). Intriguingly, it is widely accepted that the lateral zone of the dorsal telencephalon (Dl) of fish is homologous to the hippocampus, and here adult neurogenesis appears to be an evolutionarily conserved trait (Kaslin et al., 2008) with demonstrated importance for experience-based learning in mammals (Shors et al., 2012) and birds (Barnea and Nottebohm, 1994). This observation suggests that life-long neurogenesis in hippocampus-like regions of the brain is an ancient component of animal cognition. Furthermore, environmental complexity, conspecific interactions and stress all have been shown to modify adult neurogenesis in mammals (Opendak and Gould, 2015; Opendak et al., 2016; Schoenfeld and Gould, 2012; Shors et al., 2012), birds (Balthazart and Ball, 2016; Sherry and MacDougall-Shackleton, 2015) and teleost fish (Dunlap, 2016; Sørensen et al., 2013), suggesting evolutionary conservation of the mechanisms that regulate neurogenesis.

Glucocorticoid stress hormones, including cortisol, are known to regulate several stages of neurogenesis during brain development and in the adult vertebrate brain. As such, a stress-induced increase in cortisol may be an important mechanism by which adaptive behavioural and physiological responses to stress are solidified in

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List of abbreviations

D	dorsal telencephalic area
Dd	dorsal zone of D
DI	lateral zone of D
Dm	medial zone of D
TelV	telencephalic ventricle
Vd	dorsal nucleus of ventral telencephalic area
Vv	ventral nucleus of ventral telencephalic area

the central nervous system. Yet, studies that associate increased plasma cortisol with changes in adult brain cell proliferation in fish offer mixed results. For example, plasma cortisol and brain cell proliferation are negatively correlated during social subordination (Johansen et al., 2012; Maruska et al., 2012; Sørensen et al., 2012), which is a well-characterized chronic stressor (Gilmour et al., 2005; Johnsson et al., 2006; Sørensen et al., 2013). In contrast, cortisol treatment in the absence of a stressor increased (Dunlap et al., 2006), decreased (Sørensen et al., 2011) or did not change (Lindsey and Tropepe, 2014) rates of brain cell proliferation. This ambiguity, along with a paucity of direct experimental evidence, challenges our ability to assign a mechanistic role for cortisol in mediating stress-induced changes to adult neurogenesis in teleost fish. Therefore, the present study tested the hypothesis that chronically elevated cortisol during social stress reduces neurogenesis in the telencephalon of adult zebrafish.

MATERIALS AND METHODS**Experimental animals**

Male ($N=99$, mass 419.3 ± 20.5 mg, fork length 3.46 ± 0.04 cm) and female ($N=38$, mass 521.1 ± 58.9 mg, fork length 3.74 ± 0.10 cm) zebrafish, *Danio rerio* (F. Hamilton 1822), 6–12 months of age, were obtained from an in-house breeding programme in the aquatic facility of the University of Ottawa, Canada. Fish were housed in 5 or 10 l polycarbonate tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water at 28.5°C . A photoperiod of 14 h light:10 h dark was used. Zebrafish were fed three times per day with a mixture of equal parts of Zeigler zebrafish diet, Brine Shrimp Direct Golden pearls (300–500 μm) diet and Zeigler AP 100 5 diet (250–400 $\mu\text{mol l}^{-1}$), with an additional serving of brine shrimp daily. Fish were acclimated to these holding conditions for at least 1 week prior to experimentation. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching, and after approval of the University of Ottawa Animal Care Committee (protocol BL-2118).

Experiments were carried out on dominant and subordinate zebrafish obtained by housing fish in pairs to establish a social hierarchy. Fish were lightly anaesthetized by immersion in a buffered solution of MS-222 (0.24 mg ml^{-1} 3-aminobenzoic acid ethyl ester, 21 mmol l^{-1} Tris, pH 7; Sigma-Aldrich, Oakville, ON, Canada), and mass and fork length were recorded. For identification, each fish was given a unique mark using Alcian Blue (Sigma-Aldrich) (Jeffrey and Gilmour, 2016). The fish within a pair were of the same sex, size matched for length (mean difference in fork length within a pair: 0.06 ± 0.01 cm or 1.8% for $N=42$ male pairs and 0.06 ± 0.01 cm or 1.5% for $N=14$ female pairs) and mass (mean difference in mass within a pair: 56.5 ± 6.7 mg for male pairs and 93.0 ± 31.9 mg for female pairs), and obtained from different holding tanks to avoid prior exposure to each other. The members of a pair were placed in an experimental chamber (4.5 l)

supplied with flowing water as above, and were separated by an opaque divider for 24 h of recovery and isolation to ensure strong interactions within a pair (Larson et al., 2006; Oliveira et al., 2016). The divider was then removed and the fish were allowed to interact for 48 or 96 h, with pairs being observed twice a day for 2 min each time. After each observation period, fish were fed by adding small pinches of food to the tank until the dominant fish stopped feeding.

During each 2 min observation period, acts of aggression (e.g. chases, bites) and retreats from aggression were counted for each fish. Position in the tank (actively patrolling the water column versus limited movement at the top or bottom of the tank) was assessed at the end of the observation period. Finally, a pellet of food was added to the tank to determine which fish fed first. Behaviours were scored using a system similar to that of previous studies (Dahlbom et al., 2011; Filby et al., 2010; Jeffrey and Gilmour, 2016), with dominant behaviours awarded higher scores than submissive behaviours. Scores for specific behaviours were averaged over the interaction period and combined using a principle components analysis (PCA) to obtain a single behaviour score for each fish. The fish within a pair with the higher score was assigned dominant status, and only pairs with divergent behaviour scores were retained in the experiment (behaviour scores were not divergent in 14% of pairs).

Studies of social interactions frequently use sham-treated fish as a control group, i.e. fish that are handled in the same way as fish paired with a conspecific, but held in isolation (Jeffrey and Gilmour, 2016). However, forebrain cell proliferation is reduced by social isolation in zebrafish (Lindsey and Tropepe, 2014); therefore, fish housed in a stable group were chosen as the control for the present study.

Experimental protocols

Three series of experiments were carried out. First, social hierarchies in pairs of zebrafish were characterized in terms of behaviour, circulating cortisol concentrations and impact on cell proliferation in the forebrain. To determine whether neurogenesis was affected by social interaction, a second experimental series was carried out in which fish that had been held in pairs for 48 h of interaction were returned to group-holding conditions for 2 weeks to allow newborn cells to differentiate. Finally, pairs of zebrafish were exposed to metyrapone to inhibit cortisol synthesis to establish a causal link between cortisol and effects of social interaction on cell proliferation. Male and female zebrafish pairings were used for series 1, whereas only male fish were used in series 2 and 3.

Series 1: effect of social interaction on cortisol concentrations and cell proliferation in the forebrain

Plasma cortisol concentrations were assessed in pairs of male zebrafish that had been allowed to interact for 48 h ($N=7$) or 96 h ($N=8$), in pairs of female zebrafish that had interacted for 96 h ($N=9$) and in control fish sampled from a holding tank ($N=7$ male and $N=6$ female fish). Following the interaction period, the fish in a pair were killed simultaneously in a lethal concentration of MS-222 (0.72 mg ml^{-1}), mass was recorded, and blood was collected using the method of Babaei et al. (2013). Blood samples were centrifuged at 13,800 g for 15 min at 4°C , and plasma was separated and stored at -80°C . Cortisol concentration was quantified using a commercially available enzyme-linked immunoassay according to the manufacturer's instructions (Neogen, Lexington, KY, USA). The intra-assay coefficient of variability was 3.4% and all samples within a given experiment were analysed in a single assay.

Cell proliferation in the forebrain was assessed using a single pulse of 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) in pairs of male zebrafish that had interacted for a total of 48 h ($N=4$) or 96 h

($N=4$), in pairs of female zebrafish that had interacted for a total of 96 h ($N=4$) and in control fish sampled from a holding tank ($N=4$ male and $N=4$ female fish). Using the basic approach of Lindsey et al. (2012), at 23 h (for 48 h pairs) or 71 h (for 96 h pairs), the fish in a pair were lightly anaesthetized (see 'Experimental animals', above) and given a 10 mmol l^{-1} bolus of BrdU via intraperitoneal injection at a volume of $50 \mu\text{l g}^{-1}$. The pair was returned to its tank and allowed to recover for 1 h with the divider in place before removal of the divider for the final 24 h of the interaction period. At the end of the interaction period, fish were killed as above, and the brain was removed and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C (pH 7.4). All subsequent processing was also carried out at 4°C . Fixed brains were washed with PBS, immersed in 30% sucrose overnight and transferred to optimal cutting temperature (OCT; Thermo Fisher Scientific, Ottawa, ON, Canada) compound for 45 min. Brains embedded in OCT were cryosectioned (CM3050 S, Leica Biosystems, Concord, ON, Canada) at $20 \mu\text{m}$ intervals. Sections were collected onto Fisherbrand Superfrost Plus slides (Thermo Fisher Scientific), dried overnight at 4°C and stored at -20°C until immunohistochemistry was carried out.

For immunohistochemistry, slides were allowed to thaw at room temperature and sections were rehydrated in PBS ($2 \times 5 \text{ min}$). Antigen retrieval was carried out by immersing slides in 10 mmol l^{-1} sodium citrate containing 0.05% Tween (pH 6) at 65°C for 30 min. Slides were cooled to room temperature and were then exposed to 2 mol l^{-1} HCl at 37°C for 15 min. This reaction was quenched twice with 0.1 mol l^{-1} borate buffer (pH 8.5; Thermo Fisher Scientific) for 5 min each time. Sections were permeabilized using 0.05% Triton X-100 (Sigma-Aldrich) in PBS ($3 \times 5 \text{ min}$). To block non-specific binding, sections were exposed to 1% skimmed milk powder in PBS containing 0.05% Triton X-100 for 45 min at room temperature. Sections were incubated overnight at 4°C in a 1:100 dilution of mouse anti-BrdU antibody (Developmental Studies Hybridoma Bank) in 1% skimmed milk powder in PBS. This antibody was developed by S. J. Kaufman (University of Illinois) and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa (Department of Biology, Iowa City, IA, USA). Following incubation with the primary antibody, sections were washed with PBS ($3 \times 10 \text{ min}$) and then incubated for 3 h at room temperature with a 1:300 dilution of donkey anti-mouse IgG coupled to Alexa Fluor 488 (Invitrogen; Thermo Fisher Scientific). Sections were washed with PBS ($3 \times 5 \text{ min}$) and mounted using Vectashield antifade medium containing the nuclear marker 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA).

A Nikon A1RSiMP confocal microscope (Nikon Instruments, Melville, NY, USA) and NIS Elements Advanced Research Imaging Software (v4.20) were used to image brain sections. Every third section through the rostrocaudal axis of the telencephalon was examined, and telencephalic subregions were identified by comparing sections stained with Cresyl Violet (Sigma-Aldrich) with a neuroanatomical atlas of the zebrafish brain (Wullmann et al., 1996). Within each brain region, all cells exhibiting BrdU immunofluorescence (BrdU⁺ cells) were counted from a z-stack image created from $20 \mu\text{m}$ optical sections at $20 \times$ magnification. The number of BrdU⁺ cells was normalized to the total number of cells based on DAPI staining. Using the optical disector principle (West, 1999), DAPI⁺ cells were counted within a circle of $4000 \mu\text{m}^2$ that was positioned in a representative area of each brain region and kept in the same place for every section. Cell

counts were carried out by an observer who was blind to the treatment group to which the fish belonged.

Series 2: effect of social stress on neuron differentiation in the forebrain

To determine the fate of progenitor cells labelled with BrdU during social interactions, male zebrafish were established in pairs ($N=4$) and BrdU was administered, both as described above. At the end of a 48 h social interaction period, fish were returned to their original holding tanks for 2 weeks. This holding period was chosen because Lindsey et al. (2012) reported that neuronal differentiation peaked 2 weeks after BrdU treatment. Fish were killed with a lethal dose of MS-222 and brains were collected and processed as described above. In addition to immunofluorescence detection of BrdU, the neuronal nuclear protein NeuN was used as a marker of neuronal differentiation (Arslan-Ergul et al., 2016; Gusel'nikova and Korzhevskiy, 2015). Sections were incubated with a 1:1000 dilution of rabbit anti-NeuN (Abcam, Toronto, ON, Canada) followed by donkey anti-rabbit IgG coupled to Alexa Fluor 633 (1:700 dilution, Invitrogen). Sections were examined as described above, and BrdU⁺ cells as well as cells that exhibited co-localization of BrdU and NeuN were counted. The percentage of BrdU⁺ cells that also expressed NeuN was used as an index of neurogenesis.

Series 3: effect of inhibiting cortisol synthesis on forebrain cell proliferation during social stress

To establish a causal link between elevated cortisol and lowered forebrain cell proliferation in subordinate male zebrafish, cortisol synthesis was inhibited using metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; AdooQ Bioscience, Irvine, CA, USA), an inhibitor of the enzyme 11 β -hydroxylase that catalyses the conversion of 11-deoxycortisol to cortisol (Mommensen et al., 1999). Male zebrafish were randomly allocated to sham-treated and metyrapone-exposed groups that were held in static tanks (6 fish per tank). After an overnight acclimation period, the water in metyrapone-exposed tanks was replaced with $325.4 \mu\text{mol l}^{-1}$ metyrapone in system water (Vera-Chang et al., 2018). Water changes were carried out every 24 h and all tanks were covered to reduce degradation of metyrapone by light exposure. Pilot trials revealed that this metyrapone exposure abolished the cortisol response (Fig. S1) to a standardized netting stressor (Ramsay et al., 2009), and that at least 4 days of exposure were necessary to achieve full blockade. Therefore, after 4 days of exposure to sham or metyrapone treatment, pairs of length- and mass-matched fish drawn from separate exposure tanks were transferred into experimental chambers (1.5 l) containing system water with or without $325.4 \mu\text{mol l}^{-1}$ metyrapone, as appropriate, and with the members of the pair being separated by a divider for 24 h. The divider was then removed and the fish were allowed to interact for 48 h, with behavioural observations being carried out as described above. In one trial ($N=7$ sham-treated and $N=3$ metyrapone-exposed pairs), fish were killed after 48 h of interaction and blood samples collected for plasma cortisol assays. In a second trial ($N=4$ sham-treated and $N=3$ metyrapone-exposed pairs), BrdU was administered after 23 h of interaction and fish were killed after 48 h of interaction to quantify forebrain cell proliferation in subordinate fish, as described above.

Statistical analyses

Data are reported as mean values ± 1 s.e.m. Specific growth rate (SGR) was calculated as $[\ln(m_{\text{final}}) - \ln(m_{\text{initial}})] \times 100 / t$, where m is the mass of the fish in grams and t is time (the number of days that elapsed between measurements of mass). For each experimental series,

statistical analysis of plasma cortisol, total regional DAPI⁺ cell count and regional cell proliferation (BrdU⁺/DAPI⁺ cell count) or neuron differentiation (NeuN⁺/BrdU⁺ cell count) used similar approaches. Two-way repeated measures (RM) analysis of variance (ANOVA) was used to test for significant effects of interaction period (48 or 96 h) and social status (dominant or subordinate) in male zebrafish (Series 1). Paired Student's *t*-tests were used to test for significant effects of social status in female zebrafish (Series 1), and of neuron differentiation in male zebrafish (Series 2). These approaches were used to take into account the likelihood that variables measured for the members of a pair during social interaction were not independent of one another (Briffa and Elwood, 2010). Unless otherwise stated, total DAPI⁺ cell count did not differ between treatments. Where comparisons were made among group-held, dominant and subordinate zebrafish, a one-way ANOVA was used. Student's *t*-tests were used to make comparisons between sham-treated and metyrapone-exposed subordinate zebrafish (Series 3). Where underlying assumptions of normality and equal variance were not met, data were transformed to achieve normality or alternative tests were used. Statistical analyses were carried out using Sigmaplot v13 (Systat Software, San Jose, CA, USA) with $\alpha=0.05$.

RESULTS

Series 1: effect of social interaction on cortisol concentration and cell proliferation in the forebrain

Pairs of male zebrafish confined in an experimental chamber engaged in agonistic interactions that resulted in one fish becoming dominant over the other, as indicated by one fish reliably retreating from the aggressive attacks of the other. Mean scores over the interaction period for position in the tank, aggressive acts, retreats from aggression and feeding behaviour were combined by PCA into a behaviour score that distinguished dominant from subordinate fish (Fig. 1A). Dominant and subordinate male zebrafish also differed physiologically, with subordinate fish having significantly higher plasma cortisol concentrations (Fig. 1B; two-way RM ANOVA, $P_{\text{status}}=0.01$) and significantly lower SGR than dominant fish (Fig. 1C; paired Student's *t*-tests, $P<0.001$ for each of 48 and 96 h; two-way RM ANOVA was not used because data could not be transformed to meet the assumption of equal variance). Male zebrafish that engaged in social interaction for 96 h exhibited significantly higher plasma cortisol concentrations than those that experienced 48 h of interaction ($P_{\text{time}}=0.011$; $P_{\text{status}\times\text{time}}=0.401$). For comparison, plasma cortisol concentrations in fish sampled directly from the holding tank (group-held, dotted line in Fig. 1B) were 6.21 ± 1.55 ng ml⁻¹ ($N=7$).

Social status, but not interaction time, also had a significant impact on cell proliferation in most but not all regions of the forebrain (Figs 2 and 3). Using the number of BrdU⁺ cells as a measure of cell proliferation, subordinate male zebrafish exhibited significantly lower levels of cell proliferation than dominant fish in the dorsal telencephalic area (D) (Fig. 3A; two-way RM ANOVA, $P_{\text{status}}=0.001$), and the dorsal (Fig. 3B; $P_{\text{status}}=0.015$), lateral (Fig. 3C; $P_{\text{status}}=0.004$ for square root transformed data) and medial (Fig. 3D; $P_{\text{status}}=0.04$) zones of the D (Dd, Dl and Dm, respectively). The number of BrdU⁺ cells in the central and posterior zones was low in dominant and subordinate fish, and therefore not pursued. Cell proliferation did not differ between dominant and subordinate fish in the dorsal nucleus (Fig. 3E; $P_{\text{status}}=0.238$) or ventral nucleus (Fig. 3F; $P_{\text{status}}=0.091$) of the ventral telencephalic area (Vd and Vv, respectively). Neither the effect of interaction time ($P_{\text{time}}=0.615, 0.488, 0.283, 0.967, 0.221$ and 0.938 , Fig. 3A–F, respectively) nor the interaction of social status and interaction

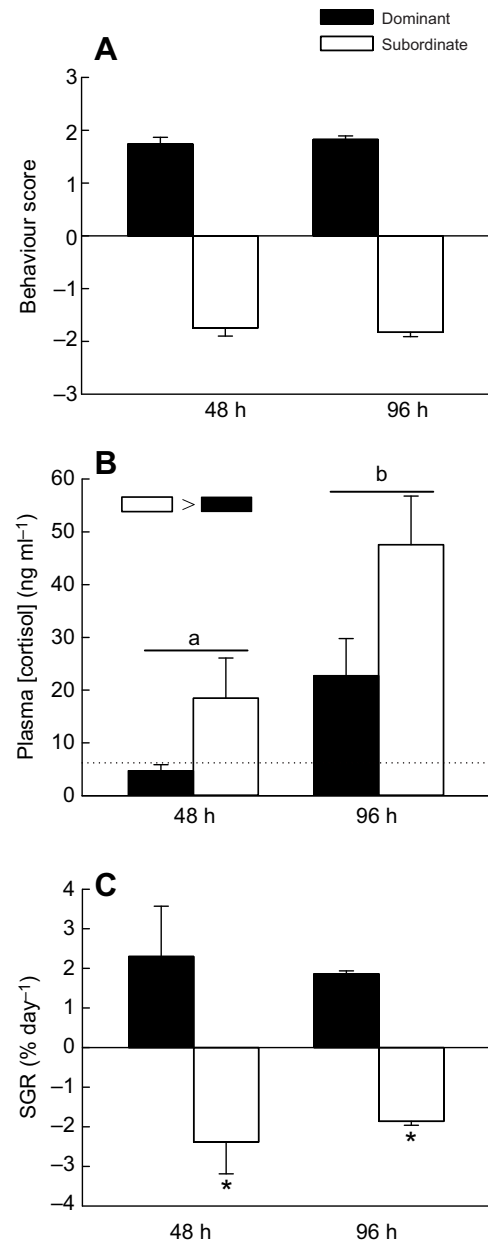


Fig. 1. Characterization of social rank in male zebrafish pairs.

(A) Behaviour score, (B) plasma cortisol concentration and (C) specific growth rate (SGR) for dominant and subordinate fish after 48 or 96 h of social interaction. Values are means \pm s.e.m., with $N=11$ – 12 pairs for behaviour scores and $N=7$ – 8 pairs for plasma cortisol concentration and SGR. The dotted line in B represents the mean cortisol concentration in group-held male zebrafish. In B, interaction times that share a letter are not significantly different from one another, and the effect of social status is indicated by symbols (two-way RM ANOVA, see Results for details). In C, an asterisk indicates a significant difference between dominant and subordinate fish (paired Student's *t*-tests, see Results for details).

time ($P_{\text{status}\times\text{time}}=0.585, 0.207, 0.833, 0.891, 0.309$ and 0.778 , Fig. 3A–F, respectively), was significant for any brain region. To provide a broader context for the effects of social interaction, values for dominant and subordinate fish were compared with those for group-held control fish (dotted lines in Fig. 3A–F). In general, levels of cell proliferation were comparable between dominant and group-held fish. However, in the Vv, both dominant and subordinate zebrafish exhibited significantly lower levels of cell proliferation than

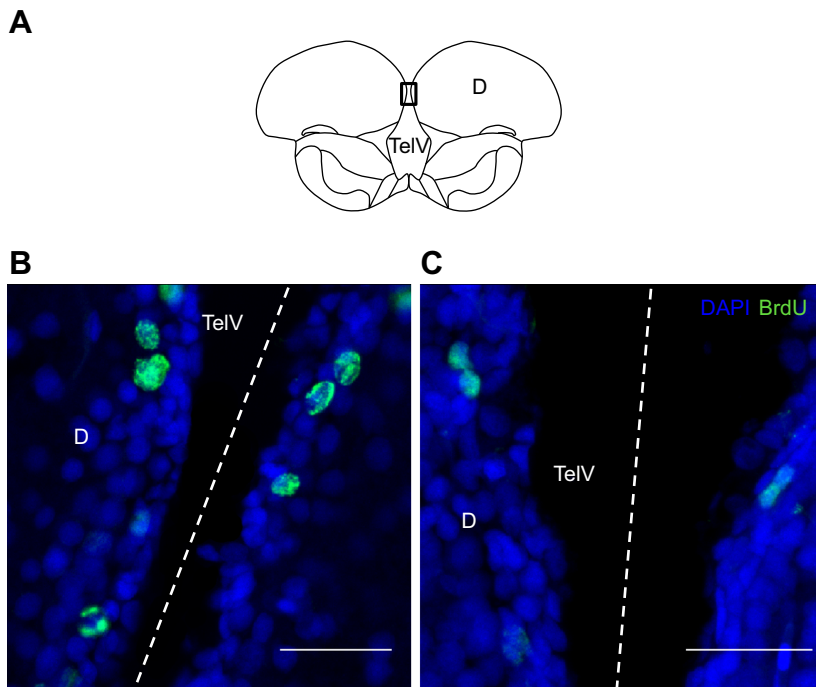


Fig. 2. Quantification of cell proliferation in the zebrafish telencephalon using immunohistochemical detection of BrdU-labelled cells (green). (A) Schematic drawing showing a cross-sectional view of the rostral telencephalon at Level 50 (adapted from Wullmann et al., 1996). The boxed region indicates the origin of the representative fluorescence micrographs for dominant (B) and subordinate (C) male zebrafish after 48 h of social interaction. D, dorsal telencephalic area; TelV, telencephalic ventricle. The dashed white line shows the midline. Scale bars: 25 μ m.

group-held fish (Table 1; one-way ANOVA, $P < 0.001$). Of note, DAPI⁺ cell number was used to normalize BrdU⁺ cell counts in each brain region under the assumption that social pairings did not impact the existing cell population. In general, this assumption held true, but two exceptions were identified: compared with dominant males, there were significantly fewer DAPI⁺ cells in the D of subordinate males at 48 h (two-way RM ANOVA, $P_{\text{interaction}} = 0.0210$), and in the Vv of subordinate males (two-way RM ANOVA, $P_{\text{status}} = 0.0230$); compared with group-housed control fish, DAPI⁺ cell counts were lower in subordinate but not dominant fish in the Vd and Vv (one-way ANOVA; $P = 0.009$ and $P = 0.007$, respectively).

Female zebrafish confined in pairs in an experimental chamber also engaged in aggressive interactions that led to the establishment of a social hierarchy in which dominant fish could be distinguished from subordinate fish on the basis of their behaviour (Fig. 4A). Subordinate female zebrafish exhibited significantly lower growth rates than dominant fish (Fig. 4B; paired Student's *t*-test, $P = 0.013$). However, neither plasma cortisol concentration (Fig. 4C; Student's *t*-test on \log_{10} -transformed data, $P = 0.504$) nor cell proliferation in the D (Fig. 4D; paired Student's *t*-test, $P = 0.702$) differed between

dominant and subordinate female zebrafish after 96 h of social interaction. The D was chosen for analysis in this experiment because this region had exhibited both relatively high rates of cell proliferation and strong effects of social stress in experiments on male zebrafish (Fig. 3A). Although levels of cell proliferation in this forebrain region were comparable between male and female zebrafish, the pronounced effect of social stress detected in male zebrafish (Fig. 3A) was absent in female zebrafish (Fig. 4D). Indeed, cell proliferation in the D of both dominant and subordinate female zebrafish was on a par with that of group-held female fish (dotted line in Fig. 4D). In the absence of an effect of social interaction on cell proliferation in female zebrafish, subsequent experiments focused exclusively on pairs of male fish, and utilized a 48 h interaction period because the duration of social interactions had not affected dominant–subordinate differences.

Series 2: effect of social stress on neuronal differentiation in the forebrain

The fate of progenitor cells in the D that labelled with BrdU during social interactions was assessed in male zebrafish that were confined in pairs for 48 h of social interaction and then returned to their original holding tanks for 2 weeks. Whereas $73.4 \pm 6.3\%$ ($N = 4$) of BrdU⁺ cells in the forebrain of dominant males also exhibited immunofluorescence for the neuronal marker NeuN, only $53.8 \pm 5.5\%$ ($N = 4$) of BrdU⁺ cells in the forebrain of subordinate males were NeuN⁺, a difference that did not quite reach statistical significance (paired Student's *t*-test, $P = 0.0596$; Fig. 5).

Series 3: effect of inhibiting cortisol synthesis on forebrain cell proliferation during social stress

Exposure of fish to waterborne metyrapone prior to and during a 48 h social interaction did not affect the establishment or nature of a social hierarchy between male fish pairs. The behaviour scores of dominant (1.7 ± 0.1 , $N = 6$) and subordinate (-1.6 ± 0.1 , $N = 6$) metyrapone-exposed fish did not differ significantly from those of sham-treated fish of the same status (dominant 1.8 ± 0.1 , $N = 11$; Student's *t*-test, $P = 0.333$; subordinate -1.9 ± 0.1 , $N = 11$; Student's

Table 1. Cell proliferation in forebrain regions of control, group-housed male zebrafish (*Danio rerio*)

Forebrain region	BrdU ⁺ cells normalized to DAPI ⁺ cell counts	<i>P</i>	Treatment groups significantly different from the control group
D	0.27 ± 0.01	0.007	48 h Sub, 96 h Sub
Dd	0.038 ± 0.022	0.128	
DI	0.060 ± 0.021	0.107	
Dm	0.078 ± 0.017	0.222	
Vd	0.12 ± 0.03	0.092	
Vv	0.25 ± 0.04	<0.001	48 h Dom, 48 h Sub, 96 h Dom, 96 h Sub

Cell proliferation was measured as the number of BrdU⁺ cells and normalized to counts of DAPI⁺ cells. Values are means \pm s.e.m., $N = 4$. For forebrain region definitions, see 'List of abbreviations'. Sub, subordinate; Dom, dominant. Data were analysed by one-way ANOVA, with *post hoc* comparisons carried out only against the control group. Bold indicates significance.

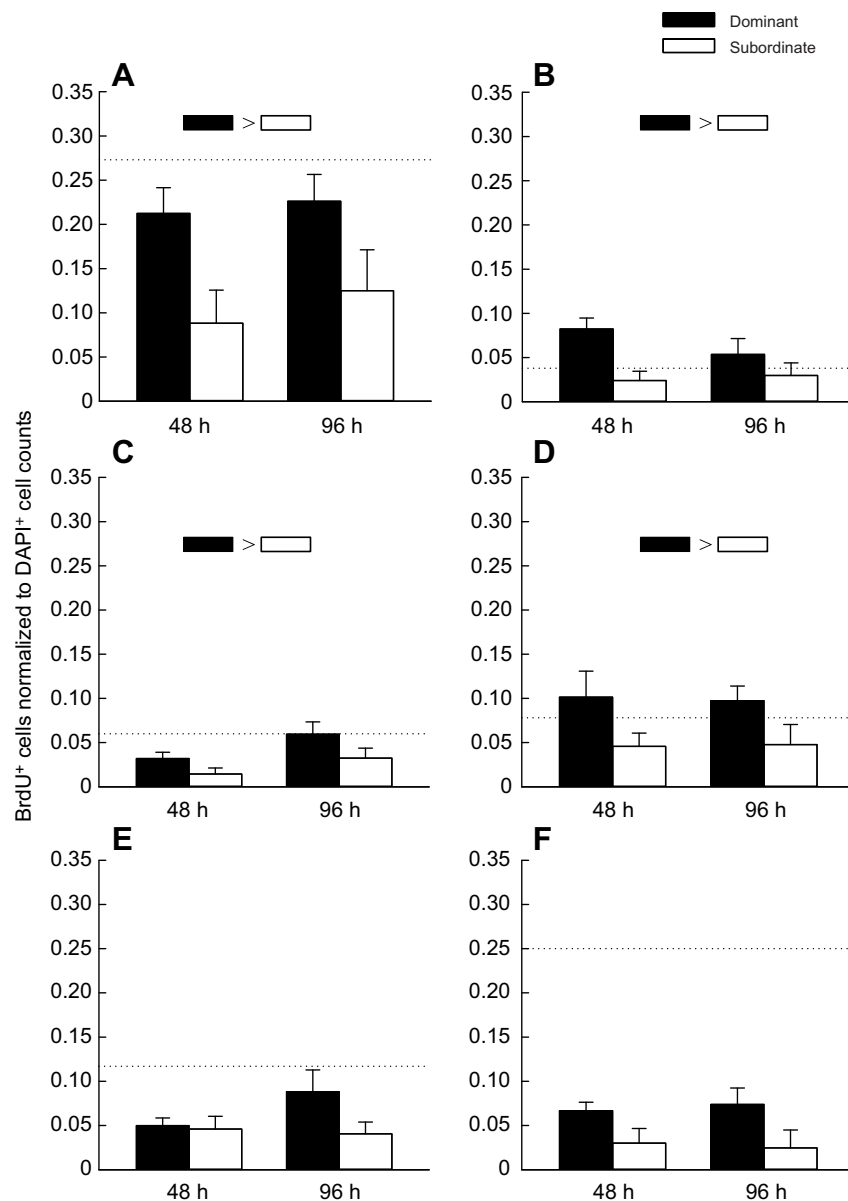


Fig. 3. Cell proliferation in forebrain regions of dominant and subordinate male zebrafish after 48 or 96 h of social interaction. Cell proliferation was measured as the number of BrdU⁺ cells and normalized to counts of DAPI⁺ cells in the D (A), Dd (B), Dl (C), Dm (D), Vd (E) and Vv (F). Values are means \pm s.e.m. for $N=4$ pairs. The dotted lines represent mean values for cell proliferation in group-held male zebrafish. A significant effect of social status is indicated by symbols, and no significant effects of interaction period were detected (two-way RM ANOVA, see Results for details). For forebrain region definitions, see 'List of abbreviations'.

t -test, $P=0.0804$). As expected, plasma cortisol concentrations in metyrapone-exposed subordinate fish were significantly lower than in sham-treated subordinates (Fig. 6A; Student's t -test, $P=0.0223$ for \log_{10} -transformed data). To provide context, the data from Series 1 for dominant and subordinate male zebrafish that had interacted for 48 h, and for group-held male fish, are indicated (lines) in Fig. 6. This comparison demonstrates that cortisol levels in metyrapone-exposed subordinates were similar to those in group-held and dominant zebrafish. Cell proliferation in the D of metyrapone-exposed subordinate fish was significantly higher than that of sham-treated subordinates (Fig. 6B; Student's t -test, $P=0.00362$), and was on a par with cell proliferation rates observed in group-held and dominant male zebrafish.

DISCUSSION

This study provides novel evidence to support a direct role for cortisol in regulating cell proliferation in the telencephalon of male adult zebrafish during the chronic stress of social subordination. Subordinate male zebrafish experienced elevated plasma cortisol and reduced incorporation of the thymidine analogue BrdU into

dorsal but not ventral zones of the telencephalon. Two lines of experimental evidence support a role for cortisol in driving this reduction in brain cell proliferation. First, inhibiting cortisol synthesis with metyrapone prevented a decrease in cell proliferation in the D of subordinate male fish. Second, subordinate female zebrafish did not increase plasma cortisol in response to aggressive interactions with a dominant female, and cell proliferation in the D was unaltered. Combined, these data establish a role for cortisol in mediating changes to brain cell proliferation during social subordination, possibly in a brain region- and sex-specific manner.

Social subordination and neurogenesis in male zebrafish

Pairs of male zebrafish rapidly formed a stable dominant/subordinate relationship, where the dominant fish was aggressive towards the subordinate fish and monopolized tank resources, including space and food. The distinctive behaviours of dominant versus subordinate fish are well described in zebrafish (Filby et al., 2010; Larson et al., 2006; Oliveira et al., 2011) and other teleost fishes (reviewed by Gilmour et al., 2005; Johnsson et al., 2006; Sørensen et al., 2013), and enable quantification of the interaction to

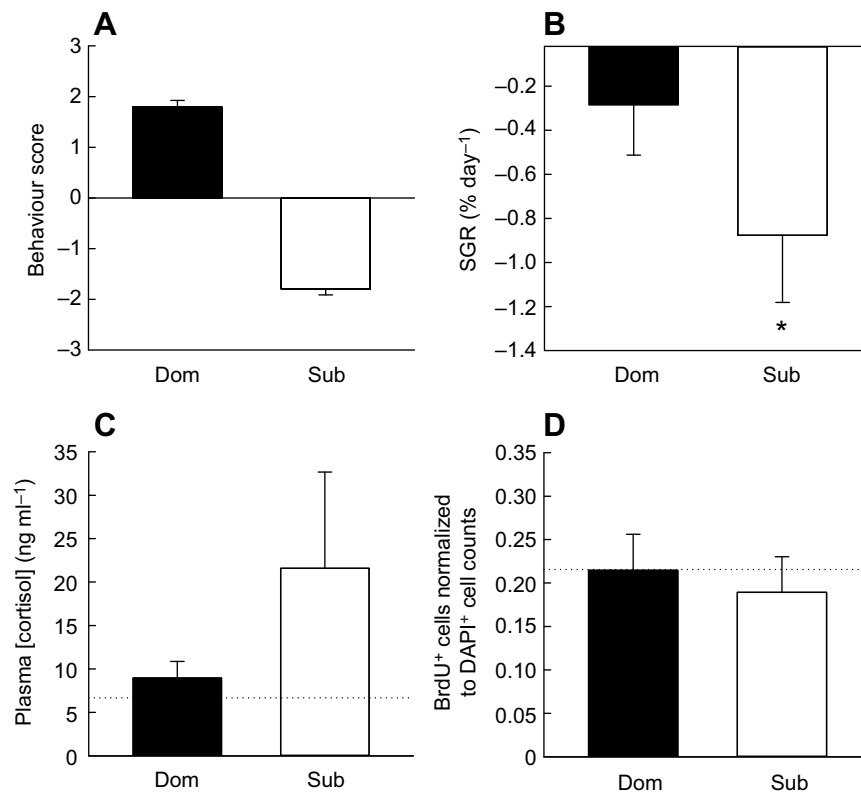


Fig. 4. Characterization of social rank in female zebrafish pairs. (A) Behaviour score, (B) SGR, (C) plasma cortisol concentration and (D) cell proliferation in the dorsal telencephalon for dominant (Dom) and subordinate (Sub) fish after 96 h of social interaction. Values are means \pm s.e.m., with $N=14$ pairs for behaviour scores and SGR, $N=8$ pairs for plasma cortisol and $N=4$ pairs for cell proliferation. The dotted lines in C and D represent mean values for group-held female zebrafish. An asterisk indicates a significant difference between dominant and subordinate fish (paired Student's t -tests or Student's t -test, see Results for details).

assign social rank. The behavioural inhibition typical of subordinate fish is frequently accompanied by physiological changes, with elevated plasma cortisol and reduced growth rate being most common (Gilmour et al., 2005; Johnsson et al., 2006; Sørensen et al., 2013). In zebrafish, reports of cortisol responses to social interaction have been mixed, probably reflecting differences in the social groups and in the measurement techniques that have been used (Filby et al., 2010; Pavlidis et al., 2011). Consistent with the significant elevation of plasma cortisol in subordinate males reported here, Filby et al. (2010) detected increased transcript abundance of corticotropin releasing factor, the neuropeptide that activates the endocrine stress axis, in male but not female

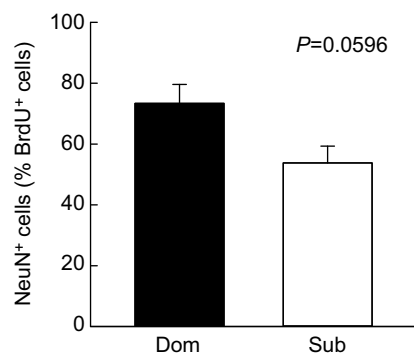


Fig. 5. Neurogenesis in the forebrain of dominant (Dom) and subordinate (Sub) male zebrafish after 48 h of social interaction. Neurogenesis was recorded 2 weeks after the period of social interaction to allow neuronal differentiation to occur (see Materials and Methods), and measured as the percentage of BrdU⁺ cells that also expressed NeuN (NeuN⁺). Values are means \pm s.e.m. for $N=4$ pairs. A paired Student's t -test revealed that the difference between dominant and subordinate fish did not reach statistical significance ($P=0.0596$).

subordinate zebrafish. Reduced growth was observed in both male and female subordinate zebrafish in the present study, and may reflect monopolization of food by dominant fish, forcing subordinate fish to rely on energy reserves. For example, reduced hepatosomatic index was reported in subordinate male zebrafish (Filby et al., 2010). In males, the catabolic effects of elevated cortisol may also contribute to lower growth, as may lower expression of growth regulators such as insulin-like growth factor-1 (Filby et al., 2010). Social status in zebrafish also has impacts on reproduction (Filby et al., 2010; Paull et al., 2010), and on neuromodulators such as vasotocin (Larson et al., 2006), serotonin and dopamine (Dahlbom et al., 2012; Teles et al., 2013).

The number of BrdU⁺ cells in male subordinate zebrafish was significantly lower in all quantified zones of the dorsal telencephalon (D, Dd, Dl, Dm) after 48 and 96 h of interaction with a dominant conspecific. Conversely, cell proliferation in the ventral telencephalic nuclei (Vd and Vv) was not significantly altered by social rank in male fish, indicating that social subordination induces region-specific changes in brain cell proliferation. Interestingly, these differences in BrdU⁺ cell number were highly consistent at both time points, suggesting that the underlying mechanisms at play during the first 23 h of social interaction (timing of earliest BrdU pulse) may be maintained but not increased through 71 h of interaction (timing of last BrdU pulse). However, it is important to note that in certain brain regions the total number of DAPI⁺ cells was also reduced in subordinate males (in the D at 48 h of interaction, and in the Vv at both interaction times). In the D, this indicates that the reduction in cell proliferation may be even greater than reported here, and leaves room for a differential effect of interaction time on cell proliferation. In the Vv, the confounding effects of subordination on the non-proliferating cell population in combination with the overall effects of the dyadic interaction itself on cell proliferation (i.e. relative to

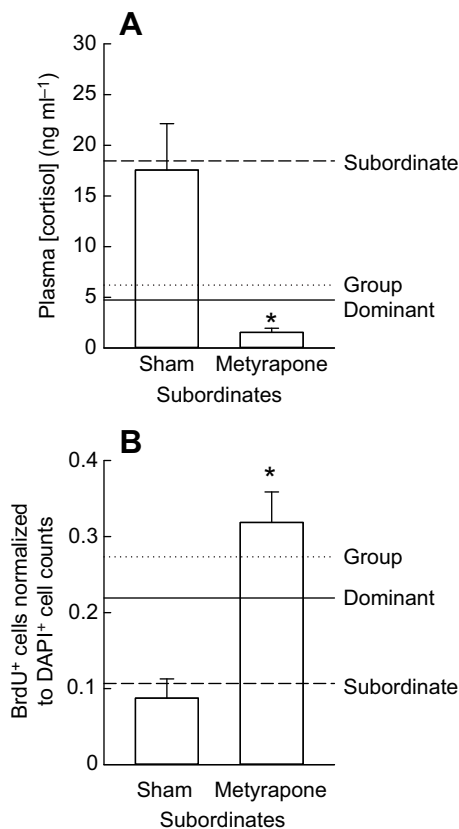


Fig. 6. Effects of metyrapone in subordinate male zebrafish. (A) Plasma cortisol concentration and (B) cell proliferation in the D for sham-treated and metyrapone-exposed fish after 48 h of social interaction. Values are means \pm s.e.m., with $N=3$ metyrapone-exposed fish in each panel, and $N=7$ or $N=4$ sham-treated fish for plasma cortisol and cell proliferation, respectively. An asterisk indicates a significant difference between treatment groups (Student's t -tests, see Results for details). For comparison, mean values for plasma cortisol (from Fig. 1B) and cell proliferation (from Fig. 3A) from male zebrafish in Series 1 experiments are included in A and B, respectively, with dotted lines for group-held males, solid lines for 48 h dominant males and dashed lines for 48 h subordinate males.

group-held fish) raise questions about the role of the Vv in governing social behaviours and the influence of social status on the heterogeneous cell population in this neurogenic niche.

The fate of newborn cells formed in the D during the social pairing was tracked for a 2 week chase period to determine the proportion BrdU⁺ cells that differentiated into neurons. During this time, dominant and subordinate fish were allowed to recover separately from each other and within their original tank of familiar conspecifics. The proportion of double-labelled cells (NeuN⁺ and BrdU⁺) was lower in subordinate relative to dominant fish at the end of the chase period. Although this result did not reach statistical significance, it suggests that neurogenesis may be reduced by social subordination in male zebrafish. It will be interesting to determine whether a decrease in neurogenesis caused by social subordination leads to long-term behavioural changes, and whether such changes represent an adaptive response to chronic stress.

These results add to a growing body of literature reporting changes in brain cell proliferation and neurogenesis in teleost fish under a variety of social and environmental contexts. For example, 96 h of social subordination in rainbow trout reduced the number of BrdU⁺ cells in the telencephalon by 40% relative to a dominant conspecific (Sørensen et al., 2012). Although sub-regions were not

discretely quantified, this response is of similar magnitude to that observed in the present study. Similarly, in a mixed-sex group of African cichlids (*Astatotilapia burtoni*), subordinate males had fewer BrdU⁺ cells than dominant males in several regions of the brain, including an ~60% reduction in the Vv (Maruska et al., 2012). In the present study, both dominant and subordinate male zebrafish showed reduced proliferation in the Vv relative to group-held fish, suggesting that social interactions rather than subordination per se may drive the proliferation response in this brain region. Zebrafish exposed to other forms of social perturbations, including social isolation and social novelty, also experienced niche-specific reductions in neurogenesis. These social contexts, however, only influenced neurogenesis in sensory structures of the brain, whereas telencephalic nuclei were largely unaffected (Lindsey and Tropepe, 2014). Clearly, the nature of the social environment has a considerable impact on how and where neurogenesis is altered, and such regional specificity implies a causal link between rates of mitotic activity and adaptive behavioural responses (Dunlap, 2016; Sørensen et al., 2013). For example, Maruska et al. (2012) showed that proliferation rapidly increased when subordinate male cichlids were presented with an opportunity to ascend in social rank, strongly supporting a functional relationship between brain cell proliferation and social status. Similarly, social novelty in brown ghost knifefish (*Apteronotus leptorhynchus*) enhances electro-communication, and coincides with increased cell addition in regions of the brain that control this behaviour (Dunlap and Chung, 2013).

Sex-specific responses to social subordination

The physiological response to social subordination differed between male and female zebrafish. In both male and female pairings, behavioural scoring clearly differentiated dominant from subordinate fish, with no obvious differences in the nature or extent of interactions (J.T., personal observation). Yet, despite repeated aggression from a dominant female, subordinate female zebrafish did not mount a significant cortisol response (present study; Jeffrey and Gilmour, 2016). This sex-specific cortisol response to social subordination presented a novel opportunity to tease apart the specific effects of cortisol and negative social interactions on brain cell proliferation. Although the entire ventricular surface of the telencephalon retains proliferating cells in the adult brain, the D contains the highest number of mitotic cells relative to the rest of the telencephalon (present study; Lindsey et al., 2012), and here the largest difference in BrdU⁺ cell numbers between social ranks in male zebrafish was observed. However, cell proliferation in the D of female fish, unlike that in subordinate males, was unchanged by 96 h of social subordination. It is unlikely that the differential response of the D to subordination in male versus female fish was a result of pre-existing sex differences in proliferation rates, because dominant male and dominant female fish had similar BrdU⁺ cell numbers to group-housed fish. Thus, social subordination only reduced D proliferation in male zebrafish. To the best of our knowledge, this study is the first to specifically address sex differences in neurogenesis in fish, because previous studies used juveniles (Johansen et al., 2012; Sørensen et al., 2012), males only (Dunlap et al., 2013; Maruska et al., 2012), or did not separate sexes during analysis (Lindsey and Tropepe, 2014). Although this study did not investigate sex specificity in other brain regions, or the functional significance of sex-specific reductions in D proliferation in response to social stress, it is reasonable to postulate a connection to behavioural and/or physiological correlates of reproduction, as is known in birds. For

example, in songbirds where only the male sings to attract a mate, the rate of neurogenesis in the region of the brain responsible for song learning (hyperstriatum ventralis pars caudalis; HVC) is far greater in males than in females, particularly during the breeding season (Guigueno et al., 2016a; Nordeen and Nordeen, 1988). Conversely, female brown-headed cowbirds (*Molothrus ater*) exhibit more complex use of space relative to males, and this higher requirement for spatial learning corresponds to an increased rate of hippocampal neurogenesis and larger hippocampus volume (Guigueno et al., 2016b).

Effect of cortisol on cell proliferation

Although the mechanisms underlying the effects of social interactions and stress on neurogenesis remain uncertain, cortisol is probably an important endocrine modulator in fish (Dunlap, 2016), as it is mammals (Schoenfeld and Gould, 2012). To date, however, studies that have linked cortisol to brain cell proliferation offer mixed results. The present study provides compelling new evidence to support a causal link between elevated cortisol and reduced cell proliferation in at least one subregion of the telencephalon during the chronic stress of social subordination. Proliferation was ~50% lower in the D of subordinate male fish but unchanged in subordinate female fish, and this difference corresponded to elevated plasma cortisol in male but not female subordinates. Further, cell proliferation rate in the D was recovered in metyrapone-treated male subordinates, where the cortisol response to social stress was eliminated by inhibiting cortisol synthesis. Importantly, metyrapone treatment was accomplished via waterborne exposure to both fish in a pair, and this treatment did not affect the nature or intensity of the social interactions that determined social rank, as indicated by behavioural scores that were consistent between metyrapone-exposed and sham-treated fish within a social rank. That is, even though metyrapone-treated subordinate fish experienced the full suite of negative encounters from the dominant conspecific and exhibited a typical subordinate behavioural phenotype, plasma cortisol was not elevated and proliferation in the D was not reduced. In line with these results, chronically elevated plasma cortisol in subordinate rainbow trout correlates with reduced forebrain BrdU⁺ cells (Sørensen et al., 2012) and reduced midbrain proliferating cell nuclear antigen (PCNA) expression (Johansen et al., 2012). Similarly, 6 days of a cortisol-laced diet yielded plasma cortisol concentrations similar to those of subordinate trout and a 50% reduction in PCNA⁺ cells in the telencephalon (Sørensen et al., 2011). However, isolation of rainbow trout increased PCNA expression throughout the midbrain despite high plasma cortisol levels in these fish (Johansen et al., 2012). In brown ghost knife fish, too, regionally specific increases in BrdU⁺ cell number were observed in the brains of fish held in pairs relative to isolated fish, and this response was replicated with a cortisol implant (Dunlap et al., 2006), and partially blocked by mifepristone, a glucocorticoid-receptor antagonist (Dunlap et al., 2011). Yet, in zebrafish, repeated cortisol injections for 3 days did not influence BrdU⁺ cell numbers across multiple neurogenic niches, including the D (Lindsey and Tropepe, 2014), and zebrafish held in enriched environments had higher cell proliferation in the telencephalon and higher cortisol levels than zebrafish held in barren environments (von Krogh et al., 2010). These apparent inconsistencies underline the fact that the effects of cortisol on the brain are not simple and linear, and may require co-occurrence of context-specific neuronal inputs. In zebrafish, then, we might conclude that for social subordination to reduce cell proliferation in the telencephalon, a fish must experience

chronically elevated cortisol in combination with the multiple sensory inputs activated by the social interaction itself. The next step will be to determine whether such stress-induced changes in brain cell addition contribute to the maintenance of an adaptive behavioural phenotype for social stress (Sørensen et al., 2013).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.L.A., K.M.G.; Methodology: J.T., S.L.A., K.M.G.; Validation: J.T., K.M.G.; Formal analysis: J.T., K.M.G.; Investigation: J.T.; Resources: S.L.A., K.M.G.; Data curation: J.T., K.M.G.; Writing - original draft: S.L.A.; Writing - review & editing: J.T., S.L.A., K.M.G.; Visualization: J.T., S.L.A., K.M.G.; Supervision: S.L.A., K.M.G.; Project administration: K.M.G.; Funding acquisition: K.M.G.

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Supplementary information

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