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Social Enhancement of Adult Neurogenesis in Zebrafish is Not Regulated by Cortisol

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Abstract—In Mammals adult neurogenesis is influenced by environmental conditions, and the glucocorticoid hormones (GC) play a major role in this regulation. In contrast in fish, the study of the effects of cortisol on the regulation of environmental driven adult neurogenesis has produced conflicting results. While in some species elevated cortisol levels impair cell proliferation, in others, it promotes cell proliferation and differentiation. This lack of consistency may be explained by methodological differences across studies, namely in the stimuli and/or cortisol treatments used. Here, we tested the effects of the social environment on adult neurogenesis, considering a positive and a negative social context, and different durations of cortisol exposure. We hypothesise that there is an interaction between the valence of the social environment and cortisol, such that elevated acute cortisol experienced during social interactions only have a detrimental effect on neurogenesis in negative social contexts. Therefore, fish were exposed to a positive (conspecific shoal) or negative (predator) social experience, and the interaction between the valence of the social context and cortisol exposure (acute and chronic) was tested. Our results indicate that adult neurogenesis is modulated by the social environment, with the number of newly generated cells being dependent on the valence of the social information (positive > negative). These effects were independent of cortisol, either for acute or chronic exposure, highlighting the social environment as a key factor in the modulation of cell proliferation in the adult zebrafish brain, and rejecting a role for cortisol in this modulation.

Key words: adult neurogenesis, cortisol, positive social environment, negative social information, zebrafish.

INTRODUCTION

Adult neurogenesis is known to be influenced to a large extent by environmental conditions. In mammals, while positive experiences such as physical activity, mating or enriched environments increase the production of new neurons, aversive events, such as social defeat, predator exposure, and stress have the opposite effect (Opendak and Gould, 2015). These environmentally modulated changes in adult neurogenesis are usually paralleled by changes in cognitive performance, such that increased neurogenesis leads to enhanced cognitive performance, whereas reduced neurogenesis compromises it, suggesting a functional significance to the production of new neuronal cells.

The association between environmental cues and cell cycling of brain cells is also observed in fishes. In species that form social hierarchies, dominant males express higher proliferation rates than subordinates, in specific brain nuclei (Maruska et al., 2012; Tea et al., 2018), and environmental enrichment also increases neurogenesis rates (von Krogh et al., 2010). In contrast, elevated predation pressure has been associated with a lower density of newborn cells (Dunlap et al., 2016).

The lack of social interactions also has a significant impact on brain structure and function. Rodents reared in social isolation showed a reduction in adult neurogenesis, altered cognitive performance, with a decrease in synaptic number and impaired myelinisation (Wongwitdecha and Marsden, 1996; Ibi et al., 2008; Liu et al., 2012). Social isolation in the electric fish *Apteronotus leptorhynchus*, reduces cell addition and radial glia density (vimentin immunoreactivity) in a regional specific manner (Dunlap et al., 2006), and zebrafish raised in social isolation (first 6 months) show proliferation deficits

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Abbreviations: DAPI, 4.6-diamidino-2-phenylindole dihydrochloride; Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; F, cortisol; GC, glucocorticoid hormones; PB, phosphate buffer; PBS, Phosphate-buffered saline; PCNA, proliferating cell nuclear antigen protein; POA, preoptic area; SDMN, social decision-making network; TBS, Tris-buffered saline; Vv, ventral nucleus of the ventral telencephalic area; Vs, supracommissural nucleus of the ventral telencephalic area.

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in specific sensory structures (i.e. periventricular grey zone and olfactory bulbs, Lindsey and Tropepe, 2014). Recent work in rainbow trout revealed for the first time in fish, the adaptive significance of the social environment by showing cognitive impairments in a learning task and reduced neuronal numbers in four weeks isolated fish (Ausas et al., 2019). Interestingly, no differences were found in proliferation rates between isolated and group living fish, a result previously found for the same species, in a different experiment where isolated fish did not differ in cell proliferation from winners of staged fights (Sørensen et al., 2012). Therefore, contrarily to mammals, the impact of social isolation and social stress in fish appears to be species-specific.

The stress-axis, and glucocorticoid hormones (GC) in particular, have been proposed as the major candidate mechanism underlying the environmental regulation of adult neurogenesis, since they are known to regulate several stages of neurogenesis during brain development, and in adult vertebrate brains (Gould and Tanapat, 1999). Stress and elevated GC levels are known to inhibit adult neurogenesis in a wide range of mammalian species (mice, rats, marmoset and macaques), and exogenous GC administration has similar effects on cell proliferation, differentiation, survival, as well as on oligodendrogenesis (Schoenfeld and Gould, 2012; Chetty et al., 2014). Effects of GC on neurogenesis have been described to follow an inverted U-shaped curve, with acute and low cortisol levels acting as stimulatory and adaptive, whereas severe and chronic exposures functioning as inhibitory and mal-adaptive (Sørensen et al., 2013; Saaltink and Vreugdenhil, 2014). However, this relationship is less clear in fish. While elevated cortisol impairs cell proliferation in some species (Sørensen et al., 2012; Tea et al., 2018) in others, it promotes cell proliferation and differentiation (Dunlap et al., 2006, 2011). This variability was recently suggested to be related to a differential expression of glucocorticoid receptors (Sadoul et al., 2018). Furthermore, for the same species, conflicting results can be found regarding the cortisol effects on adult neurogenesis (Lindsey and Tropepe, 2014; Tea et al., 2018). This lack of consistency may be explained by differences in the stimuli, stressors, and levels of cortisol treatments used in the different studies. Another, possible source for these conflicting results can be the interaction between stimulus valence and cortisol. For example, there are situations in which elevated GC levels are experienced in positive social contexts, such as the case of mating or environmental enrichment, and in these cases an increase in brain cell proliferation has been observed (von Krogh et al., 2010; Leuner et al., 2010). Thus, we hypothesize that the effects of cortisol on adult neurogenesis can be modulated by the valence of the social context in which they are experienced.

In order to test this hypothesis, here, we tested the effects of the social environment, considering a positive and a negative context, and cortisol (i.e. exposure to increased acute and chronic physiological levels) on adult neurogenesis in zebrafish. In this paper we restricted our analysis to the cell proliferation stage of adult neurogenesis using immunohistochemistry for the proliferating cell nuclear antigen protein (PCNA), which is the auxiliary protein of DNA polymerase δ, hence being essential for the replication of eukaryotic cells and commonly used as a proliferation marker in neurogenesis studies (e.g. zebrafish: Wullimann and Puelles, 1999). Our decision of not using commonly used thymidine analogues (e.g. bromodeoxyuridine, BrdU), that incorporate DNA of dividing cells and therefore allow to monitor not only cell proliferation but also other stages of neurogenesis (i.e. migration, short- and long-term survival, differentiation into neurons/glia cells, and functional integration into relevant neural circuits) aimed to avoid the handling stress associated with the required injections of these markers (Wojtowicz and Kee, 2006). Our hypothesis is that there is an interaction between the valence of the social environment (positive vs negative) and cortisol, such that elevated acute cortisol levels experienced during social interactions only have a detrimental effect on neurogenesis in negative, but not in positive, social contexts, and that chronic exposure heightens this effect. For this purpose, we first validated the effects of exogenous acute and chronic cortisol administration on circulating cortisol levels and we also validated the valence of a putative positive (conspecific shoal) and of a putative negative (predator) social context. Then, we tested the effects of exposure to a positive (conspecific shoal) vs negative (predator) social experience and its interaction with exposure to increase cortisol levels (both acute and chronic). Finally, in order to assess reference levels of brain cell proliferation experienced by individuals living in social groups under lab conditions, to which those induced by positive and negative social environment treatments could be compared, we have also measured cell proliferation and cortisol levels in group living and socially isolated fish. Five forebrain nuclei of the social decision-making network (SDMN), an evolutionarily conserved network known to respond to social stimuli (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011) and to process stimulus reward through dopaminergic signalling (Wickens et al., 2007) were used as our target tissue to assess the environmental and cortisol influences on cell proliferation.

EXPERIMENTAL PROCEDURES

Animals

Wild-type (AB) zebrafish adult males (approximately 4 months old) were used. They were bred at the Instituto Gulbenkian de Ciência and kept in mixed-sex groups in a recirculating system (ZebraTec, 93 Tecniplast) at 28 °C with a 14 L: 10 D photoperiod. We decided to focus this study only on males because of the putative effect of estradiol on adult neurogenesis that would require the hormonal monitoring of ovarian cycle in females, and sampling them accordingly, in order to avoid introducing another source of variation in the data. Subjects were fed twice a day, with *Artemia salina* in the morning and commercial food flakes in the afternoon.

This research project was ethically reviewed and approved by the ORBEA (Animal Welfare Body) of the Instituto Gulbenkian de Ciência, and by the Portuguese National Entity that regulates the use of laboratory animals (DGAV - Direção Geral de Alimentação e Veterinária). All experiments conducted on animals followed the Portuguese (Decreto-Lei no. 113/2013) and European (Directive 2010/63/EU) legislations, concerning housing, husbandry and animal welfare.

Validation of cortisol treatments

To test for the effects of acute and chronic cortisol exposures fish were immersed for 1 h ($n = 6$) or 5 days ($n = 7$), in a cortisol bath (hydrocortisone, 10 μg/ml, Sigma- Aldrich). Controls were immersed in a water bath of the same volume ($n = 6$). All animals were maintained in the setup for 5 days, to keep the three groups in the same experimental conditions, and the different cortisol exposure times applied accordingly. It has been shown previously that the cortisol concentration used (10 μg/ml) induces effects at the cellular level in adult fish (Sakamoto et al., 2001). To maintain a fresh supply of cortisol, 50% of the water volume was changed daily during the experimental period for the chronic cortisol treatment, and the same amount of water change was applied to the acute and control conditions.

Subjects were then lightly anaesthetized in tricaine solution (MS222, Pharmaq; 20–40 mg/L) and blood collected from the caudal peduncle (caudal aorta and inferior vena cava) with a heparinised pipette tip. Blood samples were kept in 1.5 ml tubes on ice until being centrifuged (10 min at 3000 rpm), the serum collected to a final volume of 110 μl of ELISA buffer (Cayman Chemical Company #500360) and the plasma was kept at − 20 °C until further processing. Cortisol (F) concentrations were quantified using a specific enzyme immunoassay kit (Cayman Chemical Company #500360) following the manufacturer's instructions. Samples were tested in duplicate, and the plasma used directly in the kit without extraction, since it has been previously demonstrated that there are no interferences of other putative immunoreactive substances with this kit in non-extracted plasma for cortisol quantification (Félix et al., 2013).

Validation of the valence of social stimuli

The positive and negative social stimuli were both composed by visual and chemical cues. The social positive stimulus consisted in a mixed-sex shoal of four males and four females (visual cue) paired with socially conditioned water (i.e. water from a stock tank = chemical cue). The social negative stimulus was a zebrafish natural predator (Indian leaf fish, *Nandus nandus*, visual cue) paired with alarm substance (chemical cue) collected from conspecifics according to (Speedie and Gerlai, 2008).

In order to validate stimulus valence, a setup with three tanks placed side by side was used. The focal fish was placed in the middle tank, and the side tanks were used for the stimuli fish (one with the positive or negative visual stimulus, and the other one kept empty). Chemical cues were always delivered to the focal fish tank using a tube connected to a syringe. For the control condition, both lateral tanks were kept empty and non-conditioned water delivered to the focal fish tank.

Three regions of interest (RoI) in the focal fish tank were defined: RoI 1 within 2.5 cm of the stimulus tank; RoI 2 within 2.5 cm of the empty tank; and a central neutral zone RoI 3 (with a length of 7.5 cm). Freezing behaviour was used as a measure of the fear response to the negative stimulus (Speedie and Gerlai, 2008). Time spent in the RoI_1 of the positive stimulus was used as a measure of preference for that stimulus. A total of 58 fish were tested ($n = 18$ for the positive stimulus, $n = 19$ for the negative stimulus, and $n = 21$ for the control condition).

Behavioural responses were recorded for 10 min before and 10 min after stimulus presentation, using video cameras (Alexa Eletro), and analyzed using a multi-event recorder software (Observer XT Noldus, *Wageningen, The Netherlands*). Fish that were immobile for >60% of the first 10 min were excluded from the analysis ($n = 9$), since this indicates anxiety behaviour prior to the test, which could influence their behavioral response.

Experimental paradigm for cortisol × social environment effects

The impact of cortisol (acute vs chronic exposures) and of the social environment (positive vs negative social stimuli) on adult neurogenesis, were tested using a two-by-two factorial design. A total of 34 fish were used for the acute condition (positive social stimulus: with cortisol $n = 9$ and without cortisol $n = 8$; negative social stimulus: with cortisol $n = 8$ and without cortisol $n = 9$) and a total of 30 fish for the chronic condition (positive social stimulus: with cortisol $n = 6$, without cortisol $n = 8$; negative social stimulus: with cortisol $n = 8$ and without cortisol $n = 8$).

Reference levels of socially induced brain cell proliferation and cortisol

Although, a positive response of cell proliferation to the social environment has already been described in zebrafish (Maruska et al., 2012; Tea et al., 2018), we used a group of fish to establish the proliferative baseline levels in socially isolated fish and group living fish. Neurogenesis baselines for both conditions were established for 12 subjects (6 subjects per treatment) and 16 subjects were used to measure plasma cortisol levels; $n = 7$ for social and $n = 9$ for isolated treatment. The measurement of the impact of social isolation on cell proliferation per se was particularly relevant, since in our experimental setup (see below) the focal fish has limited temporal access to the social stimuli (1 h for the acute condition, or 1 h/day for 5 days, for the chronic condition), hence experiencing social isolation to some degree.

Intracardiac perfusion and tissue preparation

After the experiment, the fish were deeply anaesthetized by immersion into tricaine solution (MS222, Pharmaq; 500–1000 mg/L) and intracardially perfused with a flush solution containing 0.1 M phosphate buffer (PB; pH 7.4), 0.9% NaCl, 1 ml/100 ml heparin solution (research grade, 5000 U.I; Braun), and 1 ml of 2% lidocaine (Sigma-Aldrich). After all blood had been washed out, the perfusion continued for 30 min with 2% paraformaldehyde (Sigma-Aldrich) in 0.1 M PB (Zupanc et al., 2005; Dunlap et al., 2021). The brains were removed from the skull, post-fixed in the same fixative solution for 1 h at 4 °C, and cryoprotected in 1 M sucrose in 0.1 M PB overnight at the same temperature. After embedding the brains in O.C.T. compound (Sakura), four series of 16 μm-thick coronal sections were cut in the cryostat.

PCNA immunohistochemistry and cell quantification

For detection of PCNA-labeled cells, the sections were washed with 0.1 M Tris-buffered saline (TBS; pH 7.5) and incubated in citrate buffer (pH = 6) for 20 min at 103 °C for antigen retrieval. After, the slides were cooled at room temperature (RT) for 30 min and rinsed three times in distilled water. Sections were then rinsed in TBS-T (Tween 0.1%) and blocked for 1 h in PBS (pH = 7.4) containing 1% bovine serum albumin (fraction V, nzytech) and 30% Tween (Sigma-Aldrich), (hereafter referred to as Blocking Solution) at RT. The sections were then incubated with rabbit anti-human PCNA (FL-261) primary antibody (Santa Cruz, Biotechnology, Cat. No. sc7907) diluted 1:50 in Blocking Solution overnight at 4 °C. The day after, the sections were washed three times for 10 min with PBS and incubated overnight at 4 °C with secondary antibody, Goat Alexa Fluor 568-conjugated anti-rabbit IgG (H + L) (Invitrogen; Cat. No. A11036) diluted 1:500 in Blocking Solution. Following three ten min washes in PBS, the sections were counterstained with 4.6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich; 1 mg/ml) for 20 min at RT, rinsed three times in PBS in the dark, and the slides embedded in fluorescence mounting medium (Dako, Cat. No. S3023).

Quantification of $PCNA^+$ cells was then performed in a fluorescence microscope (Leica DMRA2) using a 40× dry lens (0.75 numerical aperture). Target brain nuclei of the SDMN were identified for analysis, using DAPI counterstain and the zebrafish brain atlas (Wullimann et al., 1996): Dm, medial zone of the dorsal telencephalic area (putative homologue of the mammalian basolateral amygdala); Dl, lateral zone of the dorsal telencephalic area (putative homologue of the mammalian hippocampus); Vv, ventral nucleus of the ventral telencephalic area (putative homologue of the mammalian lateral septum); vs supracommissural nucleus of the ventral telencephalic area (putative homologue of the mammalian anterior part of the bed nucleus of the stria terminalis); and POA, preoptic area (putative homologue of the mammalian preoptic area plus paraventricular nucleus of the hypothalamus).

For quantitative analysis, only one series of alternate sections covering the telencephalon and anterior diencephalon, corresponding to one quarter of the whole brain, was analyzed. All the $PCNA^+$ cells were counted manually under the microscope for one hemisphere and the area of each nucleus measured (mm^2) using ImageJ software.

Statistical analysis

The effect of exogenous administration of cortisol on circulating cortisol levels was assessed using a one-way ANOVA, followed by planned comparisons, on log-transformed data to fulfil parametric assumptions. For the validation of the social stimuli (positive vs negative) Kruskal-Wallis tests were used, since the data did not meet parametric assumptions.

The effects of cortisol (acute vs chronic exposures), environment (positive vs negative), and brain nuclei (Dm, Dl, Vv, vs POA) in $PCNA^+$ cells, as well as the effect of social isolation on cell proliferation, were evaluated using linear mixed models with subjects as a random effect, followed by post-hoc tests (Tukey HSD). Because no effect of cortisol on cell proliferation was found, the groups of cortisol exposure and no cortisol exposure (F+ and F−), for the same environment, were pooled and the effect of the social environment analyzed independently of cortisol exposures. Linear mixed model analysis was performed to access the effects of the social environment (positive vs negative), and brain nuclei (Dm, Dl, Vv, vs POA) on $PCNA^+$ cells. Planned multiple comparisons were then used to evaluate the effect of the interaction of social environment and brain nuclei on cell proliferation within each brain nuclei. In order to meet parametric assumptions data were transformed [log(x) + 1].

Finally, to study the overall effects of the social environment on cell proliferation in the brain (i.e. for all brain nuclei together) a Kruskal-Wallis test, followed by a multiple comparison, where the mean rank of each group was compared with the mean rank of every other group was used.

Effect sizes (Cohen's d_s or d_z) for were also reported, and reference effect size values (small: $d > 0.2$, medium: $d > 0.5$, and large: $d > 0.8$) used to interpret the mean difference of the effect [11]. Parametric assumptions were confirmed by Shapiro-Wilk to test for normality, Bartlett test for homoscedasticity, combined with a visual inspection of plots of the residuals fitted values. Sample sizes varied either due to technical problems (i.e. problems with blood collection, intracardiac perfusion or immunocytochemistry) or to the removal of outlier values. Outliers were identified for each condition using the generalized extreme studentized deviate procedure with a $p = 0.05$ and a maximum number of outliers of 20% of sample size. Statistical analyses were performed in R (R CoreTeam, 2015) using the following packages: fBasics and psych (assumptions), nlme (linear mixed models), and multcomp (multiple comparisons) and Graphpad Prism software (version 8.0.1).

RESULTS

Validation of cortisol treatments

Circulating cortisol concentrations were significantly affected by exogenous cortisol administration ($F_{(2,16)}$ = 19.56, p < 0.0001). Planned comparisons analysis revealed that cortisol levels were significantly higher in the cortisol exposure group than in the control group (p < 0.05, d_s = 1.093), and were significantly higher in the chronic exposure group than acute or control groups (p < 0.01, d_s = 2.221; p < 0.0001, d_s = 2.562, respectively; Fig. 1A). Cortisol concentrations in the plasma induced by the exogenous treatments were within physiological levels previously reported in the literature (Félix et al., 2013; Teles et al., 2016).

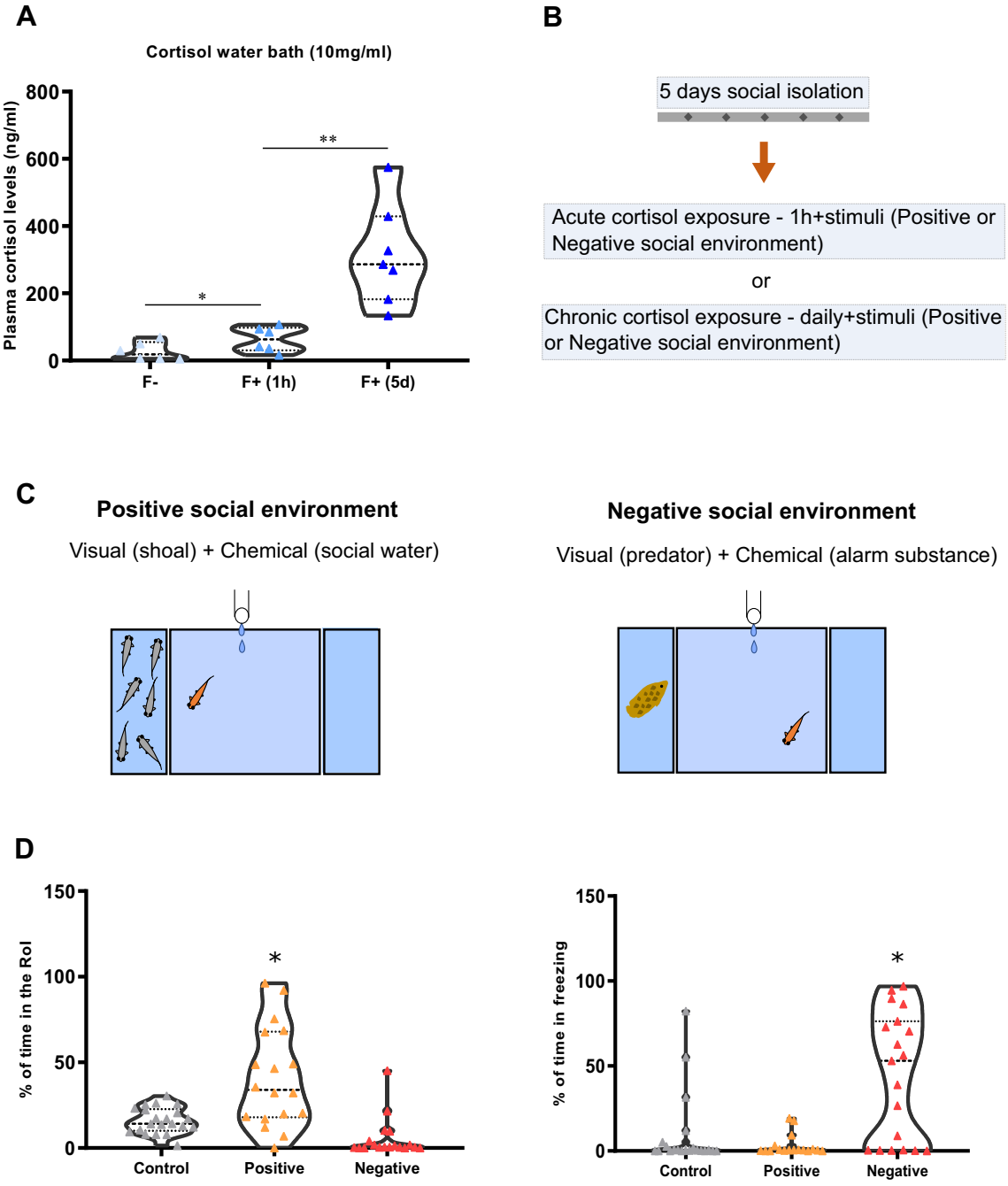

Fig. 1. Experimental setup and cortisol water bath and social stimuli validation. (**A**) Plasma cortisol (F) levels in fish with no cortisol (F−), 1 h of cortisol exposure [F + (1 h)] and 5 days of cortisol exposure [F + (5d)], (**B**) Experimental design, (**C**) Experimental setup for stimuli validation, positive social environment (shoal + socially conditioned water) and negative social environment (predator + alarm substance), respectively. (**D**) Validation of the valency of the social stimuli, percentage of time close to the positive stimulus and percentage of freezing in the presence of the negative stimulus. In the violin plots the dashed line in the middle represents the median, and the lines in the bottom and the top the 25th the 75th quartiles, respectively. Asterisks indicate significant differences: *p < 0.05.

Validation of the valence of social stimuli

Social stimulus valence, positive or negative, was assessed using appetitive and aversive behavioural responses, respectively (Fig. 1C). The results indicate overall differences for the time spent close to the positive stimulus X^2_{KW} (2) = 25.62, $p < 0.0001$, $N = 57$), and freezing behaviour for the negative stimulus, X^2_{KW} (2) = 10.96, $p < 0.01$, $N = 57$). Multiple comparisons analysis of the ranks revealed that the fish freeze more in the presence of the negative (predator) stimulus, compared to the positive (conspecific shoal, $p < 0.05$) or control (empty tank; $p < 0.05$) and spend more time close to the positive stimulus, compared to the negative ($p < 0.0001$) (Fig. 1D). There were no significant differences between the control and positive stimuli for either behaviours ($p = 1.00$, $p = 0.116$; Fig. 1D).

Effects of acute and chronic cortisol exposure and social environment valence on brain cell proliferation

For the acute treatment, we found a significant main effect of the social environment ($F_{(1,30)} = 8.24$, $p < 0.01$), with increased cell proliferation in the positive compared to the negative environment ($z = 3.04$, $p = 0.002$, $d_s = 0.581$). There was also a significant main effect of brain nuclei ($F_{(4,117)} = 7.84$, $p < 0.0001$), with higher PCNA$^+$ cell numbers in Dm and POA than in Dl, Vv, and vs (see Table 1 for detailed information). No main effects were detected for cortisol or the interaction between the two main factors.

Regarding the chronic treatment, a main effect was also found for the social environment ($F_{(1,26)} = 13.53$, $p < 0.01$), with individuals from the positive social environment presenting higher levels of adult neurogenesis than those of the negative one ($z = 3.73$, $p < 0.001$, $d_s = 0.959$). A main effect for brain nuclei was also found here ($F_{(4,100)} = 3.853$, $p < 0.05$), where Dm revealed significantly higher levels of PCNA$^+$ cells compared to Dl, vs POA, and a close to significance result to Vv (see Table 1 for detailed information). No main effect for cortisol or any of the interactions between the two main factors were found for the chronic treatment.

Since cortisol had no significant effects on neurogenesis, the groups corresponding to cortisol exposure and no cortisol exposure (F+ and F−), for the same social environment treatment were pooled, and the effects of the social environment on cell proliferation was subsequently analyzed in a new model, and planned comparisons performed (Table S1). For the acute treatment, planned comparison analysis within each nucleus, found a significant effect in vs ($z = 2.65$, $p < 0.01$, $d_s = 0.721$) and a close to significant effect in Dm ($z = 1.69$, $p = 0.009$, $d_s = 1.072$) (Fig. 2A). No other nuclei respond to the acute exposure. For the chronic exposure, planned comparison analysis found significant difference between positive and negative social environments in vs ($z = 3.66$, $p < 0.001$, $d_s = 0.828$) and a close to significant difference in POA ($z = 1.710$, $p = 0.08$, $d_s = 1.216$ (Fig. 2B) (for a detailed description on this model see Supplementary Table S1). We should note that the differences in the multiple comparisons results for the brain nuclei main effect between Table 1 and Table S1 result from the fact that data for the analysis reported in Table S1 needed to be transformed to meet parametric assumptions.

Brain cell proliferation in group living and sociality isolated fish

There are main effects of group living ($F_{(1,10)} = 8.40$, $p < 0.05$) and brain nuclei ($F_{(4,39)} = 2.75$, $p < 0.05$) on

Table 1. Effect of acute and chronic cortisol exposures on adult neurogenesis, interactions and multiple comparisons were calculated using linear mixed models

	Acute			**Chronic**		
	F	*p*-value		*F*	*p*-value	
Environment	**8.236**	**<0.01**		**13.531**	**<0.01**	
Cortisol	0.133	0.718		1.662	0.209	
Brain nuclei	**7.838**	**<0.0001**		**3.853**	**<0.05**	
Environment * Cortisol	0.172	0.681		1.649	0.21	
Environment * Brain nuclei	0.385	0.819		0.914	0.458	
Cortisol * Brian nuclei	1.12	0.35		1.081	0.37	
Environment * Cortisol * Brain nuclei	0.365	0.833		0.571	0.684	
Multiple comparisons (Brain nuclei)	***z*-value**	***p*-value**	***d*$_s$**	***z*-value**	***p*-value**	***d*$_s$**
Dm-Dl	**3.727**	**<0.001**	0.932	**3.178**	**<0.01**	0.722
Dm-Vv	**3.334**	**<0.01**	0.743	2.081	0.09	0.351
Dm-Vs	**4.25**	**<0.001**	0.883	**3.661**	**<0.01**	0.588
Dm-POA	0.197	0.844	0.031	**2.989**	**<0.01**	0.642
Dl-Vv	−0.365	0.795	0.0756	−1.084	0.464	0.198
Dl-Vs	0.549	0.729	0.1368	0.521	0.669	0.122
Dl-POA	**−3.562**	**<0.001**	0.71	−0.158	0.875	0.036
Vv-Vs	0.906	0.5213	0.181	1.588	0.224	0.244
Vv-POA	**−3.166**	**<0.01**	0.591	0.917	0.513	0.201
Vs-POA	**−4.08**	**<0.001**	0.721	−0.672	0.627	0.08

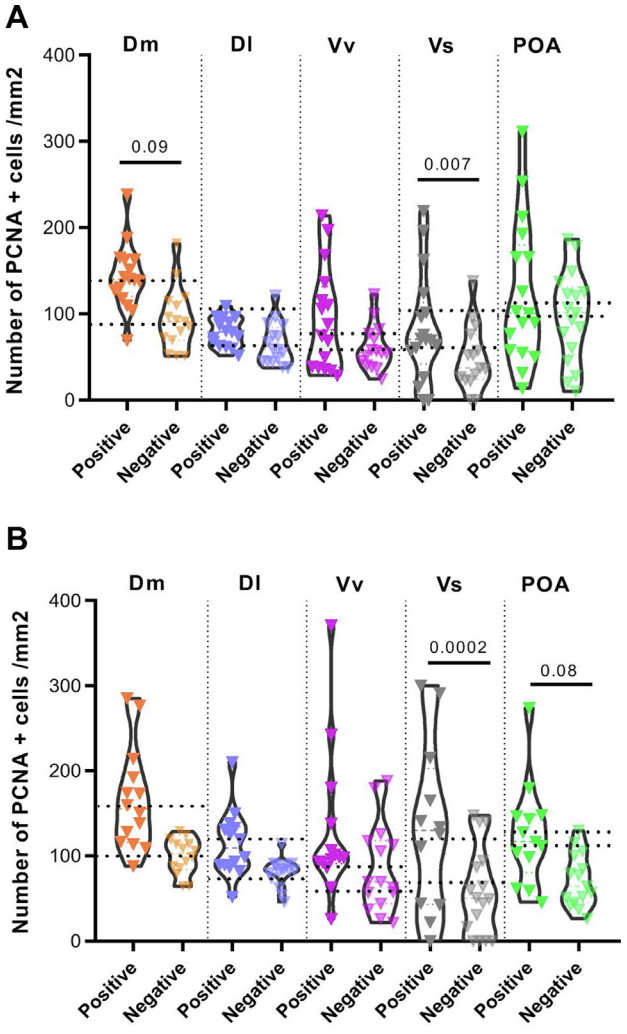

Fig. 2. Number of newly generated cells in the social decision-making network (SDMN) nuclei, in a socially positive and socially negative environment. (**A**) Acute environmental exposure, (**B**) Chronic environmental exposure. Dashed lines indicate the basal proliferation levels for group living (upper dashed line) and socially isolated fish (lower dashed line) for each nucleus. Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; vs supracommissural nucleus of the ventral telencephalic area; POA, preoptic area. In the violin plots the dashed line in the middle represents the median, and the lines in the bottom and the top the 25th the 75th quartiles, respectively.

Table 2. Effect of social isolation on adult neurogenesis, interactions and multiple comparisons using linear mixed models

	F	*p*-value	
Environment	**8.399**	**<0.05**	
Brain nuclei	**2.749**	**<0.05**	
Environment * Brain nuclei	0.555	0.697	
Multiple comparisons (Brain nuclei)	***z*-value**	***p*-value**	**d_s**
Dm-Dl	1.971	0.121	1.007
Dm-Vv	**3.099**	**0.019**	**1.507**
Dm-Vs	2.12	0.113	0.632
Dm-POA	0.573	0.629	0.199
Dl-Vv	1.052	0.409	0.531
Dl-Vs	0.096	0.923	0.01
Dl-POA	0.1413	0.263	0.539
Vv-Vs	−0.979	0.409	0.3
Vv-POA	**−2.527**	**0.057**	**0.914**
Vs-POA	−1.548	0.243	0.4

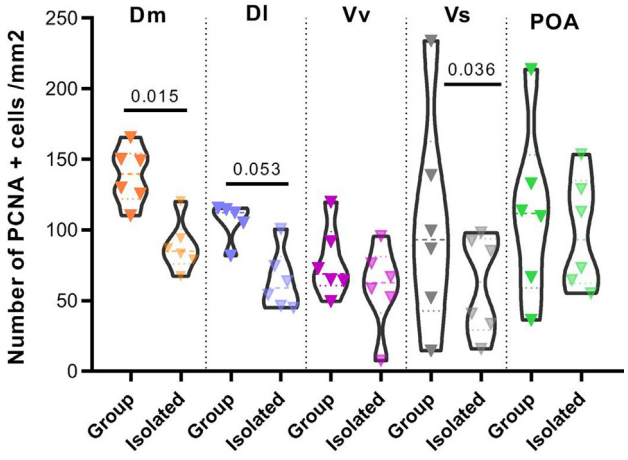

Fig. 3. Number of newly generated cells in group living and socially isolated fish in the SDMN nuclei. Dm, medial zone of the dorsal telencephalic area; Dl, lateral zone of the dorsal telencephalic area; Vv, ventral nucleus of the ventral telencephalic area; vs supracommissural nucleus of the ventral telencephalic area; POA, preoptic area. In the violin plots the dashed line in the middle represents the median, and the lines in the bottom and the top the 25th the 75th quartiles, respectively.

adult cell proliferation. A post-hoc analysis identified a negative effect of social isolation on adult neurogenesis, with a decrease in the proliferation rate compared to standard social group living conditions ($z = -3.17$, $p < 0.01$, $d_s = 0.819$). Regarding the brain nuclei, a significant difference was found in Dm compared with Vv ($z = 3.09$, $p < 0.05$, $d_s = 1.507$), and a close to significance difference was detected between POA and Vv ($z = 2.53$, $p < 0.05$, $d_s = 0.914$), with lower levels of newly generated cells in the Vv. All the other comparisons were non-significant (for detailed information see supplementary Table 2).

The effects of environment on cell proliferation within each nucleus, were analysed by planned comparisons (Fig. 3). The results indicate that different nuclei respond distinctively to social isolation: Dm and vs show a significant decreased on cell proliferation ($z = 2.42$, $p = 0.015$, $d_z = 2.630$, $z = 2.09$, $p = 0.036$, $d_z = 0.729$, respectively), whereas Dl present a close to significance difference ($z = 1.94$, $p = 0.052$, $d_z = 2.287$); Vv and POA did not show a significant response to social isolation ($z = 0.85$, $p = 0.39$, $d_z = 0.644$, $z = 0.68$, $p = 0.49$, $d_z = 0.274$) (Fig. 3). There was no difference in circulating levels of plasma cortisol between group living and socially isolated zebrafish ($F_{(1,14)} = 0.28$, $p = 0.61$).

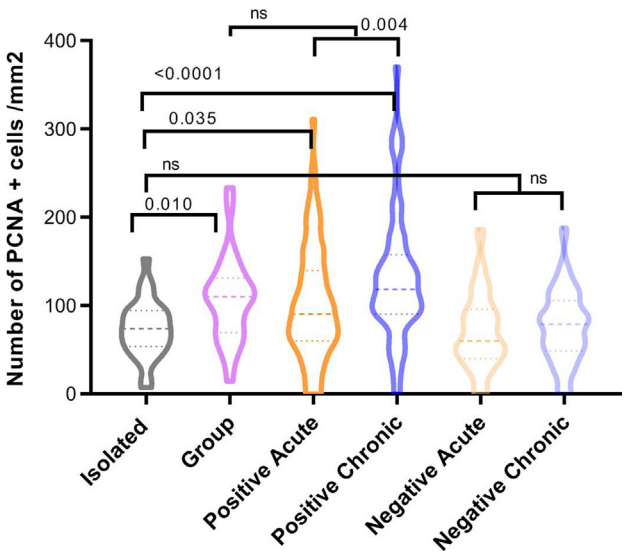

Fig. 4. Overall environmental effects on cell proliferation numbers in the different experimental conditions. Isolated, group living, acute positive environment, chronic positive environment, acute negative environment, and chronic negative environment. Error bars represent the standard error of the mean. P-values are indicated in the figure and "ns" indicates non significant comparisons. In the violin plots the dashed line in the middle represents the median, and the lines in the bottom and the top the 25th the 75th quartiles, respectively.

Comparison of the effects of positive and negative social valence to group living/ social isolation on brain cell proliferation

The impact of positive and negative social environment on brain cell proliferation was compared to reference levels of group living and socially isolated zebrafish (Fig. 4). For this purpose we compared the six groups described above (group living vs social isolated vs acute positive social environment vs chronic positive social environment vs acute negative social environment vs chronic negative social environment). Our results indicate overall differences between groups X^2_{KW} (6) = 52.50, $p < 0.0001$, $N = 372$). A multiple comparison post-hoc analysis revealed that fish who experience negative social environments (acute or chronic) did not differ in brain cell proliferation from those who were socially isolated ($p = 0.622$; $p = 0.845$) (Fig. 4). For cell proliferation in fish exposed to positive social environments, significant differences were observed between acute and chronic exposures ($p < 0.05$), but these two social valence treatments did not differ from the group living reference group ($p = 0.304$; $p = 0.272$) and are significantly higher than the social isolation group ($p < 0.05$ and $p < 0.0001$, respectively). The overall number of newborn cells was also significantly higher in the group living than in the socially isolated fish ($p < 0.05$) as previously shown in the nuclei specific analysis (Figs. 3 and 4).

DISCUSSION

The results of this study indicate that adult neurogenesis is modulated by the social environment, with the number of newly generated brain cells being dependent on the valence of the social information (positive > negative). When compared to reference groups, individuals exposed to negative social environments (i.e. predator), either acute or chronically, show similar levels of cell proliferation to those of socially isolated fish, whereas individuals exposed to positive social environments (conspecific shoal) show similar levels of cell proliferation to those of group living fish, with the latter showing higher cell proliferation than socially isolated ones. These effects of the social environment were independent of circulating cortisol levels, either for acute or chronic exposures, leading to a rejection of our initial prediction that cortisol would have only a detrimental effect on adult neurogenesis in negative social contexts, but not in positive ones. In the following sections, we will discuss first the absence of cortisol effects on cell proliferation in zebrafish, and then the specificity of the social environment regulation of adult neurogenesis.

Role of cortisol on zebrafish adult neurogenesis

A linear rule for the role of cortisol on adult neurogenesis is not consistently found in fish, with mixed results present in the literature. For example, in the context of social status in dominance hierarchies, subordinate individuals have been documented to have reduced brain cell proliferation (Maruska et al., 2012; Tea et al., 2018) which in some studies have also been shown to be paralleled by higher circulating cortisol levels (rainbow trout, *Oncorhynchus mykiss*, Sørensen et al., 2012; zebrafish: Tea et al., 2018). Moreover, in zebrafish treatment of subordinates with metyrapone, a cortisol synthesis inhibitor, attenuates the suppression of neurogenesis (Tea et al., 2018). In contrast, social isolation reduces cell proliferation independently of cortisol regulation in zebrafish (Lindsey and Tropepe, 2014). Moreover, in rainbow trout environmental enrichment increases cell proliferation despite also increasing circulating cortisol levels (von Krogh et al., 2010), and in electric fish both, cortisol administration and social interactions, when compared to social isolation, increase adult neurogenesis (Dunlap et al., 2006). Finally, in rainbow trout individuals of an artificially selected line that responds to stress with high cortisol levels have been described to have higher levels of adult neurogenesis, both before and after exposure to a long-term stressor, than those of an also selected low-response line (Johansen et al., 2012), but cortisol treatment in the absence of a stressor decreases cell proliferation (Sørensen et al., 2011).

It could be concluded from the data described above that cortisol only has detrimental effects on adult neurogenesis in the context of social stress associated with social defeat (e.g. Tea et al., 2018), and that increased levels of cortisol that parallel positive experiences (e.g. enriched physical environment or group living/ social interactions) have no such negative effects. To test this hypothesis, we have exposed zebrafish to both positive (exposure to conspecific shoal) and negative (predator) social environments, to test the prediction that elevated cortisol levels would have only detrimental effects in the negative social environment. However, despite the exposure to a stressor (predator) we found

no effect of cortisol of the detrimental effect of the negative social environment. Therefore, we have analysed other sources of variation that may explain these divergent results. A possible explanation for the differences found between our study and Tea et al. (2018) results, could be the plasma cortisol levels induced by the different social contexts used in the two studies. In our study, basal cortisol levels (i.e. no cortisol exposure) were ca. 28 ng/ml, and the acute and chronic exposures induced circulating levels of 63 ng/ml and 300 ng/ml, respectively, which are within the physiological range of response reported for this species (Félix et al., 2013; Teles et al., 2016). In the social status study (Tea et al., 2018), subordinates from chronic agonistic interaction (48 h of social stress), had circulating levels of cortisol of ca. 20 ng/ml, resembling our baseline levels. However, in that study the baseline was ca. 6 ng/ml, which could be related to the use of a different zebrafish strain. Thus, if we compare the fold change, in our study we have a 2.25-fold change from the baseline to the acute cortisol treatment, and a 10.7-fold change for the chronic treatment. Thus, the cortisol fold change experienced by subordinate fish in Tea et al. (2018) study (4×) is well within the cortisol fold-changes used in this study (2–10×). However, a major difference between the two studies was the duration of the stressor, and despite the scope of response being equal, the duration of the exposure was different, which may have led to a differential activation of the mineralocorticoids (MR) and glucocorticoid receptors (GR). Thus, the dynamics of cortisol signalling can play a major role in the detrimental effects of cortisol on neurogenesis in face of negative social stimuli, and future studies should address the temporal and dose response effects in more detail. As for high levels of cortisol in the presence of positive stimuli, our results confirm that they do not seem to have a detrimental effect. In this respect, it is interesting to note that *in vitro* experiments in human cells targeting GR and MR show a non-linear dose–response, with low and high cortisol levels inducing an MR-dependent increase and a GR-dependent decrease in cell proliferation, respectively (Anacker et al., 2013).

Future studies should address the mechanisms through which positive environmental stimuli (enriched environment/social interactions) buffer adult neurogenesis against the detrimental effects of cortisol. One potential candidate for such role is oxytocin, which has been shown in rats to stimulate adult neurogenesis even when given exogenous GC or exposed to a stressor, hence suggesting that oxytocin buffers adult neurogenesis against the suppressive effects of GCs (Leuner et al., 2012; Sánchez-Vidaña et al., 2016). At the level of molecular mechanisms, both morphogen signalling pathways and neurotrophic factors have been shown to play a major role in the cell cycle regulation of neural stem and progenitor cells (Egeland et al., 2015). The morphogens sonic hedgehog (SHH) and wingless (WNT) are key regulators of early stages of progenitor proliferation and differentiation, hence playing a critical role for the maintenance of the neurogenic pool, and both pathways have been implicated in stress-induced changes in adult neurogenesis (Matrisciano et al., 2011; Egeland et al., 2015). Neurotrophic factors, such as brain derived neurotrophic factor (BDNF), also play a key role in the differentiation and survival of developing neurons, and also respond to environmental factors, hence subsequently regulating adult neurogenesis by both direct and indirect pathways (Waterhouse et al., 2012; Gray et al., 2013; Egeland et al., 2015). Therefore, environmentally-driven glucocorticoid or oxytocin effects on adult neurogenesis, most probably will be mediated by the regulation of these molecular mechanisms. In this respect it is important to note that GRs, which are expressed in progenitor cells as well as in late stages of neuronal differentiation, crosstalk with BDNF signalling, which may account for their roles in neurogenesis regulation (Jeanneteau et al., 2008; Egeland et al., 2015). In contrast, the enhancing effects of oxytocin on adult neurogenesis in mice, are non-cell autonomous since oxytocin receptors are not expressed in neural progenitor cells (Lin et al., 2017), and oxytocin has been shown to crosstalk with BDNF signalling to facilitate its actions through receptor transactivation (Mitre et al., 2022).

The impact of social isolation on adult neurogenesis

Given that in our experimental design, both the control and treatment fish were isolated for 5 days for the cortisol treatments, we also sampled group living animals to serve as reference. We found that social isolation had a detrimental effect in the number of cycling cells in specific nuclei of the SDMN, namely Dm, vs and Dl. Based on gene expression analysis, Dm is considered the fish homolog of the mammalian basolateral amygdala (BLA), which together with other pallial-subpallial regions forms the amygdaloide complex in teleost fish, that mediates associative and emotional learning (Porter and Mueller, 2020). Studies in zebrafish on the expression of the cannabinoid receptor 1 (Cb1) in the Dm supports this comparability. The Cb1 location in the telencephalic periventricular matrix, is also suggestive of its involvement in neurogenesis (Lam et al., 2006). Other studies provide functional support for Dm to be involved in decision making, reward, fear learning and the endocannabinoid signaling critical for its function (Lau et al., 2011; Trotha et al., 2014; Ruhl et al., 2017). The supracommissural (Vs) and postcommissural (Vp) nuclei of medial amygdala (MeA) are considered the homologues of the anterior and posterior bed nucleus of the stria terminalis (BSTa/BSTpd) (Porter and Mueller, 2020), and these regions are activated in response to stressful stimuli and also emotional responses (Davern and Head, 2011). Finally, the other responsive nucleus, Dl, is the fish homologue of the hippocampus in tetrapods (Portavella et al., 2002; Ganz et al., 2014).

In prairie voles (*Microtus ochrogaster*), it has been shown that long-term social isolation decreases adult neurogenesis levels and altered cell death in the hippocampus, and the amygdala (Lieberwirth et al., 2012). Four weeks of social isolation in rainbow trout, also decreased by 40% neuronal differentiation in Dm. However, both the proliferation and survival of neuronal progenitors were unchanged (Ausas et al., 2019). Our data suggests, an involvement of these same nuclei (hip-

pocampus and amygdala) in response to the social isolation stress in zebrafish. Interestingly, after an acute agonistic encounter, which is another type of social stress, winners and losers regulate distinctively the gene expression of the neurogenic genes *wnt3* and *neurod* in Dm and Vv (Teles et al., 2016), suggesting a central role of the amygdala in the regulation of the cell cycle response to the social environment. Surprisingly, developmental social isolation (6 months) targeted mostly sensory niches (PGZ and OB), instead of higher processing order centres, and no differences were found within any of this telencephalic areas for cell proliferation (Lindsey and Tropepe, 2014). The differences between these studies are most probably related with the period and duration of sensory deprivation.

Neurogenesis modulation by the social environment

In baseline conditions (5 days of social isolation), positive or negative social stimuli were presented to the fish, with different exposure durations (acute or chronic). The negative stimuli did not impact cell numbers in most of the analysed nuclei either for acute or chronic conditions, since the neurogenic levels were on the same threshold imposed by social isolation (lower dashed line Fig. 2) on the nuclei that respond to isolation (Dm, Dl and Vs). There was no change in Vv in response to the social environment, and the POA followed the same pattern for the acute exposure. However, in the chronic condition, the negative environment prompted to a reduction in the number of cycling cells. The POA involvement in the modulation of adult neurogenesis was also observed in prairie voles in chronic social isolation (Lieberwirth et al., 2012). Given the important role that POA plays in complex social behaviour it is very interesting that in our study this nucleus only responds to negative chronic stimuli (with a reduction in cell proliferation), and did not respond to the positive social context. In zebrafish, POA is among the most active regions during social reward stimuli (Kelly, 2019; Nunes et al., 2020), and a recent report has shown that POA has the greatest percentage of newly generated neurons responding to a brief exposure to a social rewarding stimulus (Dunlap et al., 2021). Nevertheless, in this study changes in proliferation rate only occurred in response to the social negative context. Together, these might indicate that changes in cell proliferation are not directly connected with the functional neuronal response. Further studies are needed to unravel the contribution of proliferation, migration and the function of newly born neurons to the functional activity of the SDMN.

For the rewarding stimulus, we found that acute (1 h) rewarding environment was enough to rescue cell numbers to levels found in the control conditions (Fig. 2, upper dashed line). To our knowledge, these results demonstrate for the first time that a short-term exposure to a positive social environment is sufficient to revert the negative effect imposed by a negative social situation, and these effects are not affected by cortisol. Thus, the mechanism underlying these changes are related to the emotional valency of the stimulus, and processed by the nuclei of the amygdaloid complex, at least at the initial stages of adult neurogenesis.

Positive chronic environment, also increase cell proliferation in vs indicating the central role of this teleost homologue of the bed nucleus of the stria terminalis on the emotional evaluation of the environment, since it was implicated in the reduction and also in the increase of the cell numbers (Figs. 2 and 3).

By pooling all the brain areas to investigate the overall environmental effect, we can conclude that a single exposure (1 h) to a socially rewarding stimulus (Fig. 4: Positive acute) was sufficient to rescue the deficit on cell proliferation caused by social isolation to numbers found in group-living fish. This recovery effect appears to be cumulative, as the number of newborn cells increases with increased exposures (Fig. 4: Positive chronic). We also found that, in general, our aversive stimuli did not surpass the negative effect of social isolation, either for acute or chronic exposures, since it did not differ from the isolated control indicating a possible floor effect of social isolation (Fig. 4). This result was surprising given the previously described robust neurobehavioural response induced by the alarm substance (Faustino et al., 2017), which was used here as part of the aversive stimulus. When a fish receives this social signal, there is an increase in neuronal activity in Dm, Dl and POA (Faustino et al., 2017), and on the other hand, Dm, Dl and vs were the nuclei responding to social isolation. So, the same areas are shared in the response to different social stressors, probably given lower or null scope of response in these nuclei.

In conclusion, this study demonstrates that changes in socially-driven adult neurogenesis do not respond to changes in cortisol levels and are independent of its concentration (low, high) and duration (acute, chronic). On the other hand, the valence of the social environment proved to have a high impact, since a single acute exposure to a social positive context was enough to rescue cell proliferation levels, and a chronic exposure to increase even further cell proliferation cell numbers. Hence, the social environment is key factor in the modulation of cell proliferation in the adult zebrafish brain.

CONTRIBUTIONS

RFO, MCT and FF design the study; FF and MCT conducted the experiments; FF, MCT, CC, AC, and ME collected and analysed data; MCT, FF, and RFO wrote the first version of the paper; all authors revised the paper and contributed to its final version.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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ETHICAL STATEMENT

This research project was ethically reviewed and approved by the ORBEA (Animal Welfare Body) of the Instituto Gulbenkian de Ciência, and by the Portuguese National Entity that regulates the use of laboratory animals (DGAV – Direção Geral de Alimentação e Veterinária). All experiments conducted on animals followed the Portuguese (Decreto-Lei no. 113/2013) and European (Directive 2010/63/EU) legislations, concerning housing, husbandry and animal welfare.

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APPENDIX A. SUPPLEMENTARY MATERIAL

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