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SHORT COMMUNICATION

Social stimuli increase activity of adult-born cells in the telencephalon of zebrafish (*Danio rerio*)

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ABSTRACT

Fish have particularly high levels of adult neurogenesis, and this high neurogenic capacity may contribute to behavioural plasticity. While it is known that adult-born cells can differentiate into neurons and incorporate into neural circuits, it is unclear whether they are responsive to external stimuli and are thereby capable of contributing to behavioural change. We tested whether cells born in the telencephalon of adult zebrafish are activated by social stimuli. We marked cell birth with BrdU and, 40 days later, exposed fish to brief (15 min) visual social stimuli and assayed cellular activity through immunolocalization of phospho-S6-ribosomal protein (pS6). $BrdU^+/pS6^+$ co-labelled cells were found in six brain regions, and, in four regions [dorsal (D), dorsomedial (Dm) and dorsolateral (Dl) zones of the dorsal telencephalon and pre-optic area (POA)], the number of co-labelled cells and fraction of $BrdU^+$ cells that labelled positive for pS6 increased during social stimulation. These results are consistent with the hypothesis that adult-born neurons play a role in regulating social behaviour.

KEY WORDS: Social, Adult neurogenesis, Zebrafish, Telencephalon, pS6, BrdU

INTRODUCTION

Compared with adult birds and mammals, adult teleost fish add new cells at especially high rates to many brain regions (Zupanc and Horschke, 1995; Ganz and Brand, 2016, Augusto-Oliveira et al., 2019). Part of this abundant and widespread brain cell proliferation in adulthood probably relates to their pattern of growth. Most fish species grow continuously, and high rates or cell proliferation in adulthood combined with low rates of cell death contribute to the lifetime growth of their brains (Zupanc, 2011; Traniello et al., 2014; Dunlap et al., 2019; Labusch et al., 2020). However, researchers have also proposed that this high degree of cell addition may confer greater structural plasticity in the adult brain, which in turn, contributes to behavioural flexibility (Dunlap, 2016; Øverli and Sørensen, 2016; Ausas et al., 2019; Narita et al., 2018; Labusch et al., 2020).

It is clear from many studies that the cell proliferation rate in the adult brain of fish is influenced by diverse environmental stimuli (Dunlap et al., 2006; von Krogh et al., 2010; Lindsey and Tropepe, 2014; Dunlap, 2016; Tea et al., 2019). Most adult-born cells differentiate into neurons (Zupanc et al., 2005; Dunlap, et al., 2013), and some adult-born neurons show long-term persistence in the brain (Hinsch and Zupanc, 2007) and incorporate into neural circuits (Rothenaigner et al., 2011). However, it is still unclear whether neurons that differentiate in adulthood respond to sensory stimuli and could thus participate in behavioural outputs. In the present study, we address two specific questions. (1) Do adult-born cells in the teleost telencephalon show signs of activity? And (2) are these cells responsive to social stimuli from a conspecific?

One technique used to address similar questions in mammals has been to assay markers of cell activity, for example, immediate early genes (*cfos*, *egr-1*), to see if adult-born cells express these markers in response to sensory stimuli (Huang and Bittman, 2002; Carlén et al., 2002; Jessberger and Kempermann, 2003; Clark et al., 2011; De Miguel et al., 2019; Kedrov et al., 2019). Although many studies in fish have used such molecular markers to map patterns of neuronal activity during behaviour (Teles et al., 2015, 2016; Cabrera-Álvarez et al., 2017; Fischer et al., 2018; Kent and Bell, 2018), none has addressed whether adult-born cells express these markers and if their pattern of expression changes during behaviour.

Using zebrafish, we examined the expression of a neuronal activity marker, phospho S6 ribosomal protein (pS6), in 40-day-old cells of the forebrain immediately after the fish were exposed acutely to a conspecific fish. Electrical activity in neurons causes phosphorylation of the S6 ribosomal protein, and diverse sensory and physiological stimuli increase pS6 expression in behaviourally relevant brain regions (Knight et al., 2012; Biever et al., 2015; Kelly, 2019). We found that a small fraction (~7%) of newborn cells expressed pS6 when fish were socially unexposed, but this proportion of cells more than doubled (~15–30%) in several telencephalic regions when fish received social visual stimuli. The number of active newborn cells is especially high in the pre-optic area (POA), a brain region known to play a particularly active role in social behaviour. Thus, our data are consistent with the hypothesis that adult-born cells contribute to the regulation of social behaviour in fish.

MATERIALS AND METHODS

Animals and behavioural protocol

Ten adult zebrafish, *Danio rerio* (TL background; 0.22–0.28 g, age ~120 days) were housed in a mixed sex group in an 8 litre aquarium at 28°C under 12 h:12 h light:dark conditions. This study complied with the relevant regulations for the care and use of animals in research and was approved by the competent Portuguese authority (Direcção Geral de Alimentação e Veterinária).

The time course of the experiment is illustrated in Fig. 1. After acclimating to group housing conditions for 1 week, fish were lightly anesthetized by immersion in 3-aminobenzoate methanesulfonate (MS222; 100–200 mg l^{-1}), and injected intraperitoneally with labelling reagent [aqueous solution of 5-bromo-2′- deoxyuridine (BrdU; 3 mg ml^{-1}) and 5-Xuoro-2′-deoxyuri- dine (0.3 mg ml^{-1}), supplied with the Cell Proliferation Kit (Amersham, UK)] at a dosage

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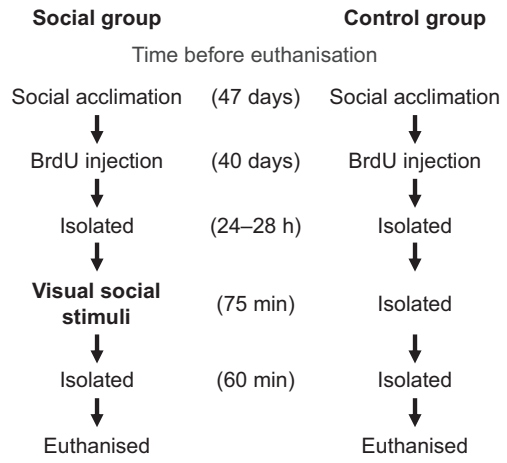

Fig. 1. Time course of experiment in zebrafish exposed to social stimuli versus empty tank (control). Social fish were exposed visually to a single conspecific for 15 min then euthanised 60 min later. Control fish remained isolated.

of 50 µl g^{-1} body mass (15–20 µl injection volume). Fish were then returned to their group aquarium.

Thirty-nine days after BrdU injection, we exposed fish to social stimuli using an acute social stimulus protocol that reliably induces pS6 activation in zebrafish (Nunes et al., 2020 preprint). Fish were removed from the group tank and placed individually in 1 litre isolation tanks (10×10×10 cm). Tanks were separated with white opaque barriers to block visual contact between fish. After 24–28 h of isolation, fish were then divided randomly into two treatments: one group (n=5) received visual social stimuli and a control group (n=5) received no social stimuli. Experimental fish were exposed visually to one other conspecific by removing the barrier between tanks for 15 min. The barriers were then returned, and the fish remained isolated for an additional 60 min. For control fish, the barriers were removed similarly, but the fish were presented an empty tank. All fish were then euthanised by overdose in MS222 (500–1000 mg l^{-1-1}).

Fish were immediately perfused intracardially with paraformaldehyde (2% in 0.1 mol l^{-1} phosphate buffer) for 30 min, using a procedure described previously (Teles et al., 2012). Brains were then dissected, fixed again in paraformaldehyde (2%, 60 min at 4°C), washed (2×15 min) in PBS, cryoprotected in sucrose (25% overnight at 4°C), frozen with pulverized dry ice and stored at −80°C. Brains were then cryosectioned (16 µm) and air dried.

We used immunohistochemistry for BrdU to identify adult-born cells and for pS6 to identify active cells in the brain, using protocols similar to those previously reported (Teles et al., 2012; Nunes et al., 2020 preprint). Sections were washed in Tris buffered saline (TBS, 2×7 min), treated with HCl (2 mol l^{-1}, 37°C, 10 min), buffered (sodium borate 0.1 mol l^{-1}, 15 min) incubated (4°C, overnight) simultaneously with the primary antibodies (rat anti-BrdU, 1:200, Oxford Biotechnology cat. no. OBT0030 and rabbit anti-pS6, 1:400, Cell Signaling Technology) and followed by appropriate secondary antibodies.

We quantified $BrdU^+$ labelled cells in coronal sections at axial levels 50, 60, 71, 85, 92, 98 and 107 of the zebrafish brain atlas (Wulliman et al., 2012). Sections were viewed and photographed with Leica SP5 confocal microscope. Confocal Z-series stacks were acquired using a 63×1.3 NA oil immersion objective, the 488 nm and 568 nm laser lines, and spectral detection was adjusted for the emission of the Alexa 488 and Alexa 568 fluorochromes, using HyD detectors in Standard Mode. All $BrdU^+$ cells were counted in the following telencephalic regions: dorsal (D), dorsomedial (Dm), and dorsolateral (Dl) zones of the dorsal telencephalon, the ventral zone (Vv) and dorsal zone (Vd) of the ventral telencephalon and the anterior preoptic nucleus (POA). For each $BrdU^+$ cell, we examined whether it colabelled for pS6.

Statistics

We analysed log-transformed data using a general linear model in R (https://www.r-project.org/) with treatment (social versus control) and brain region as factors and individual as a random effect. Separate analyses were conducted on the number of $BrdU^+$ cells, the number of co-labelled cells ($BrdU^+/pS6^+$) and the percentage of $BrdU^+$ cells that co-labelled for $pS6^+$. *Post hoc* Tukey's multiple comparisons were used to compare labelling in each brain region.

RESULTS AND DISCUSSION

We found that adult-born ($BrdU^+$) cells in the zebrafish telencephalon were active, as indicated by their expression of pS6. Moreover, an acute exposure (15 min) to visual social stimuli increased the number of active cells in adult-born cells in specific subregions of the telencephalon. These results demonstrate for the first time in fish that adult-born brain cells are indeed responsive to external stimuli and are consistent with the hypothesis that adult-born cells contribute to social behaviour in fish.

Pooled across the telencephalon, there were no differences between fish exposed to visual social stimuli and unexposed control fish in the total number of $BrdU^+$ cells (Fig. 2A; means±s.e.m.: social=176±12 cells; control=162±26; $F_{1,9}$=12.4, P>0.05), but visual social exposure induced a greater number of $BrdU^+/pS6^+$ co-labelled cells (Fig. 2B, social=21±3 cells; control=9±2 cells; $F_{1,9}$=102.3, P<0.001) and a greater fraction of all $BrdU^+$ cells that co-labelled with $pS6^+$ (Fig. 2C social=13.4±1.9%, control=6.7 ±1.4%; $F_{1,9}$=34.9, P<0.01).

Among telencephalic brain regions, the pattern of $BrdU^+$ labelling resembled that reported previously (Fig. 3; Hinsch and Zupanc, 2007), with the greatest number of newborn cells in the dorsal telencephalon (especially the Dm and Dl), intermediate levels in the ventral telencephalon (Vd and Vv) and the least in the POA (Fig. 2A). Fish exposed to visual social stimuli and control fish did not differ in the number of $BrdU^+$ cells in any telencephalic region (Fig. 2A; $F_{5,44}$=2.4, P>0.05). This was expected, since both groups had the same experience except for 15 min during the final hour before sacrifice.

By examining the different brain regions, we found that there was a significant treatment×brain region interaction, with visual social stimuli significantly increasing the number (Fig. 2B; ($F_{5,44}$=7.3, P<0.01) and percentage of $pS6^+/BrdU^+$ co-labelled cells (Fig. 2C; $F_{5,44}$=2.8, P<0.05) in the D, Dm, Dl and POA, but not in the Vd and Vv. The POA showed the greatest percentage increase in co-labelled cells in response to social stimuli. There were no clear and consistent concentrations of co-labelled cells within each brain region.

Previous studies have shown that cells born in the telencephalon of adult zebrafish express markers of neuronal differentiation (HuC/D) (Zupanc et al., 2005; Grandel et al., 2006), and form synapses within 28 days of cell birth (Rothenaigner et al., 2011). Moreover, by 49 days, they develop the capacity to fire spontaneous action potentials (Rothenaigner et al., 2011). The age at which adult born cells demonstrate electrical activity is similar to the age of cells (40 days) in which we detect activity in $BrdU^+$ cells via pS6 expression. Even in control fish not exposed to the social stimuli,

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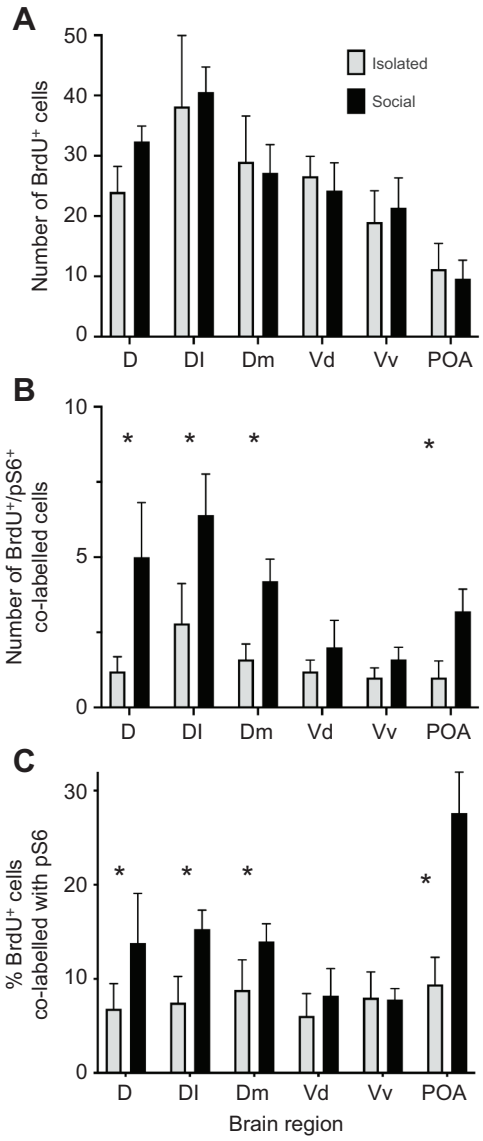

Fig. 2. BrdU and pS6 labelling in subdivisions of telencephalon in control and socially stimulated zebrafish. (A) Number of adult-born, $BrdU^+$ cells. (B) Number $BrdU^+/pS6^+$ co-labelled cells. (C) Percentage of all $BrdU^+$ cells that co-labelled with $pS6^+$. Asterisks indicate significant difference ($P<0.05$) between treatment groups. Data are presented as mean ±s.e.m. For both treatment groups, $n=5$. D, dorsal; Dl, dorsolateral; Dm, dorsomedial zones of the dorsal telencephalon; Vd, dorsal zone; Vv, ventral zone of the ventral telencephalon; POA, anterior preoptic nucleus.

$pS6^+/BrdU^+$ cells were found, albeit in low numbers, throughout the telencephalon, indicating that adult-born cells activate either spontaneously or in response to minimal background stimuli.

In addition to documenting this background activity in adult-born cells, we show that the number of active adult-born cells in the zebrafish telencephalon increases in response to visual social stimuli. Many studies in teleosts, including zebrafish, have demonstrated that social stimuli over a broad temporal range influence several processes in adult neurogenesis, from gene expression to proliferation to survival and differentiation. In the zebrafish telencephalon, 30 min of paired social interaction alters telencephalic expression of neurogenic genes, including *wnt3* and *neurod* (Teles et al., 2016), 2–3 days of paired social interaction decreases cell proliferation (Tea et al., 2019), and 28 days of group

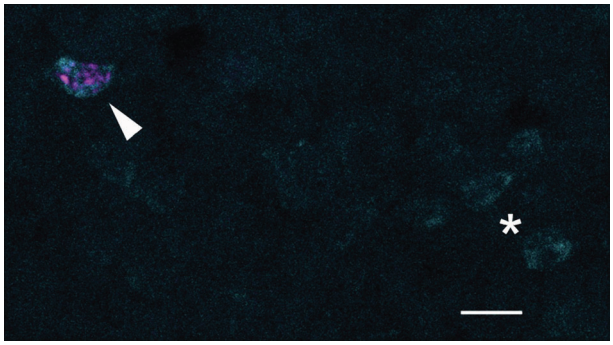

Fig. 3. Confocal micrograph of $BrdU^+$/ $pS6+$ co-labelling. Arrowhead shows a cell double immunolabelled for $BrdU^+$ (magenta) and $pS6^+$ (blue-green). Asterisk shows a cluster of cells labelled only for $pS6^+$. Cells are located in the dorsomedial telencephalon. Scale bar: 20 µm.

interaction enhances neuronal differentiation (Lindsey and Tropepe, 2014). We showed that 15 min of visual social interaction increased the activity of adult-born cells in four telencephalic regions in the social decision-making network (*sensu* O'Connell and Hofmann, 2012). Our present results are among the first to demonstrate that adult-born neurons participate in the short-term expression of social behaviour in fish.

Interestingly, the regional specificity of this effect differs considerably from that found in social stimulation of neurogenic genes. For example, in the present study, social stimuli increase $BrdU^+/pS6^+$ to the largest extent in the POA and not at all in the Vv, but, in another study using a similar aggression behavioural protocol (Teles et al., 2016), social stimuli increase *wnt3* and *neurod* mRNA expression in the Vv but not at all in the POA. These results show that brain regions that are most apt to activate adult-born cells during social interactions are not necessarily those that most respond with changes in neurogenic genes during social interaction and suggests that the later stages of functional integration of adult-born cells are not directly linked to the initial stages of socially induced neurogenesis.

The particularly high fraction of socially responsive adult-born cells in the POA is interesting given its important role in social behaviour. In several teleosts (Cabrera-Álvarez, et al., 2017; Fischer et al., 2018; Baran and Streelman, 2020), including zebrafish (Kelly, 2019; Nunes et al., 2020 preprint), the POA is among the regions with the most activation during social stimuli. It is possible the greater percentage of $BrdU^+/pS6^+$ in this region that we describe simply reflects overall high level of activity of the POA during social interaction. Future studies should examine whether newborn cells are disproportionately represented among all socially responsive cells. The POA contains two primary cell types (magnocellular and parvocellular) and likely regulates social behaviour through its production of the nonapeptides vasotocin and isotocin (Godwin and Thompson, 2012; Larson et al., 2006; Kelly, 2019). To better understand the role of adult-born cells in the POA during social interaction, future studies should examine their detailed morphology and neurochemical phenotypes.

Our study raises several other questions for future research. First, from our study, we are not certain about the cell phenotype of $BrdU^+/pS6^+$ cells. These cells appeared morphologically to be neurons. However, from other studies, it is known that glia are born in the telencephalon of adult zebrafish and can contribute to visually mediated behavior (Mu et al., 2019; Diotel et al., 2020). Thus, future studies should positively identify the phenotype of socially active newborn cells with immunomarkers. Secondly, with our limited

sample, we could not evaluate sex differences in the distribution or abundance of socially active newborn cells. Males and females differ in the density of newborn cells certain telencephalic nuclei at 21days after BrdU treatment (Ampatzis et al., 2012) and in the patterns of coactivation among telencephalic regions during social interaction, so it is possible that they also differ in the social activation of newborn cells.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.D.D., M.C.T., R.F.O.; Methodology: K.D.D., M.C.T.; Formal analysis: K.D.D.; Investigation: K.D.D.; Resources: M.C.T., R.F.O.; Data curation: K.D.D.; Writing - original draft: K.D.D.; Writing - review & editing: K.D.D., M.C.T., R.F.O.; Supervision: R.F.O.; Project administration: K.D.D., R.F.O.; Funding acquisition: K.D.D., M.C.T., R.F.O.

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