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Social isolation impairs active avoidance performance and decreases neurogenesis in the dorsomedial telencephalon of rainbow trout

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A B S T R A C T

Alterations in the social environment, such as isolating an individual that would normally live in a social group, can generate physiological responses that compromise an individual's capacity to learn. To investigate this, we tested whether social isolation impairs learning skills in the rainbow trout. We show that rainbow trout can achieve an active avoidance (AA) learning program with inter-individual variability. Moreover, c-Fos expression in dorsomedial telencephalon (Dm) correlates with the AA performance, indicating that this structure is involved in this cognitive task. Given that Dm participates in AA learning and this region is under plastic remodelling by addition of new-born neurons, we tested whether social isolation impinges on adult neurogenesis and, consequently, on the Dm cognitive outcome. Trout were reared for four weeks in control or isolated conditions. We found that social isolation diminished the percentage of adult-born neurons that are being incorporated into Dm network. Interestingly, the same isolation treatment also induced a severe deficit in the AA performance. Our results demonstrate a structure-to-function relationship between the Dm and the learning ability in an AA task, indicate that social isolation reduces the incorporation of adult-born neurons into Dm, and show that social isolation impairs the Dm-related cognitive function.

1. Background

During life, the social environment significantly influences the neurochemical balance and connectivity of the brain and in consequence the organisation of behaviour. This phenomenon seems to be conserved across vertebrates evolution. For example, rodents reared in social isolation show altered cognitive performance, with a decrease in the synapse number, impaired myelination, reduction of adult neurogenesis, and perturbation on neurochemical balance [1–5]. On the other hand, it has been shown that social isolation impairs a T-maze associative learning performance in a social cichlid fish [6], and alters thigmotaxis and whole-brain serotonin levels in adult zebrafish [7]. Moreover, rainbow trout under social stress exhibit a decrease in the proliferation of telencephalic neuronal progenitors [8], whereas social isolation decreases cell addition in the diencephalic ventricles of adult electric fish [9]. However, these studies did not addressed the problem in a circuit-to-function context, making it difficult to understand how social isolation affects neural networks. In this paper we aimed to relate a rainbow trout telencephalic region with a behavioural outcome, in order to explore plastic modifications induced by social isolation in both cognitive function and the related neuronal substrates.

Experimental data have shown that learning and memory systems are more conserved throughout evolution than previously thought, with teleost fish relying on homologous neural substrates for learning and memory processes as mammals [10]. For example, the amygdala and the hippocampus play an important role in the acquisition and retention of conditioned avoidance behaviour [11–15]. In teleosts, molecular and behavioural studies suggest that the telencephalic dorsomedial (Dm) region shares homology with the basolateral amygdala of mammals [10,16–18], whereas the dorsolateral (Dl) telencephalic region is proposed to share homology with the mammalian hippocampus [17,19,20].

The active avoidance (AA) paradigm is a delay conditioning variant procedure, that involves both emotional and temporal associations in order to relate a conditioning signal (neutral cue) with a subsequent and overlapped unconditioned stimulus (aversive cue). In consequence, fish learn to avoid the aversive side of an experimental tank by swimming to the safe one. This behavioural paradigm has been tested on a number of teleost fish [16,21–24]. Interestingly, the Dm region has been found to be relevant to achieve an associative learning in the AA paradigm [16,25,26], and also is known to be subjected to a continuous network remodelling by the addition of adult-born neurons [8,27–29].

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For these reasons, in this work we evaluated the rainbow trout's ability to learn an AA task, we linked this cognitive task to Dm region activity, and finally we assessed whether isolation modifies adult neurogenesis in Dm, and consequently the behavioural performance in the AA test.

2. Methods

2.1. Subjects

Adult hatchery-bred rainbow trout (*Oncorhynchus mykiss*) were supplied by the Centro de Salmonicultura Bariloche, Universidad Nacional del Comahue, Río Negro, Argentina. Adult trout of approximately 1.5 years old were used (228 ± 22 g, mean weight ± SD). Experimental individuals were reared in constantly aerated water tanks under natural photoperiod, and fed daily with 1% of body weight. During experimental time water temperature ranged from 10 to 15 °C.

2.2. BrdU administration

Fish were anaesthetised by immersion in a low dose (50 mg/l) of benzocaine (Ethyl 4-aminobenzoate) and then BrdU (50 μg/g body weight) was administered by i.p. injection. BrdU was diluted at 20 mg/ml in sterile PBS-DMSO (1:1).

2.3. Housing

Experimental individuals were maintained under constant aerated stream water. Two different treatments were used: *Isolation (I)* and *Control (C)*. The *I* experimental individuals were housed individually in small net cages (in cm, l = 36 x w = 26 x h = 18) that allow fish to turn and change swimming direction. The *C* fish were housed in large tanks (in cm, l = 150 x w = 150 x H = 60), in groups of 10, and supplemented with artificial shelters. After 4 weeks of treatment, fish were placed again in the common tank during one extra week. At the end of the fifth week, swimming performance was evaluated in the "Novel Tank" test, and then cognitive ability was assessed in the AA paradigm.

2.4. Novel Tank (NT)

Fish were placed on a novel tank (in cm, l = 80 x w = 40 x h = 30) and swimming performance was recorded for 20 min. The upper portion of the tank was defined at 13,3 cm from the bottom of the tank. Swimming performance was analysed using IdTracker [29,30] and Matlab R2017a software.

2.5. Active avoidance (AA)

Individuals were trained in a delay fear conditioning variant, with a variable overlap time paradigm. In this protocol, the fish have to associate a white led light as a neutral stimulus (conditioned stimulus) with a mild electric shock as an aversive stimulus (unconditioned stimulus). For stimulation, we selected 3.5 mV as the lowest voltage that evoked an aversive response. Experimental tank (in cm, l = 90 x w = 45 x h = 55) was divided in two identical compartments by a 10 × 10 cm hurdle. The training protocol was adapted from other AA learning tasks described for different teleost species [16,23,31]. Experimental individuals were subjected to one training session per day during 3 successive days. Each learning session consisted of a maximum of 60 trials, each trial had a maximum duration of 30 s, and a 30 s intertrial interval (ITI). To avoid the shock, fish had to cross the hurdle within 15 s after the onset of the light (Avoidance response). If the trout did not change to the safe side after 15 s, then an electric shock was delivered for a maximum of 15 s. When fish changed to the safe compartment, the light or the light + electric shock were immediately turned off. Note that both stimuli culminate at the same time. Each session concluded when the subject reached an 80% of avoidance responses during the last ten trials or a maximum of 60 trials. The criterion for considering a fish as good learner, was to reach an 80% of avoidance responses in < 30 trials during the third session. Latencies, CS-US overlap, as well as the SD for the CS-US overlap for all learner fish used in this manuscript are shown in Supplementary Fig. 1C.

Short-term memory (STM) was evaluated 30 min after the third session, whereas long-term memory (LTM) was tested 24 h later. Memory tests consisted in a maximum of 30 trials in which only the conditioned stimulus (light) was presented.

2.6. Tissue fixation and Immunohistochemistry

Ninety minutes after memory test, fish were deeply anaesthetised with 100 mg/l benzocaine and were intracardially perfused with 10 mM phosphate buffer (PBS) at 4 °C followed by 4% paraformaldehyde in 10 mM PBS. Brains were dissected, progressively dehydrated in 15% and 30% sucrose, frozen and stored at −20 °C. Telencephalic sections of 10 or 40 μm were cut on a cryostat (Microm, HM 550), and mounted on positively charged slides. Slides were rinsed three times with TBS (pH = 7.4) for 5 min, incubated with 0.05% sodium borohydride in ice-cold TBS for 15 min and rinsed three more times with TBS for 5 min. Then, slides were blocked with 6% bovine serum albumin in TBS for 1 h and incubated overnight at 4 °C with primary antibody diluted in 6% BSA in TBS. Then, they were washed with TBS four times for 5 min, and incubated with secondary antibody coupled to Cy2 or Cy3 (1:2000 dilution using 6% BSA in 0.3%TritonX-100/TBS) for 2 h at room temperature (RT). Sections were then washed six times (5 min each) in 0.3% TritonX-100/TBS, and mounted using DABCO-glycerol mounting medium. Finally, sections were dried overnight in the dark, and subsequently stored at 4 °C. When double immunostains were performed both primary antibodies were incubated at the same time. For BrdU immunodetection antigen retrieval was performed before blocking: 90 min in 50% formamide in 10 mM citrate buffer at 65 °C, then washed with sodium citrate for 15 min and incubated with 2 N HCl for 20 min at 37 °C, followed by 10 min neutralization in 0,1 M borate buffer (pH = 8,5). For PCNA immunostaining antigen retrieval was performed for 1 h (sodium citrate 10 mM at 60 °C, 3 × 5 min TBS wash) before incubation with the blocking solution. Antibodies used were: rabbit polyclonal anti-*c-Fos* 1:1000 (sc-253, Santa Cruz Biotechnology, Inc., raised against a peptide mapping within an internal region of c-Fos of human origin), rat monoclonal anti-*BrdU* 1:500 (ab6326, Abcam), mouse monoclonal anti-*PCNA* 1:400 (PC10, Dako, raised against a recombinant PCNA of human origin), rabbit monoclonal anti-*NeuN* 1:500 (ab177487, Abcam, raised against a synthetic peptide within human NeuN aa 1–100). Secondary antibodies were obtained from Jackson Immunoresearch Labs, Inc. For quantification analysis 5 telencephalic sections spaced 200 μm apart were used for each fish. Anatomical telencephalic regions were depicted in accordance with Folgueira and coworkers, 2004 [32,33].

2.7. Western blot

After 90 min of behavioural assays, rainbow trout were euthanised and telencephalon were dissected and mechanically lysed at 4 °C in TNE buffer (25 mM Tris-ClH [pH 7.4]; 1 mM EDTA; 137 mM NaCl) containing 0.5% Triton X-100, plus protease and phosphatase inhibitors. Proteins lysates were clarified by centrifugation and analysed by western blotting in a SDS-PAGE. The blots were scanned in a Storm 845 PhosphorImager (GE Healthcare Life Sciences).

2.8. Ethics

Experimental procedures were conducted in accordance with the National regulations and following the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory and other Research Animals [34]. Fish are at the centre of our research

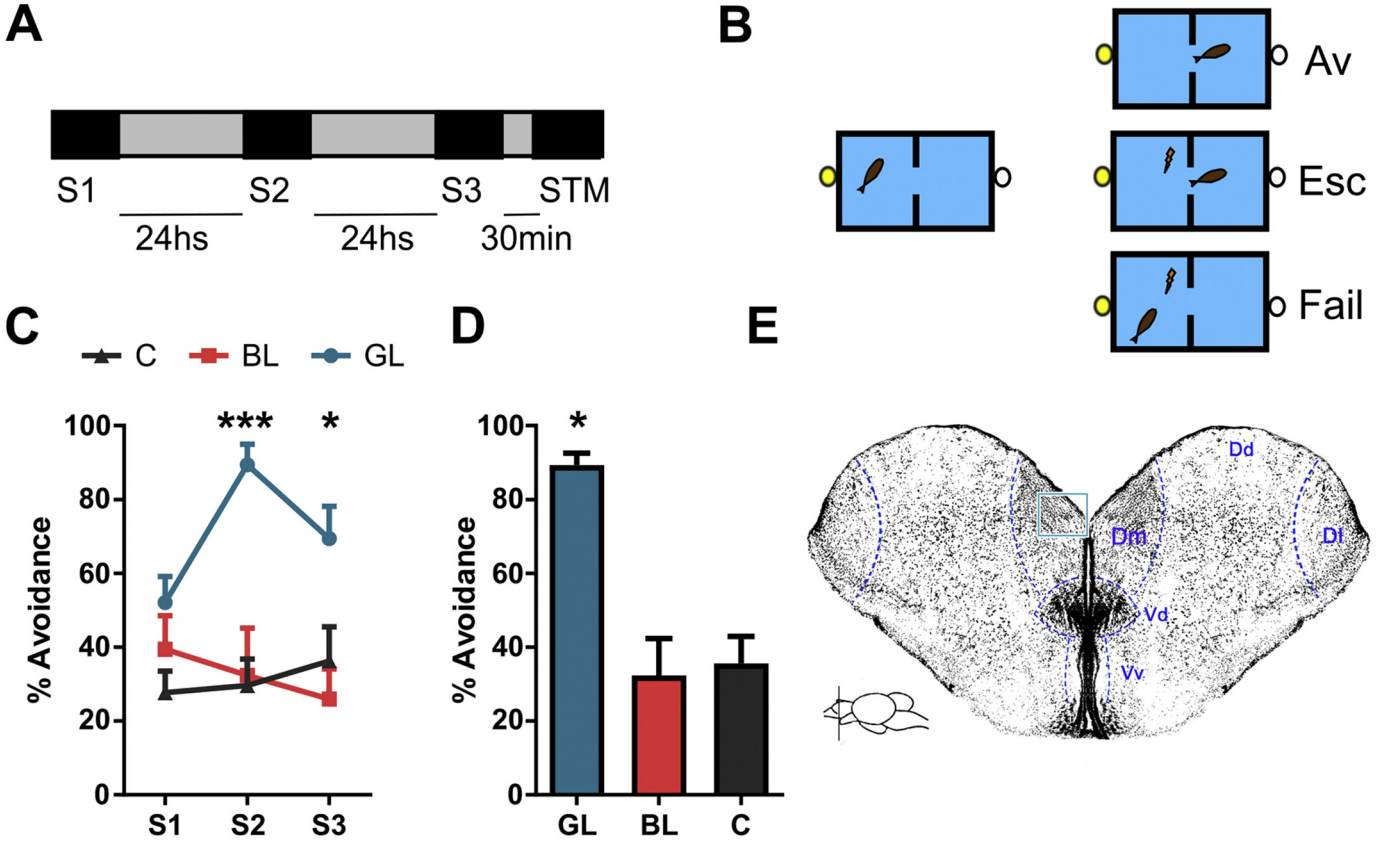

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Fig. 1. Active avoidance (AA) learning in rainbow trout.
A) Time schedule for AA training. Each fish was trained for three daily sessions. Each session consisted of a maximum of 60 trials, separated by a 30-s ITI. The tests to analyse retrieval of trained avoidance behaviour was performed 30 min after the last training.
B) Schematic top view of the AA training. A LED light was presented as the conditioned cue for a maximum of 15 s in the compartment where fish were located. Three reaction types were observed. "Av": the fish successfully crossed the hurdle avoiding the mild electric shock delivered as punishment. "Esc": describes fish that received the punishment but subsequently escaped from that compartment within the 15 s. "Fail": describes fish that neither avoided nor escaped during the whole trial of 30 s.
C) Percentage of Avoidance responses throughout learning sessions. C, control group; GL, good learners; BL, bad learners. Two-way ANOVA for repeated measures, group effect $F_{(2, 12)} = 12.22$, $p = .0013$, Sidak's multiple comparisons test: *** $p < .0001$ for GL vs C, and for GL vs BL; * $p < .05$ for GL vs C, and for GL vs BL.
D) Short Term Memory Test (STM). Percentage of Avoidance responses 30 min after S3 in the absence of the aversive stimulus. One-way ANOVA, group effect F (2,12) = 18.85, p = .0002, Holm-Sidak's multiple comparisons test * denotes p < 0,001 for GL vs C and for GL vs BL.
E) Schematic view of cross section of rostral telencephalon labelled with fluorescent Nissl stain NeuroTrace®. Blue dashed lines delimit anatomical regions. Sagital view of the telencephalon (bottom left) indicates the level of cross section. Boxed area is shown magnified in (F).
F) Optical sections of rainbow trout's Dm telencephalon (left hemisphere) depicting c-Fos expression 90 min after the STM session. Scale bar: 50 μm.
G) Number of cells expressing c-Fos in Dm and Dl of GL, BL and C trout. As no differences were found along the rostro-caudal axis the analysis show the mean value of Dm and Dl c-Fos along this axis. Two-way ANOVA for repeated measures, group effect F(2,12) = 72.89 with p < .0001, region effect F(1,12) = 148.7 with p < .0001. Sidak's multiple comparisons test, *** denotes p < .0001 for GL vs C and BL, whereas * denotes p < .005 for GL vs C.
H) The number of cells expressing c-Fos correlates with AA learning performance. Three learning parameters were assessed: number of trials, latency to change compartment, and percentage of Avoidance responses. Pearson's correlation index is informed for each analysis, *** denotes p < .0005 and ** denotes p < .001.

and we have taken all possible measurements to reduce the number of experimental organisms. The authors declare no conflict of interests in this article.

3. Results

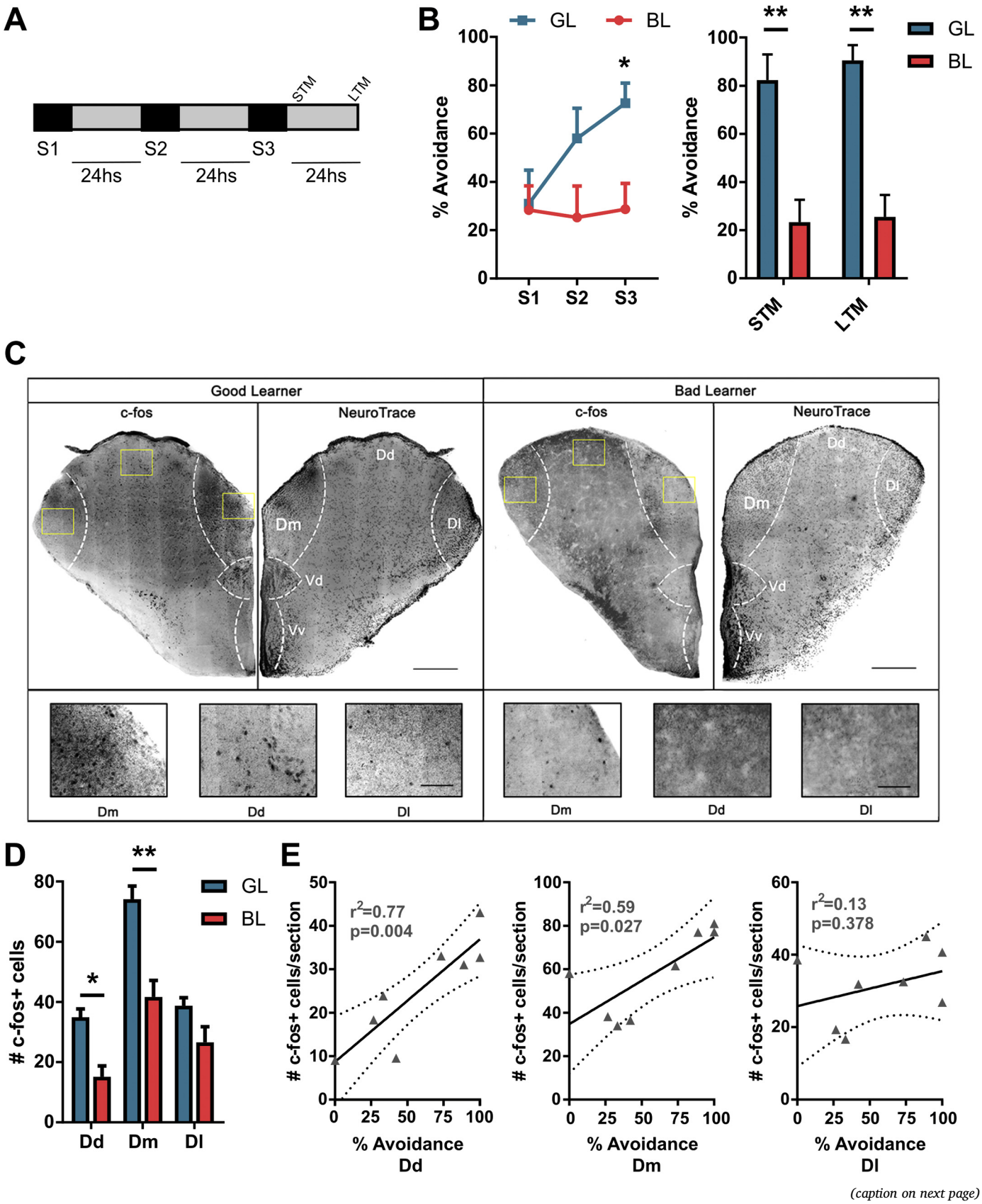
To assess rainbow trout's cognitive ability, individuals were trained in an active avoidance (AA) paradigm, see the Methods section for a detailed description (Fig. 1A, B). As control condition (C), trout were subjected to the AA training but the aversive stimulus was omitted. The C group showed no improvement in performance across sessions and failed the memory test. In the AA trained group, we found that a 50% of individuals exhibited a good learner (GL) performance whereas the other half performed as bad learners (BL) (Two-way ANOVA for repeated measures, group effect $F_{(2, 12)} = 12.22$, $p = .0013$, $n = 5$ for each group), see Fig. 1C, D. Thirty minutes after the third session, a short- memory test (STM) was performed by omitting the aversive stimulus. We found that GL retained the learned rule and performed an 89.33 ± 3.17% of avoidance responses, whereas the C and BL groups exhibited less that a 36% of avoidance responses (ANOVA $F_{(2, 12)} = 18.85$, $p = .0002$, n = 5 for each group), see Fig. 1D (and Supp. Fig. 1C). Also, GL subjects performed better that the C and BL, and showed improvements in AA STM when different parameters were evaluated: number of trials to reach criterion, latency to switch into the safe chamber, and number of errors (Supp. Fig. 1A, B).

Given that Dm telencephalic region was demonstrated to be necessary for AA behaviour in goldfish [16,25,35] and zebrafish [26], we evaluated the involvement of Dm telencephalon during this cognitive process. For this purpose, 90 min after the memory test we evaluated the expression of the immediate early gene c-Fos, as a marker of neuronal activity [18,36]. The specificity of the c-Fos antibody was tested by western blot on telencephalic extracts from control and stressed trout, 90 min after each experimental condition (Supp. Fig. 2a). Fig. 1E illustrates the anatomical divisions of trout telencephalon, as well the rostro-caudal position of the telencephalic section. We found that 90 min after AA STM test, only GL fish exhibited an enhanced c-Fos expression in Dm, whereas BL exhibited c-Fos levels similar to the C group (Fig. 1F, G). Moreover, in Dm the c-Fos expression correlated with all the AA learning parameters evaluated in the memory test (Pearson correlation: # trials: $r^2 = 0.84$, latency: $r^2 = 0.62$, and % Avoidance: $r^2 = 0.70$, all with $p < .01$ and $N = 10$), whereas it did not correlate in Dl telencephalic region (Fig. 1H). We found no differences in c-Fos expression along the Dm rostro-caudal axis (Supplementary Fig. 2B). Telencephalic c-Fos expression is also enhanced by stress, nevertheless in our AA paradigm we did not find a relationship between the number of aversive stimuli and the levels of c-Fos label (Supplementary Fig. 2C).

Here we demonstrated that in trout, Dm is activated after an AA short memory test. In zebrafish, it was shown that a dorsal telencephalic region located at the middle of the rostro-caudal axis increases neuronal activation when the subjects recall the learned task after 24 hs (that is a long memory test) [37]. Interestingly, the dorsal telencephalic region Dd has been proposed to be relevant for memory encoding in both electric fish and goldfish [38–40], see the Discussion for a detailed elaboration on this topic. On this basis, we decided to assess c-Fos expression after an AA LTM test (24 hs after learning), in rainbow trout. The Fig. 2A depicts the behavioural training program, after which c-Fos was evaluated (90 min after the LTM). As seen before, half of the trained subjects exhibited a GL performance, whereas the other half performed as BL, see Fig. 2B (Learning curve: Two-way ANOVA for repeated measures, group effect $F_{(2, 12)} = 4.24$, $p = .040$, $n = 4$ for each group); LTM test: Two-way ANOVA for repeated measures, group effect $F_{(1, 6)} = 45{,}31$, with $p = .0005$, $N = 4$ for each group), and Supplementary Fig. 3A. Ninety minutes after the LTM test, subjects were euthanised and c-Fos expression was analysed in different telencephalic regions (Fig. 2C-E). We observed an increase of c-Fos immunoreactivity on Dm and Dd regions in GL when compared to BL subjects, whereas Dl does not show significant differences (Two way ANOVA for repeated measures, region effect $F_{(2, 12)} = 36.82$, with $p < 0{,}0001$; group effect $F_{(1, 6)} = 35.53$, with $p = .0010$; N = 4). Interestingly, in Dd and Dm, but not in Dl, c-Fos expression correlates with the memory ability assessed by the LTM test, see Fig. 2E (Pearson's correlation: for Dd $r^2 = 0.758$ with $p = .0049$; for Dm $r^2 = 0.514$ with $p = .0454$: for Dl $r^2 = 0{,}187$ with $p = 0{,}2850$; $N = 8$), and Supplementary Fig. 3B.

In mammals, neuronal proliferation in the adult brain has been related to the circuital activity of the progenitor's environment. Here we assessed if enhanced Dm neuronal activity, as a result of AA learning, induces an increase in the proliferation of neuronal progenitors. To that end, we evaluated the expression levels of the proliferating cell nuclear antigen (PCNA). We found that PCNA was increased in Dm of GL fishes, compared to the C group (Two-way ANOVA for Repeated Measures, group effect $F_{(2, 12)} = 6.013$, with $p = .0155$, $n = 5$ for each group), see Supplementary Fig. 4. These results highlight the participation of Dm telencephalic neuronal circuit during the AA cognitive test.

As we determined this circuit-to-function relationship, we evaluated how social isolation impinges on both Dm levels of adult neurogenesis and the Dm-related AA behaviour. We assessed the proliferative activity in Dm by evaluating PCNA expression after 3 days of rearing in control (C) or isolated (I) conditions, and we did not found any differences (Mann Whitney test, $p = .70$, with $n = 3$ for each group), see Fig. 3A. Then, we labelled neuronal progenitors with the thymidine analogue



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BrdU and studied if trouts after four weeks of isolation showed alterations in the survival of neuronal progenitors when compared to controls. After four weeks of isolation both C and I subjects exhibited similar numbers of BrdU+ cells (Mann Whitney test, $p = .743$, with $n = 4$ for each group), see Fig. 3B and C. As proliferation and survival of neuronal progenitors were not affected by the experimental conditions, we assessed the neuronal identity of the BrdU labelled cells by analysing the co-expression with the neuronal marker NeuN. Interestingly,

Fig. 2. Active avoidance learning enhances c-Fos expression in Dm telencephalon.
A) Time schedule for AA training. Each fish was trained for three daily sessions. Each session consisted of a maximum of 60 trials, separated by a 30-s ITI. The tests to analyse retrieval of trained avoidance behaviour were performed 30 min and 24 h after the last training, short-term memory (STM) and long-term memory (LTM), respectively.
B) Left panel: Percentage of avoidance responses throughout learning sessions. GL, good learners; BL, bad learners. Two-way ANOVA for repeated measures, group effect $F(2, 12) = 4.24$, $p = .040$, $n = 4$ for each group. Sidak's multiple comparisons test, * denotes $p < .05$ for GL vs BL in S3. Right panel: Percentage of avoidance responses during STM and LTM for GL and BL subjects. Two-way ANOVA for repeated measures, group effect $F(1, 6) = 45{,}31$, with $p = .0005$, $N = 4$ for each group. Sidak's multiple comparisons test, ** denotes $p < .005$ for GL vs BL.
C) Top panel: Optical sections of GL (left panel) and BL's telencephalon (right panel). For each section is shown the left hemisphere depicting c-Fos expression 90 min after the LTM session and the right hemisphere labelled with fluorescent Nissl stain (NeuroTrace®). White dashed lines delimit anatomical regions. Boxed areas (yellow) indicates the regions magnified at the bottom of each section. Scale bar: 500 µm. Bottom panel: Magnified view of the square regions in top panel, showing c-Fos expression in Dm, Dd and Dl regions. Scale bar: 100 µm.
D) Number of cells expressing c-Fos in Dd, Dm and Dl of GL and BL trouts. Two-way ANOVA for repeated measures, region effect $F(2, 12) = 36.82$, with $p < .0001$; group effect $F(1, 6) = 35.53$, with $p = .0010$; $N = 4$. Sidak's multiple comparisons test, * denotes $p < .05$, whereas ** denotes $p < .001$, both for Gl vs BL.
E) Correlation between number of cells expressing c-fos and the percentage of avoidance responses in Dd, Dm and Dl. Dotted lines indicate 95% confidence intervals. Pearson's correlation index and p-value are informed for each analysis.

C subjects evidenced that 70.25% of BrdU positive cells also expressed the neuronal marker NeuN, whereas for I group there was only a 42% of co-localization. Thus, 4 weeks of isolation diminish in approximately a 40% the Dm adult neurogenesis levels (Mann Whitney test, $p = .029$, with n = 4 for each group), see Fig. 3B and D.

Since 4 weeks of rearing in isolation decrease the adult neurogenesis contribution to Dm, we wanted to test whether this treatment has any effect on the AA performance (Fig. 4A). For this purpose, adult subjects were reared for 4 weeks under C or I condition. In order to discard any possible anxiety- or stress-related bias to the cognitive outcome, both experimental groups were housed for an extra week in a common control-like social condition. The novel tank (NT) test has been used in zebrafish and trout to assess anxiety-related behaviours [41–44]. One day before the AA training, trouts were evaluated in the NT test (Fig. 4A). We found no significant differences in the NT test among both groups, suggesting that, at this moment, both groups have similar exploratory and motor activity performances (Supplementary Fig. 5). The next day, we started the AA training. As seen previously, in both groups 50% of individuals reached a good learning criterion, whereas the other half exhibited a poor learning performance (Supplementary Fig. 6). We only compared the good learner groups for both C and I treatments. We found that both groups exhibited a proper learning curve, with a session effect analysed by two-way ANOVA ($F_{(2,14)} = 7{,}63$, $p = .006$, $n = 5$ for I and $n = 4$ for C). However it must be noticed that only C subjects showed significant differences in avoidance responses throughout learning sessions (Tukey's multiple comparisons test S1 vs S2: $p = .010$ and S1 vs S3: $p = .002$, n = 5 for I and n = 4 for C), see Fig. 4B. A similar profile response was observed for number of trials and for latency of response as quantifiable variables, see Supplementary Fig. 7. Interestingly, when we evaluated AA memory retention, we found that I trouts failed on both short-term (30 min) and long-term (24 h) memory tests, and only C subjects evidenced a strong memory (Two-way ANOVA for Repeated measures, treatment effect $F_{(1,\ 7)} = 61.6$, with $p = .0001$, n = 5 for I and n = 4 for C), see Fig. 4C.

Our results indicate that rearing rainbow trout in isolation induced a severe deficit in the cognitive performance related to an AA task. We propose that adult neurogenesis in the Dm telencephalic circuit plays a relevant role in the processing of learning related signals during this task.

4. Discussion and conclusions

In this work we demonstrated that rainbow trout can achieve an associative learning task in the AA paradigm, which is widely used among vertebrates to evaluate emotional learning. It must be noticed that our training schedule evidenced that only a 50% of individuals achieved the learning program. Akin individual variability in the cognitive performance was also observed on different teleost species [45], for a recent review see Lucon-Xiccato and Bisazza, 2017 [46]. In fact, in a similar AA training program, only a 37% of trained zebrafish accomplished the learning criterion [31]. Moreover, differences in learning ability were observed between conspecifics of Buenos Aires tetra fish (*Hemigrammus caudoviattus*), with outstanding learners ranging from ~16% to ~46.15% according to the strain [47]. Interestingly, this inter-individual differences in the performance of an AA learning were also described for mammals, where subjects with different levels of fearfulness and coping strategies exhibited dissimilar performance in this task [48–51]. Altogether, these studies suggest that individual differences in AA performance are as common in fish as in other vertebrates. Remarkably, it has been shown that cognitive abilities in fish may vary with brain size, where fish selected for larger brains achieved a greater cognitive performance [52,53]. It would be of interest for further studies to address the contribution of adult neurogenesis to the brain size and therefore to the cognitive ability of different individuals.

In this work we assessed the rainbow trout performance in a behavioural paradigm of emotional learning, the AA task. In mammals, it is reported that the AA task involves different regions of the amygdalar complex [54–57]. In teleosts is proposed that the amygdalar complex is composed by different pallial and subpallial regions. Based on gene expression analysis, it has been proposed that the supracommissural nucleus of the area ventralis telencephali (Vs) is homologous to the dorsal and ventral part of the central amygdala and that the postcommissural nucleus of the area ventralis telencephali (Vp) is homologous to the dorsal part of the central amygdala [58]. Furthermore, in rodents, the cannabinoid receptor (Cb1) is expressed in the basolateral amygdala neurons [59]. Whereas, in the weakly electric fish *Apteronotus leptorhynchus* and zebrafish, Cb1 is strongly expressed in Dm [60,61], thus supporting the model that Dm is homologous to the pallial amygdala. Thus, it is plausible that Vs, Vp (subpallial amygdala) and Dm (pallial amygdala) constitute the amygdaloid complex in teleosts [17]. Furthermore, it has been shown that the rainbow trout Dm region receives afferents from the ventral nucleus of the ventral area (Vv) and from Vs, whereas it projects to the hypothalamic lobe and to Vs [32,33]. An efferent connection from a Dm subregion to the hypothalamus has also been described by Giassi and coworkers [62]. Based on this information it is plausible that the Dm pallial region could share homology with the basolateral amygdala of mammals. It has been proven that Dm is involved in emotional learning and motivated behaviour in different teleost species [18,19]. Experiments in goldfish (*Carassius auratus*), revealed that an experimental ablation of Dm, and not Dl, abolishes the cognitive abilities acquired during a delay conditioning AA protocol [25]. In fact, a recent publication identifies a glutammatergic neuronal population in zebrafish Dm that is essential for AA and fear conditioning learning [26]. Here we tested whether Dm neuronal activity is enhanced by the AA test. After a short memory test (30 min after the last learning session) we evidenced a correlation between the AA cognitive ability and the expression of the neuronal

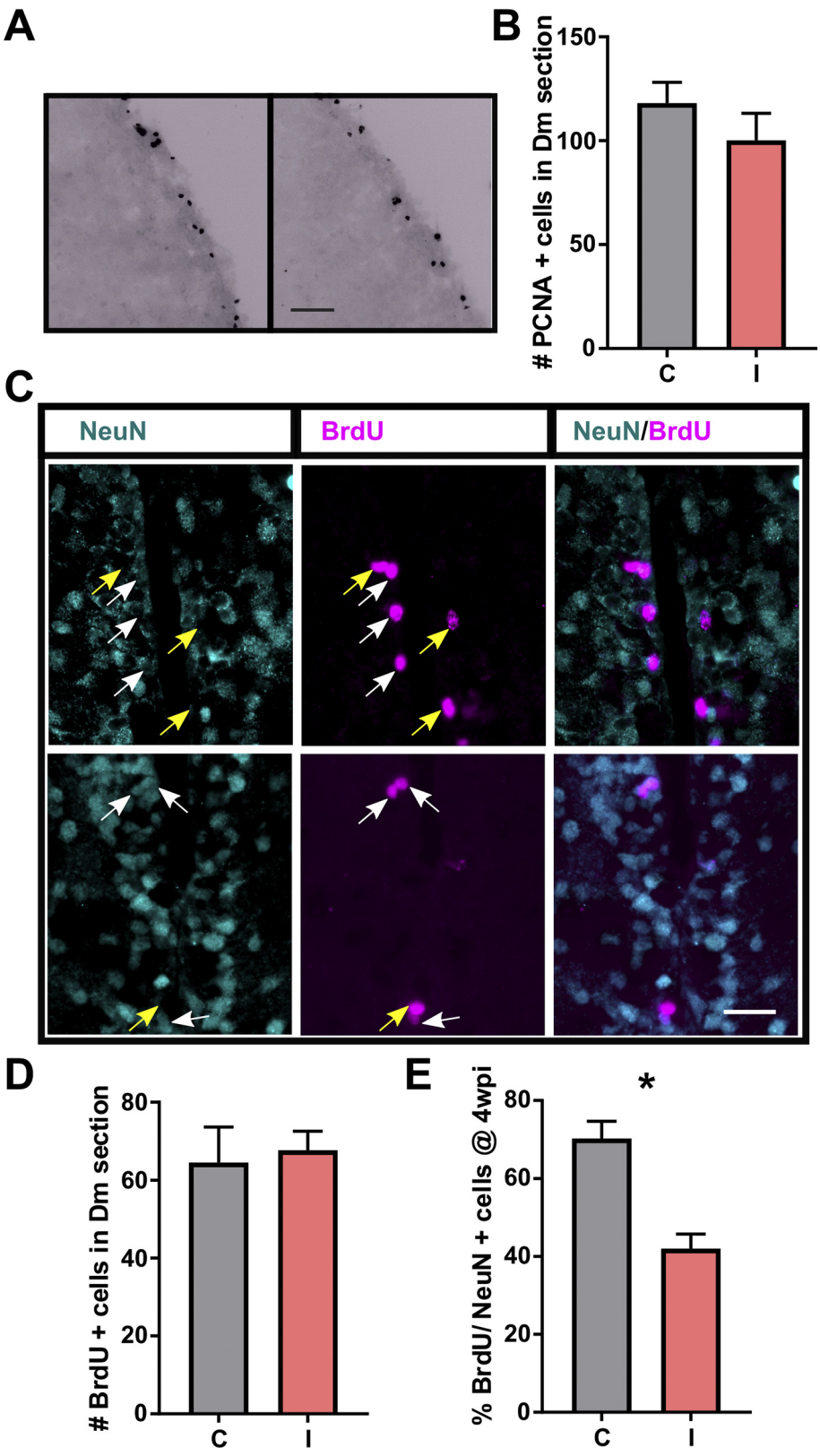

Fig. 3. Social isolation downregulates adult neurogenesis in Dm telencephalon.
A) Optical sections of rainbow trout's Dm telencephalon (left hemisphere) depicting PCNA expression after 3 days of rearing in control (C) conditions (left) or in social isolation (I) (right). Scale bar: 50 μm.
B) Number of PCNA cells in Dm section of trout subjected to 3 days in I or C conditions. Mann Whitney test $p = .70$, $n = 3$.
C) Optical sections of Dm telencephalon from rainbow trouts reared for 4 weeks under C (up) or I conditions (bottom), depicting BrdU incorporation (magenta) and NeuN expression (cyan). White arrows denote BrdU+ and NeuN+ cells, whereas yellow arrows denote cells that are only BrdU+. Sacale bar: 35 μm.
D) Total number of BrdU+ cells in Dm telencephaalon of rainbow trouts under 4 weeks of I or C conditions. Mann Whitney test $p = .68$, $n = 4$.
E) Percentage of BrdU+ cells that express NeuN in the Dm telencephalic region of trouts under 4 weeks of I or C conditions. Mann Whitney test, * denotes $p = .0286$, with $n = 4$.

activity marker c-Fos, only in the Dm region. To our knowledge this is the first report linking Dm neuronal activity to teleosts AA performance. This result is consistent with the lesion experiments in goldfish [16,19,63] and with the finding of a neuronal population in Dm necessary for AA in zebrafish [26].

We further explored the participation of the dorsodorsal (Dd) region encoding a long-term memory task evaluated 24 hs after the last learning session. Our results indicate that c-Fos is overexpressed on Dd and Dm of GL subjects, and the expression of this activity-related protein correlates with the cognitive performance (Fig. 2). Interestingly, Elliott and coworkers described Dd as a global recurrent network with complex reciprocal connections with different pallial and subpallial

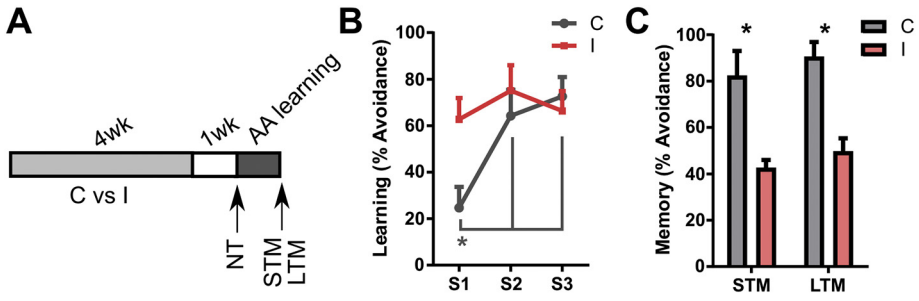

Fig. 4. Social isolation impairs cognitive performance in active avoidance task.
A) Schematic representation of the experimental design. Trout were subjected to control (C) or isolated (I) conditions for 4 weeks. Then both groups were maintained under C conditions for an extra week in order to avoid social isolation acute effects. At the end of this week, C and I trout were tested for motor performance and anxiety-like behaviour in the novel tank (NT) test. Finally, individuals were subjected to AA learning and short- and long-term memory (STM, LTM) were evaluated.
B) Percentage of avoidance responses throughout learning sessions for C and I trouts. Two-way ANOVA for repeated measures, session effect $F(2,14) = 7.53$ with $p = .006$. Tukey's multiple comparisons test: * $p < .05$ for S1 vs S2, and for S1 vs S3. For C: $n = 4$ and for I: $n = 5$.
C) Percentage of avoidance responses in the short- and long-term memory tests (STM, LTM). Two-way ANOVA for repeated measures, group effect $F(1,7) = 61.6$ with $p = .0001$. Bonferroni's multiple comparisons test: * $p < .05$. For C: $n = 4$ and for I: $n = 5$.

regions in the electric gymnotiform fish, *Apteronotus leptorhynchus* [40]. In the rainbow trout, neuronal tracing studies were performed to describe the connectivity of several telencephalic structures [32,33]. In these works, Folgueira and coworkers show that trout Vs, Vv and Vp subpallial regions are strongly interconnected between each other, and all these regions project terminals to Dm, to Dc and to Dd pallial structures, whereas Dd connects to Dm and Dc. It is very likely, that this recurrent loop involving the proposed teleost amygdaloid complex (Vs, Vv, Vd and Dm), could be involved on emotional memory processing. Moreover, Aoki and coworkers identified a dorsal region in the zebrafish telencephalon in which neurons are activated by the conditioning stimulus 24 hs after the last learning session on an AA paradigm, similar to the one employed in this manuscript [37]. In that work the authors identified the memory-involved pallial region as Dc, however the localisation of the responsive region throughout their manuscript points to denote this region as Dd. Finally, Vargas and coworkers showed that Dd lesions also disrupt trace but not delay conditioned AA memory retrieval [38]. However, it must be noticed that in that work the authors did not test the memory retention in an effective manner, since they repeat exactly the same procedure as performed during the acquisition task, instead of testing memory retention by omitting the aversive stimulus. Thus, in this case subjects with lesion in Dd are allowed to re-learn the AA rules. Taken together, this findings propose Dd as a memory engaged circuit in the teleost pallium.

For rodents, it was demonstrated that housing in isolation decreases adult neurogenesis levels [3,64]. Since adult trout are able to learn an AA task, c-Fos expression is enhanced in Dm in relation to the cognitive performance, and the periventricular region of Dm possesses neuronal progenitors that generate new-born neurons, we decided to evaluate if social isolation impacts on Dm adult neurogenesis. After 4 weeks under isolation, the subjects exhibited a ~40% decrease in the Dm adult neurogenesis level. However, both proliferation and survival of neuronal progenitors (which were labelled at the beginning of the treatment) were unaltered when compared to controls. These findings suggest that isolation impinges on the pathway that leads progenitors to neuronal commitment. It would be of relevance to study in which way social isolation acts on different neurotrofins and trophic signals involved on neuronal differentiation.

In this work, we found that rearing in isolation diminishes Dm adult neurogenesis and that Dm activity is essential for AA learning, then we tested whether isolated trout were able to accomplish the AA cognitive task. Here we show that isolated trout can learn an AA task in an intra-session manner, but evidence severe memory deficits to recall the learned task. This was highlighted by the failure in the short- and long-term memory tests (Fig. 4) and by the poor retention exhibited during each session (Supplementary Fig. 6). Interestingly, a similar cognitive deficit was described for cichlid fish that were maintained for ten days under isolation before being trained in a spatial task [6], suggesting that isolation leads to neuronal plasticity defects and in turn to cognitive deficits.

Social interactions plays a significant role in shaping animal physiology and behaviour [65,66]. It has been shown that rainbow trout can adapt to cope with changes in their social environment [67]. A study by Øverli et al. (2002) found that only six days of social isolation were sufficient to induce chronic stress in rainbow trout [68]. It has also been shown that social isolation for only 24 h in the cichlid fish *Archocentrus nigrofasciatus*, results in an increase of cortisol levels [69]. Moreover, social stressors have been found to directly reduce cell proliferation in the telencephalon of rainbow trout [8]. However, in this work we found diminished adult neurogenesis in the Dm without alterations in the proliferation of neuronal progenitors, suggesting an altered niche signalling for neuronal differentiation.

Social isolation has been found to alter stress-related signalling and locomotor activity in different fish [7,8,70,71]. To discard stress-related effects in the cognitive evaluation we decided to keep the isolated group under control conditions (in a social environment) for one week before beginning the AA training. We evaluated anxiety and swimming behaviour in the isolated and control groups by recording the fish performance on the NT test. This test revealed no differences in the swimming distance, the time that fish remained still in the same place, the time that fish spent in the upper portion of the tank, the number of transitions to the upper region, and the latency to cross to the upper region (Supplementary Fig. 5). Together, these results suggest that there were no overall differences in the motivation or exploratory activity of the isolated trout. Thus, we conclude that memory deficits evidenced by the isolated fish would be related to alterations in neuronal plastic mechanisms that are responsible for memory storage and recall. We propose that the incorporation of adult born neurons to the Dm circuitry could be one of such plastic mechanisms. However, further studies should be directed to confirm this hypothesis.

Our results demonstrate a structure-to-function relationship between the Dm neuronal activity and the learning ability in an AA task in a teleost fish. Furthermore, we show that four weeks of social isolation reduces the incorporation of adult-born neurons into Dm, and also impairs a Dm-related cognitive function.

Authors' contributions

Active avoidance experiments were carried out by MSA. Adult neurogenesis experiments were carried out by LSM-F. Novet tank experiments were performed by LSM-F, FRR and SAC. BrdU administration and histological measurements were performed by MSA and LSM-F. Data analysis and discussion were performed by all the authors. APDV and FRR performed the western blot validation of c-Fos antibody. LAM conceived, designed and coordinated the study, and wrote the manuscript. All authors gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.physbeh.2018.10.006.

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