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# An *Astyanax mexicanus* mao knockout line uncovers the developmental roles of monoamine homeostasis in fish brain

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1 Submission for Development Growth and Differentiation

2 Special issue

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5  
6 **An *Astyanax mexicanus mao* knockout line uncovers the**  
7 **developmental roles of monoamine homeostasis in fish brain**

8  
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18  
19 **Short title: MAO and fish brain development**

20  
21 **Key words: monoamine oxidase, serotonin, dopamine, locomotion, proliferation, cell**  
22 **cycle**

23

## 24 **Abstract**

25 Monoaminergic systems are conserved in vertebrates, yet they present variations in  
26 neuroanatomy, genetic components and functions across species. MonoAmine Oxidase, or  
27 MAO, is the enzyme responsible for monoamine degradation. While mammals possess two  
28 genes, *MAO-A* and *MAO-B*, fish possess one single *mao* gene. To study the function of MAO  
29 and monoamine homeostasis on fish brain development and physiology, here we have  
30 generated a *mao* knockout line in *Astyanax mexicanus* (surface fish), by CRISPR/Cas9  
31 technology. Homozygote *mao* knockout larvae died at 13 days post-fertilization. Through a  
32 time-course analysis, we report that hypothalamic serotonergic neurons undergo fine and  
33 dynamic regulation of serotonin level upon loss of *mao* function, in contrast to those in the  
34 raphe, which showed continuously increased serotonin levels - as expected. Dopaminergic  
35 neurons were not affected by *mao* loss-of-function. At behavioral level, knockout fry showed  
36 a transient decrease in locomotion that followed the variations in the hypothalamus serotonin  
37 neuronal levels. Finally, we discovered a drastic effect of *mao* knockout on brain progenitors  
38 proliferation in the telencephalon and hypothalamus, including a reduction in the number of  
39 proliferative cells and an increase of the cell cycle length. Altogether, our results show that  
40 MAO has multiple and varied effects on *Astyanax mexicanus* brain development. Mostly, they  
41 bring novel support to the idea that serotonergic neurons in the hypothalamus and raphe of  
42 the fish brain are different in nature and identity, and they unravel a link between  
43 monoaminergic homeostasis and brain growth.

44

45

## 46 Introduction

47 The characiform fish, *Astyanax mexicanus*, exists under two very different forms (Mitchell et  
48 al., 1977; Elliott, 2018): a surface morphotype ("Surface Fish", SF), which lives in Central  
49 American rivers, and a cave blind and depigmented morphotype ("Cavefish"), which has  
50 colonized northeastern Mexican caves about 20,000 years ago (*Fumey et al., 2018; Policarpo*  
51 *et al., 2020*). During their adaptation to life in darkness, cavefish have evolved both regressive  
52 and constructive features. The comparison between the two forms of the species allows  
53 unravelling changes in genetic networks underlying adaptive modifications that occurred  
54 within a relatively short period of evolution (*Keene et al., 2016; Gross et al., 2023; Retaux et*  
55 *al., 2023*).

56

57 One of the known genetic differences between the two morphotypes is the existence of a  
58 partial loss-of-function mutation in the coding sequence of the Monoamine oxidase (*MAO*  
59 *P106L*), which reduces its enzymatic activity by 50%, and that is shared by several cavefish  
60 populations (*Elipot et al., 2014; Pierre et al., 2020*). Consequently, as MAO is the enzyme  
61 responsible for the degradation of monoamines, cavefish carrying the mutation in the  
62 homozygous state have high brain serotonin, dopamine and noradrenaline levels (*Pierre et*  
63 *al., 2023*).

64

65 MAO is a mitochondrial enzyme that catalyzes the oxidative deamination of biogenic amines  
66 (monoamine neurotransmitters and dietary amines). Mammals possess two MAO  
67 isoenzymes, *MAO-A* and *MAO-B*, in their genomes. In humans, only two families with *MAO-A*  
68 mutations are known, and they show severe behavioral and cognitive deficits with borderline

69 mental retardation and maladaptive regulation of impulsive aggression (*Brunner et al., 1993;*  
70 *Piton et al., 2013*). In mice also, *MAO-A/B* knockouts display developmental alterations with  
71 weight and size at birth significantly lower than wildtype littermates. On the behavioral side,  
72 *MAO* knockout mice show aberrant emotional response to novelty, high levels of anxiety-like  
73 behaviors in select tasks and low latency to attack in the resident-intruder paradigm (*Chen et*  
74 *al., 2004*). These findings underscore the importance of *MAO* function for developmental,  
75 neurophysiological and behavioral control.

76

77 Interestingly, only one *mao* gene is present in the genomes of other eukaryotes, from fungi  
78 (*Schilling and Lerch, 1995*) to invertebrates (*Boutet et al., 2004*) and teleost fish (*Chen et al.,*  
79 *1994; Setini et al., 2005; Anichtchik et al., 2006; Elipot et al., 2014*). This is despite the teleost-  
80 specific genome duplication that usually confers two gene paralogs in this vertebrate group  
81 (*Jaillon et al. 2004*). Whereas mammalian *MAO-A* and *MAO-B* have preferences for serotonin  
82 (5-HT) and noradrenaline (NA) *versus* dopamine (DA) substrates, respectively, the single fish  
83 *MAO* displays a strong affinity for 5-HT but a modest one for DA (*Anichtnik 2016; Pierre 2023*).  
84 In larval zebrafish, the inhibition of *MAO* increases 5-HT levels, which causes decreased  
85 locomotion, increased heart rate and altered vertical positioning (*Sallinen et al., 2009*).

86

87 A zebrafish knockout line for *mao* was recently generated. The *mao* knockout larvae are  
88 hypoactive at 6 days post-fertilization (dpf) and they have stronger serotonin  
89 immunoreactivity in their brains at 10 dpf (*Baronio et al., 2021*). In *A. mexicanus* on the other  
90 hand, the *mao P106L* mutation, which affects brain monoamines levels (*Pierre et al., 2023*),  
91 has a tendency to decrease basal anxiety in a familiar environment, but strongly increases the

92 amplitude of stress response after environmental change. This is associated to changes in  
93 locomotion and food intake in juveniles and young adults (*Pierre et al., 2020*).

94

95 To explore further the role of MAO enzyme on brain and behavioral homeostasis in *A.*  
96 *mexicanus*, here we have generated a surface fish line knockout for *mao*, by CRISPR/Cas9  
97 technology. This line will be useful to characterize the drastic effects of complete loss of MAO  
98 function, to compare with the partial loss-of-function *mao P106L* mutation, and to observe  
99 the consequences on fish development and physiology. Here, we adopted an integrated  
100 approach to analyze the effects of *mao* knockout in *A. mexicanus* throughout the available  
101 developmental time (*mao* knockout fry die before reaching 2 weeks). First, we explored the  
102 development of monoaminergic neurons and their neurotransmitter contents through  
103 immuno-labelling. Next, we analyzed the locomotor activity of fish over the same period.  
104 Finally, we quantified proliferation of neural progenitors and cell cycle kinetics in the fry  
105 telencephalon and hypothalamus. We found that *mao* knockout has profound effects on brain  
106 developmental homeostasis.

107

108

109

## 110 **Materials and Methods**

111

### 112 **Fish husbandry**

113 Laboratory stocks of *Astyanax mexicanus* (*De Filippi 1853*) surface fish (origin: Texas) were  
114 obtained in 2004–2006 from the Jeffery Laboratory at the University of Maryland, College  
115 Park, MD, USA and were then bred in our local facility (Gif-sur-Yvette and Saclay, France). Fish  
116 were maintained at 23–26°C on a 12 h:12 h light:dark cycle and they were fed twice a day with  
117 dry food. The fry were raised in Petri dishes and fed with an unlimited quantity of micro-worms  
118 (*Panagrellus* sp., obtained from the local amateur aquarist network), after opening of the  
119 mouth. Animals were treated according to the French and European regulations for handling  
120 of animals in research. SR's authorization for use of *A. mexicanus* in research is 91-116 and  
121 the Paris Centre-Sud Ethic Committee protocol authorization number related to this work is  
122 2017- 05#8604. The animal facility of the Institute received authorization 91272105 (for Gif-  
123 sur-Yvette) in 2015 and authorization B91272108 (for Saclay) in 2021 from the Veterinary  
124 Services of Essonne, France.

125

### 126 **Fish lines**

127 The knockout (KO) lines for *mao* were obtained using CRISPR-Cas9 technology. RNA sgRNAs  
128 were designed to target the *mao* genomic sequence containing the polymorphic SF/CF  
129 position (Fig. 1A), using the CRISPOR-TEFOR website (<http://crispor.tefor.net/>). Two sgRNAs

130 were tested, and both generated indels at the targeted sequence as follows: Sg1 5'-  
131 AGGGGCCTTTCCCACCGATGTGG-3'; Sg3 5'-CCCACCGATGTGGAACCCTCTAG-3'.

132 A mix of Cas9 mRNA (200ng/μL), sgRNA1 or sgRNA3 (500 ng/μL) and phenol red (0.05%) was  
133 injected into 1-cell stage *A. mexicanus* surface embryos. Injected embryos (G0; founders) were  
134 reared to adulthood and their progeny (G1) screened and genotyped for indel mutations (Fig.  
135 1B). Genotyping for screening of these progenies was performed on Labchip GX touch (Perkin  
136 Elmer). The *mao* KO line analyzed herein had an 11pb deletion in exon 4.

137 The only rigorous and definitive way to control off-target is genome sequencing, which we  
138 didn't perform. We generated two different *mao* KO lines by CRISPR. The first one that we  
139 used in the manuscript induced a deletion of 11 nucleotides. The second one induced one  
140 nucleotide deletion and frame shift. Our preliminary results with the second line confirm the  
141 results obtained with the first one, suggesting that the phenotype we observed are specific  
142 to *mao* KO.

143 We used the second and third generation for all the experiments.

144 To establish the *mao* KO line we incrossed the heterozygotes *mao* KO.

145

#### 146 **Immunohistochemistry, immunofluorescence and imaging**

147 These experiments were performed on embryos (36 hpf, 48 hpf; hpf: hours post-fertilization)  
148 and fry (4 to 12 days post-fertilization (dpf)) wild-type (WT), knockout (KO) and heterozygote  
149 (het) surface fish. All samples were fixed in 4% paraformaldehyde in phosphate-buffered  
150 saline (PBS), gradually dehydrated in methanol and stored at -20°C. For the  
151 immunohistochemistry, the fry were permeabilized for 30 min in 1:1 ethanol: xylene at room



152 temperature (RT), then 10 min in 1:2 ethanol: acetone at  $-20^{\circ}\text{C}$ . The samples were rehydrated  
153 in PBS and 1% Tween (PBST). Next, brains were dissected out and incubated in PBST with 6%  
154  $\text{H}_2\text{O}_2$  for 30 min at RT. The samples were incubated in blocking buffer [10%sheep serum, 1%  
155 Triton, 1% Tween 20, 1% DMSO, 1% bovine serum albumin (BSA) in PBST] for 2 hours, then  
156 incubated with the primary antibody (rat anti-serotonin, MAB352, 5/1000, Chemicon; rabbit  
157 anti-tyrosine hydroxylase, SAB2701683, 2/1000, Sigma Aldrich) in buffer (1% sheep serum, 1%  
158 Triton, 1% DMSO, 1% BSA in PBST) for 3 days, followed by the secondary antibodies  
159 (biotinylated goat anti-rat antibody, 112-066-072, 1/500, Jackson ImmunoResearch). The  
160 revelation was performed using an avidin/biotin complex coupled with peroxidase (ABC kit,  
161 Universal Vectastain kit PK6200) and diaminobenzidine (Sigma Aldrich). Brains were mounted  
162 in glycerol. Images were taken on a Nikon Eclipse 800. Images were corrected for brightness  
163 and contrast in ImageJ or Photoshop, but no other corrections were made.

164 For the immunofluorescence experiments, the samples were rehydrated in PBS and 1% Tween  
165 (PBST). Embryos were processed *in toto*. For the fry samples, we dissected the brains. The  
166 samples were incubated in blocking buffer [10%sheep serum, 1% Triton, 1% Tween 20, 1%  
167 DMSO, 1% bovine serum albumin (BSA) in PBST] for 2 hours , then incubated with the primary  
168 antibody (rabbit anti-PH3, Thermofisher 1/500; rabbit anti-caspase activated, BD pharmigen  
169 1/500; mouse anti-PCNA, Santa Cruz 1/300) for 3 days, followed by secondary antibodies (goat  
170 anti-rabbit Alexa Fluor 486, 1/1000, goat anti-rabbit Alexa Fluor 546, 1/1000) and DAPI  
171 (4/1000). The samples were mounted in Vectashield (Vector Laboratories). Images were taken  
172 on a Leica confocal SP8 microscope. The images were processed using Fiji software. All labelled  
173 cells were counted over the entire thickness of the tissue structure (telencephalon and  
174 hypothalamus) at  $10\ \mu\text{m}$  intervals, i.e. the average thickness of the cells.

175 All the experiments, including the cell counts, were carried out blindly, without knowledge of  
176 the larvae's genotype. At the end of all the experiments, samples were retrieved from  
177 mounted slides and genotyping was carried out.

178

### 179 **EdU pulse-chase**

180 A pulse of Edu 10 mg/mL in EM (with 0.01%DMSO) was carried out by bath during 1 hour. The  
181 larvae were washed in EM and fixed in 4%PFA after a chase of 6 hours. Edu-positive cells were  
182 detected with the Click-iT™ EdU Alexa Fluor™ 647 (Thermo Fisher Scientific, C10340). Samples  
183 were whole-mounted in Vectashield medium (Vector, H1000) and imaged with a confocal  
184 microscope (Leica TCS SP8). EdU revelation was performed first, followed by the  
185 immunofluorescence labelling procedure.

186

### 187 ***In situ* hybridization**

188 To clone *A. mexicanus tph1* and *tph2*, total RNA from 48 hpf embryos was reverse-transcribed  
189 using the iScript cDNA synthesis kit (Bio-Rad) and amplified using the following primers: *tph1a*  
190 forward, 5'-CATCGGCCTCACTCTGGAAG-3'; *tph1a* reverse, 5'-GCCCTTCGACCCCAAAGTTA-3';  
191 *tph2* forward: 5'-TCCGGGAGCTGACTAACTGTR-3'; *tph2* reverse : 5'-  
192 GTGATGAGGCACTCCTGGTAG-3'. PCR products were cloned in pGEM-T vector and sequenced.  
193 Digoxigenin- riboprobes were prepared using PCR products as templates. ISH was carried out  
194 as previously described (Pottin *et al.*, 2011). These experiments were performed on embryos  
195 (36 hpf) from WT, KO and het surface fish. All samples were fixed in 4% paraformaldehyde in  
196 phosphate-buffered saline (PBS), gradually dehydrated in methanol. For probe penetration,  
197 embryos were treated with proteinase K (10 µg/ml) for 20 minutes at 37°C. Embryos were

198 incubated at 65°C for 18 h in 2 ng/μl DIG-labelled mRNA probes *tph1* and *tph2*, then with POD-  
199 conjugated anti-DIG (sheep, Roche, 1:500). All *in situ* hybridization experiments used  
200 NBT/BCIP (Sigma).

201

## 202 **Image acquisition and cell counting**

203 For immunohistochemistry and *in situ* hybridization, all images were taken on Nikon Eclipse  
204 E800 microscope using 20X and 40X air objective. For immunofluorescence, all images were  
205 taken on a Leica SP8 confocal microscope using, 20X air or 40X water objectives, then the  
206 images were processed using Fiji software. Sections are presented as single confocal planes  
207 except for Fig. 7, which corresponds to maximum intensity projections of two adjacent  
208 confocal planes. All labelled cells were counted throughout the thickness of the structure by  
209 counting all the cells at 10 μm intervals.

210

## 211 **Aggressiveness assay**

212 Behavioral tests were performed on 3 months post-fertilization (mpf) wild-type SF and  
213 heterozygotes. Fish were used in a resident-intruder test, as in *Elipot et al.* (2013). Fish were  
214 placed individually in tanks (12 cm×9 cm) with 200 ml of water for the night. In the morning,  
215 intruders were transferred into tanks of residents, and a video of 1 h was recorded. Attacks  
216 between the two fish were counted manually using ODREC software (Observational Data  
217 Recorder; <https://sourceforge.net/projects/odrec/>).

218

## 219 **Locomotion**

220 The measures of locomotion on larvae between 6 and 12 days old were performed in a  
221 Zebrabox (Viewpoint), a soundproof box isolated from light in which fish were placed  
222 individually in multiwell plates (24 wells up to 8 dpf and 12 wells between 9 and 13 dpf). The  
223 plates were then placed on an infrared floor, under an infrared-sensitive camera. This system  
224 allows following the movement of the fish thanks to their pigmentation during 30 min every  
225 day, during their activity phase. Locomotor activity was recorded using ZebraLab software  
226 (Videotrack; ViewPoint Life Sciences). The recording session was preceded by a 2-hour  
227 habituation phase in the room. After recording, the fish were transferred to another well plate  
228 where the water was changed every day, and they were fed micro-worms. The plates were  
229 kept in an incubator at 24°C between recording sessions.

230 For 2-month-old fish, the experiments were performed on wild-type SF and heterozygote  
231 siblings only. Fish were placed individually in 12×9 cm tanks (200 ml). Tanks were placed on  
232 an infrared-emitting floor. Videos were recorded with an infrared detecting camera placed  
233 above the tanks, after 1 h of habituation. Videos were recorded and locomotion was measured  
234 with ViewPoint software. All these experiments was performed on the PSI-CO platform of our  
235 Institute (UMR 9197 CNRS NEUROPSI 91400 Saclay, France).

### 236 **Gut motility assay**

237 The experiments were performed on 8dpf WT and KO. Larvae were anesthetized in a 140 ppm  
238 tricaine solution. After 5 minutes of habituation, we recorded movies with 1 image every  
239 second for 15 minutes under a dissection microscope (Olympus SZX 16 ), as in Riddle et al.,  
240 2018.

### 241 **Cardiac rhythm**

242 The experiments were performed on 8 dpf WT, het and KO. Larvae were anesthetized in a 140  
243 ppm tricaine solution, and the cardiac beats were counted manually under a binocular  
244 microscope. We cannot rule out that tricaine had a bradycardic effect. However, if any, the  
245 effect was the same on all fish lines. Note that the concentration of tricaine used here was low  
246 and this method was preferred to low temperature for example, which had a clear and  
247 immediate effect on cardiac frequency.

248

### 249 **Genotyping**

250 After carrying out the various experiments in blind, the genotype of the samples was  
251 determined. The slides were opened and the whole embryos or the dissected brains placed in  
252 25 $\mu$ L of lysis buffer (10 mM Tris-HCl pH8; 50 mM KCl; 0.3% Tween20; 0.3% NP40; 4 mM EDTA).  
253 The tubes were heated at 98°C for 10 min before adding 1.25  $\mu$ L of proteinase K. The tubes  
254 were heated at 55°C for 18h, then 98°C for 10 min to denature the proteinase K. 3  $\mu$ L of this  
255 solution was used as template DNA to amplify the *mao* sequence by PCR by using sense (5'-  
256 GGGAAATCATATCCATTCAAGGGG-3') and antisense (5'-CTCCATGCCCATCTTGTCCATAG-3')  
257 primers. The PCR products were then purified (NucleoSpin Gel and PCR Clean-up, Mini kit for  
258 gel extraction and PCR clean up, REF 740609.50, Macherey-Nagel) and sent to Eurofins  
259 Genomics for sequencing.

260

### 261 **MAO enzymatic activity assay**

262 For enzymatic activities: MAO (EC.1.4.3.4.) enzymatic activities were determined on brain  
263 homogenates as described (*Elipot et al., 2014; Pierre et al., 2023*) by radioenzymology using  
264 [<sup>14</sup>C]-5-hydroxytryptamine creatinine sulphate (serotonin, 5-HT, 1.96 GBq mmol<sup>-1</sup>, Amersham

265 GE Healthcare, Saclay, France) as substrate. In brief, 20- $\mu$ l aliquots of the homogenates were  
266 pre-incubated (possibly with an inhibitor) in a total volume of 220  $\mu$ l at 37 °C for 30 min. At  
267 the end of the pre-incubation period, the enzyme reaction was started by addition of 80  $\mu$ l of  
268 MAO substrate and the incubation continued for 15 min at 37 °C. Then, the reaction was  
269 stopped by addition of 200  $\mu$ l of HCl 2N, and the deaminated metabolites were extracted by  
270 vigorous shaking for 10 min with 5 ml of diethylether. After centrifugation (1,000 *g*; 30 s) the  
271 water phase was frozen in dry ice and the organic layer poured into plastic vials containing  
272 5 ml of scintillation cocktail. Finally, the radioactivity was determined in a scintillation  
273 spectrometer. Reaction mixtures as described above, but lacking the brain homogenate,  
274 served as blanks.

275

## 276 **Statistics**

277 Data shown are representative of a minimum of two independent biological replicates.  
278 Statistical analyses were performed with R studio. All data were analyzed in appropriate pair-  
279 wise comparisons using non-parametric Mann–Whitney tests. For **Fig. 3**, Mann–Whitney  
280 pairwise comparisons were completed by a Kruskal–Wallis analysis, accounting for all  
281 comparisons of independent means across morph and genotypes ( $p < 0.0001$ ).

282

283

284

## 285 Results

286

### 287 ***A mao* knockout (KO) *Astyanax mexicanus* surface fish line**

288 We used the CRISPR/Cas9 technology to generate a *mao* KO *Astyanax mexicanus* line. The line  
289 studied below had an 11bp deletion, creating a shift of the reading frame from the 106<sup>th</sup> amino  
290 acid of the protein. This gave rise to a STOP codon and generated a truncated protein of 127  
291 amino acids (out of 522 amino acids total; **Fig. 1 A and B, CC'**). Accordingly, MAO enzymatic  
292 activity in 6dpf embryos *mao*<sup>-/-</sup> KO heads was reduced down to almost zero as compared to  
293 WT (**Fig. 1D**). The morphological appearance of the KO individuals was similar to WT and het,  
294 and they could not be recognized by any simple phenotypic trait (even though they may be  
295 slightly smaller) (**Fig. 1 E**). The *mao*<sup>-/-</sup> KO fry died abruptly at 13 days post-fecundation. Thus,  
296 all analyses below were performed between 1 and 12 dpf.

297 Serotonin is implicated in intestinal function, particularly in the motility of the gut (*Bülbring*  
298 *and Crema, 1958; Gershon, 2013; Mawe and Hoffman, 2013*). To check whether a deficit in  
299 intestinal function could cause the defect of KO fish and their death, we performed gut  
300 motility measurements in 8dpf embryos (i.e., 2 days after mouth opening and the beginning  
301 of independent feeding in *Astyanax*). The intestinal motility of KO fry was qualitatively  
302 indistinguishable from that of WT (**Suppl Fig. 1 A-D**).

303 Similarly, serotonin affects cardiac rhythm, development and function in mice (*Abzalov et al.,*  
304 *2015; Mialet-Perez et al., 2018; Nebigil and Maroteaux, 2001; Nebigil et al., 2000; Stoyek et*  
305 *al., 2017*). Yet, the KO fry had the same cardiac rhythm at 8dpf as the WT or heterozygotes

306 **(Suppl Fig.1 E)**. These data suggest that *mao* KO fry die for reasons that are not related to  
307 intestinal motility or cardiac beating defects.

308

### 309 **Monoaminergic systems in *mao*<sup>-/-</sup> KO embryos and larvae**

310 As the MAO enzyme catalyzes the oxidative deamination of monoamine neurotransmitters,  
311 we first assessed the developmental anatomy of dopamine, noradrenalin and serotonin  
312 neurons in *mao* KO brains.

313 We first analyzed the effect of the loss of MAO function on the DA and NA system at 6 dpf by  
314 immunofluorescence staining against tyrosine hydroxylase (TH), their biosynthesis rate-  
315 limiting enzyme. The organization and the number of DA neurons (corresponding to all  
316 neuronal clusters in Figure 2 except LC) and NA neurons (LC, locus coeruleus) did not differ in  
317 KO as compared to WT and het larvae (**Fig. 2A-C**). The staining intensity for TH in neurons was  
318 not different either in brains from the three genotypes.

319 We next analyzed the serotonergic system by immunostaining against 5-HT. Different spatio-  
320 temporal effects of MAO loss of function were observed, in the hypothalamus and in the raphe  
321 5-HT neuronal clusters, between 36 hpf and 12 dpf.

322 At all stages, WT and heterozygotes fish showed similar 5-HT labeling and were together  
323 assimilated as controls. Early in development at 36 hpf, 5-HT labeling was stronger in KO than  
324 in controls, both in the hypothalamus (note that only the anterior paraventricular nucleus, or  
325 PVa, is observed at this stage) and in the raphe nucleus (**Fig. 3 A, B**). Then, labeling intensity  
326 progressively decreased in the hypothalamic region in KO individuals, reaching its lowest level  
327 at 6 dpf (**Fig. 3 F, H, J**). This progressive decrease in labeling involved the anterior regions of



328 the hypothalamus, the anterior paraventricular nucleus (PVa) and the intermediate nucleus  
329 (PVi) between 4 and 6 dpf, and also affected the posterior nucleus (PVp) at 6 dpf (**Fig. 3 F, H,**  
330 **J**).

331 At 8 dpf, in *mao* KO brains there was a progressive reappearance of 5-HT immunolabeling in  
332 PVp and in PVi (**Fig. 3 L**), and labeling subsequently returned to normal in the 3 hypothalamic  
333 5-HT clusters at 10 and 12 dpf as compared to controls (**Fig. 3 N, P**). Thus, the pattern of 5-HT  
334 immunoreactivity in the hypothalamic region seems highly dynamic in *mao* KO, with spatial  
335 and temporal variations during development. In the raphe however, at all stages studied, 5-  
336 HT neurons remained much more strongly labelled in KO than in control fry (**Fig. 3 A-P**). Overall,  
337 these data show that serotonergic neurons in the hypothalamus show finely tuned and  
338 dynamic regulation of serotonin levels across developmental stages upon loss of MAO  
339 function, in contrast to the raphe nucleus where serotonin levels are constantly heightened  
340 (**Fig. 3 Q**).

341 Finally, we have previously found that upon partial loss of MAO function due to the *mao P106L*  
342 mutation, the increase in brain 5-HT levels caused by lack of degradation through  
343 malfunctioning MAO is partially compensated for by a decrease in the activity of TPH  
344 (tryptophane hydroxylase), the 5-HT biosynthetic enzyme (*Pierre et al., 2023*). Moreover, in  
345 surface fish this compensation is observed in the hypothalamus but not in the raphe (*Pierre et*  
346 *al., 2023*). Thus, we sought to test the possibility of a differential regulation of TPH in the  
347 hypothalamus and in the raphe in the context of *mao* KO. In fish, there are two different *tph*  
348 genes, *tph1a* and *tph2*, specifically expressed in the hypothalamus and raphe, respectively.  
349 We performed *in situ* hybridizations for *tph1a* and *tph2* at 36 hpf, with the aim of determining  
350 whether there are variations in *tph* expression related to the *mao* KO genotype (**Suppl Fig. 2**

351 **G, H).** *Astyanax tph1a* was expressed in two discrete bilaterally symmetrical areas in the  
352 ventral forebrain, corresponding to the developing hypothalamus, in the position of the PVa  
353 nucleus. The localization, the intensity and the number of labeled cells did not vary according  
354 to the different *mao* genotypes at 36 hpf (**Suppl Fig.2 G**). *Tph2* was expressed more posteriorly  
355 than *tph1a*, in two discrete areas in the hindbrain at the level of the raphe nucleus. Again, no  
356 difference was observed between *mao* genotypes at 36 hpf (**Suppl Fig. 2 H**).

357

### 358 **Locomotion in *mao*<sup>-/-</sup> KO embryos and larvae**

359 Serotonin and MAO have demonstrated roles in the control of locomotor activity in 6 dpf  
360 zebrafish larvae (*Sallinen et al., 2009; Baronio et al., 2022*). As 5-HT content in neurons  
361 followed important variations as a function of age in *Astyanax mao* KO fry (**Fig. 3Q**), we  
362 recorded the locomotion 30 min per day, at the time of their activity phase, throughout the  
363 available developmental time between 6 and 12 dpf, to gain insight into the evolution of  
364 locomotion as a function of development. Of note, we initially wished to carry out this  
365 experiment on the whole duration of the developmental period studied above, however  
366 before 6 days *Astyanax* larvae were not sufficiently large and pigmented for proper tracking  
367 in the Zebrabox system. Moreover, before this stage the larvae had a rather limited  
368 locomotion, independently of their genotype.

369 The distances traveled by WT and het fry were identical at all developmental stages recorded.  
370 Between 7 and 10 dpf, the KO fish swam significantly less than WT and het, covering about  
371 100 cm during the 30 min of recording, compared to about 250 cm for the controls over the  
372 same period (**Fig. 4 A, B, C**). The comparison of trajectories illustrates well the differences in  
373 locomotion between the different genotypes (**Fig.4 B, C**). At 11 and 12 dpf however, the

374 locomotion of *mao* KO was not different from controls anymore (**Fig. 4 A**). We also analyzed  
375 locomotion in juveniles and compared the available het and WT fish. At 2mpf, the het covered  
376 a longer distance in 10 minutes than the WT (**Fig. 4 D**). In sum, MAO inactivation affected  
377 locomotion in a stage-dependent manner in *Astyanax*.

378 Finally, as the double *MAO-A/MAO-B* KO mice are very aggressive (*Chen et al., 2004*), we  
379 analyzed the aggressiveness in 3 month old fish, using a resident-intruder assay. There was no  
380 difference between WT and het in the number of attacks performed in one hour between two  
381 fish of the same genotype (**Fig. 4 E**).

382

### 383 **Proliferation of brain progenitors**

384 The distribution of serotonergic neuronal nuclei and their fibers overlap with highly  
385 proliferative areas of fish brain (*Grandel et al., 2006*). Moreover, studies on zebrafish have  
386 shown that the inhibition of serotonin synthesis modifies the proliferation pattern in the  
387 periventricular hypothalamic regions (*Perez et al., 2013*). Thus, we hypothesized that *mao* KO  
388 and associated neuronal 5-HT level variations may influence the proliferation or, more  
389 generally, the biology of brain progenitors.

390 Fluorescent immunostaining against Phospho-histone H3 (PH3), which labels all cells in M  
391 phase of the cell cycle, was performed to measure the amount of proliferation in the brain. In  
392 36 hpf and 48 hpf embryos, the number of proliferating cells did not differ between the three  
393 genotypes (WT, het, KO), whether the analysis was performed globally in the brain or focused  
394 on specific regions (telencephalon and hypothalamus) (**Suppl Fig. 2 A-C**).

395 At later larval stages (6dpf, 8 dpf and 10 dpf), we next monitored the number of PH3-positive  
396 cells in the telencephalon, a richly proliferative region receiving neuronal projections from the  
397 raphe, and in the hypothalamus (which contains 3 groups of serotonergic neurons). The  
398 proliferative PH3-positive cells were present in the ventricular zone of the telencephalon (**Fig.**  
399 **5A-C**) and in a large and continuous proliferation zone lining the periventricular hypothalamus  
400 (**Fig. 6A-C**). At 6dpf, the number of PH3 cells was identical in KO and WT larvae, both in the  
401 telencephalon (**Fig.5A-C**) and in the hypothalamus (**Fig. 6A-C**). At 8dpf, in KO we observed a  
402 reduction of the number of proliferative cells in the telencephalon (**Fig.5A-C**) and in the  
403 hypothalamus (**Fig. 6A-C**) as compared to the WT. At 10 dpf, KO fry showed a dramatic  
404 reduction of proliferative cells in both the telencephalon (**Fig. 5 A – C**) and hypothalamus (**Fig.**  
405 **6 A-C**). In fact, at this stage the number of proliferative cells in both regions was almost zero.  
406 These results suggested a potential link between MAO and brain proliferation in *Astyanax* fry.  
407 Next, we reasoned that the reduction of the number of cells in M phase (PH3-positive) in the  
408 8-10 dpf KO brains might be due either to a longer cell cycle length, or to fewer cells entering  
409 the cell cycle, or both. To discriminate between these possibilities, we performed an  
410 experiment in which 8 dpf larvae were incubated for 1 hour with EdU (which incorporates in  
411 cells during the S phase of the cell cycle), followed by a chase of 6 hours. We estimated that  
412 within 6 hours the cells in S phase should have progressed in the cell cycle to reach the M  
413 phase. In addition to the EdU marker, we used a general proliferation marker (PCNA) to label  
414 all the cells in proliferative state as well as a specific marker to label the cells in M phase (PH3).  
415 In *mao* KO, we observed a drastic reduction of the number of cells labelled for all three  
416 markers of proliferation: EdU (S phase), PH3 (M Phase) and PCNA (entire cell cycle), both in  
417 the telencephalon (**Fig.7 A-B**) and in the hypothalamus (**Fig.7 D-E**). These results suggest a

418 strong overall decrease in brain proliferation in *mao* KO larvae, including very few cell entering  
419 the cell cycle.

420 Then, to assess a potential difference in cell cycle length, we calculated how many cells that  
421 have incorporated the EdU 6 hours before fixation of the larvae, have reached the M phase.  
422 In the telencephalon, there was a strong tendency, albeit non-significant, towards a reduction  
423 of this number in the KO fry as compared to WT and het (**Fig. 7 C**). In the hypothalamus, the  
424 percentage of cells in M phase (PH3+) 6 hours after they were in S phase (EdU+) was  
425 significantly decreased in KO compared to WT and het (**Fig.7 F**). These data suggest that cell  
426 cycle length is also affected and is longer in *mao* KO larvae.

427

#### 428 **Cell death in the brain of *mao*<sup>-/-</sup> embryos**

429 Finally, in parallel to an effect of loss of MAO function on proliferation, we tested a potential  
430 effect of MAO on apoptosis, which we assessed through activated caspase 3  
431 immunofluorescence. In 36 hpf embryos, we observed no overall difference in cell death  
432 between WT, het and KO brains (**Suppl Fig. 2 D**). The same was true at 48 hpf (**Suppl Fig. 2 E**).  
433 Looking more specifically at the different forebrain regions, the telencephalon and the  
434 hypothalamus, there was also no difference between the three genotypes, neither at 36 hpf  
435 nor at 48 hpf (**Suppl Fig. 2F**). These results suggest that the loss of MAO has no effect on cell  
436 death in the brain of *A. mexicanus* surface fish embryos at these stages.

437

438

439 **Discussion**

440

441 Here, we have investigated the developmental outcomes of loss of MAO function in embryos  
442 and larvae of the fish *Astyanax mexicanus*. Although the effects on larval locomotion could  
443 have been anticipated (see *Baronio et al., 2021*), those we discovered on the fine regulation  
444 of serotonin content in different brain neuronal clusters on one hand, and on the control of  
445 brain progenitor proliferation on the other hand, were unexpected and open novel research  
446 opportunities.

447

448 ***mao* knockout is lethal in fish**

449

450 *Astyanax* individuals that are KO for *mao* die abruptly at larval stage, between 13 dpf and 15  
451 dpf. This observation is similar to what was reported for zebrafish *mao* KO, i.e., death within  
452 20 days post-fertilization (*Baronio et al., 2021*). MAO KO mice (MAO-A, MAO-B, or double  
453 MAO-A/B knockouts) do not die but display several abnormalities. MAO-A KO mice show  
454 elevated levels of 5-HT and hyperactivation of 5-HT<sub>1B</sub> receptor which induce  
455 neurodevelopmental alterations (*Cases et al., 1995; Vitalis et al., 1998; Salichon et al., 2001*).  
456 Further, MAO-A KO mice display elevated aggressiveness (*Cases et al., 1995; Scott et al., 2008*)  
457 and reduction in exploratory activity (*Godar et al., 2011*). MAO-B KO mice show increased  
458 novelty-seeking and reduced anxiety (*Bortolato et al., 2009*). Double MAO-A/B knockouts  
459 exhibit aberrant emotional response to novelty, high levels of anxiety-like behaviors and low  
460 latency to attack in the resident-intruder paradigm (*Chen et al., 2004*). All these adult  
461 phenotypes cannot be assessed in *mao* KO fish which die prematurely.

462 Although serotonin is implicated in the control of intestinal function and cardiac rhythm, we  
463 did not find any evidence for an involvement of these processes in the lethality of the loss of  
464 MAO function in our model. Death might be due to multiple dysfunctions, some unknown and  
465 some related to the major brain homeostasis defects that we have uncovered and that are  
466 discussed below.

467

#### 468 ***mao* knockout affects differentially the fish serotonergic and dopaminergic systems**

469

470 In zebrafish, pharmacological experiments had suggested that 5-HT is the preferred substrate  
471 for MAO, and that DA and NA would be poor substrates for the enzyme (*Sallinen 2009*;  
472 *Anichtnik 2006*). In *Astyanax*, direct measurements of the affinity of MAO for 5-HT and DA  
473 further demonstrated a ~ 30-fold difference, the  $K_m$  for 5-HT being in the order of  $12\mu\text{M}$  and  
474 the  $K_m$  for DA being  $\sim 370\mu\text{M}$  (*Pierre et al., 2023*). Thus, the affinity of the single fish MAO  
475 enzyme for its preferred substrate 5-HT is similar to the affinities of MAO-A for 5-HT and NA,  
476 and of MAO-B for PEA and DA, respectively, in mammals (*Bortolato et al., 2011*; *Peters et al.,*  
477 *2011*; *Darryle et al., 1981*). The lower affinity of the fish MAO for DA and NA poses the  
478 unsolved question of the catabolic pathway that is used in fish to degrade these  
479 catecholamines. Furthermore, based on these features, one can anticipate or predict that the  
480 effects of MAO perturbation in fish should be stronger on the serotonergic than on the  
481 dopaminergic and noradrenergic systems.

482

483 Accordingly, a complete loss of MAO enzyme activity after *mao* knockout in fish is expected  
484 to give rise to a dramatic increase in the amount of serotonin levels in the brain. This is what  
485 we consistently observed in the raphe nucleus throughout the developmental period where

486 we had access to homozygote knockout specimens, and using the intensity of 5-HT  
487 immunoreactivity as a proxy of neurotransmitter content. However and surprisingly, in  
488 contrast to those in the raphe, the serotonergic neurons of the hypothalamus showed very  
489 fine and dynamic serotonin level regulation upon loss of MAO function. While at 36hpf  
490 hypothalamic 5-HT neurons contained more serotonin in KO than in WT (consistently with  
491 expectations), at 48hpf the difference disappeared. Further, between 4dpf and 8dpf a drastic  
492 and unexpected reduction of 5-HT levels occurred in the three serotonergic nuclei composing  
493 the PVO in KO fish. In addition and surprisingly, starting at 8dpf, the serotonin signal re-  
494 increased to reach control levels at 12dpf. Of note, Baronio et al. (2021) described a decrease  
495 in the number of hypothalamic serotonergic neurons in 10dpf zebrafish in *mao* knockouts.  
496 Our detailed time-course analysis rather suggests that 5-HT neurons are transiently not  
497 detectable anymore because their neurotransmitter content is extremely low, but that they  
498 are still present, as demonstrated by their “re-appearance” a few hours later. Our results  
499 further suggest that loss of MAO function does not affect the development and the number  
500 of 5-HT neurons in the hypothalamus, but that it strongly affects the serotonergic content and  
501 signaling pathway. The highlighting, thanks to the *mao* KO line, of such fine regulation strongly  
502 suggests that the homeostasis of 5-HT signaling in the hypothalamus is crucial for proper brain  
503 and behavior development. The mechanisms underlying the rapid fluctuation in 5-HT content  
504 in the hypothalamus (but not the raphe) are unknown and can only be discussed.

505

506 The hypothesis that 5-HT is able to regulate its own biosynthetic pathway by negative  
507 feedback on the expression or the activity of the TPH enzyme would explain the observed  
508 wave profile of hypothalamic 5-HT immunoreactivity. As soon as serotonin reaches higher  
509 levels than the “control” level (at 36 hpf), a negative feedback loop would start functioning



510 and the 5-HT content would rapidly return to “normal” (here at 48 hpf). The inertia of this  
511 regulation could lead to a continuation of the decrease in 5-HT level until 6 dpf, the stage at  
512 which 5-HT labelling is the lowest in the three hypothalamic nuclei. Finally, from 8 days  
513 onwards, the very low level of 5-HT in neurons would stop the negative feedback, allowing  
514 the production of serotonin again and explaining the progressive re-increase in 5-HT staining  
515 observed between 8 and 12 dpf. This hypothesis can be implemented at two main levels:  
516 either at the level of regulation of the transcription of the enzyme, or at the level of its activity.  
517 Regarding transcriptional control, here we have some preliminary evidence that *tph*  
518 expression is unchanged in *mao* KO, at least at 36hpf, a stage when the expression of *tph1a*  
519 would be expected to decrease in response to elevated levels of 5-HT. Later stages should be  
520 examined as well. Regarding enzymatic activity control, we have not attempted yet to assay  
521 TPH activity due to technical limitations on the amount of tissue and samples needed to  
522 measure enzymatic activity on tiny parts of embryonic brains. However, we favor this  
523 hypothesis of a feedback loop controlling MAO enzymatic activity as a function of 5-HT  
524 neuronal levels, because we have recently demonstrated the existence of this mechanism at  
525 work in adult *Astyanax* brains carrying the partial loss-of-function *mao* P106L mutation (*Pierre*  
526 *et al.*, 2023).

527

528 Finally, the striking difference in serotonergic regulation in the hypothalamus and in the raphe  
529 nucleus after *mao* knockout adds to the many differences already described between 5-HT  
530 neurons in these two regions of the fish brain – at the difference of mammalian brain where  
531 5-HT neurons are exclusively located in the raphe. At all levels - specification, metabolism,  
532 regulation - raphe and hypothalamic 5-HT neurons appear distinct (*Lillesaar, 2011*). Their  
533 development requires the function of different ETS-domain transcription factors: *pet1* in the

534 hindbrain, *etv5b* and *fezf2* in the forebrain (Bosco *et al.*, 2013; Rink and Guo, 2004; Berberoglu  
535 *et al.*, 2009). They express different *tph* enzymes and serotonin transporter (*SERT*): *tph2* and  
536 *SERTA* in the raphe, *tph1a* and *SERTB* in the hypothalamic clusters (Herculano and Maximino,  
537 2014; *this paper*; Norton *et al.*, 2008; Wang *et al.*, 2006; Wen *et al.*, 2008; Yamamoto *et al.*,  
538 2011). Finally, they respond differently to metabolic or genetic perturbations (Pierre *et al.*,  
539 2023; *this paper*). In sum, 5-HT neurons of the hypothalamus and the raphe, although they all  
540 use serotonin as a neurotransmitter, are distinct in identity (and probably in function). They  
541 highlight the idea of convergent mechanisms leading to monoaminergic specification and  
542 differentiation in the fish brain.

543

544 The dopaminergic system on the other hand was unchanged between the *mao* KO and WT  
545 larvae at 6dpf, neither in the organization of neuronal clusters nor in the intensity of TH  
546 immunostaining. The same holds true for noradrenergic neurons. This is in line with the idea  
547 discussed above of DA and NA being poor substrates for fish MAO. In zebrafish too, a reduction  
548 in the number of Th1-immunoreactive cells in only one DA cluster was reported in *mao*  
549 knockouts, without overall difference in *th1* transcript levels in whole larvae at 10 dpf (Baronio  
550 *et al.*, 2021). Although the assessment of 5-HT itself (i.e., the neurotransmitter) to study  
551 serotonin is not equivalent to the assessment of TH (i.e., the biosynthetic enzyme)  
552 immunoreactivity or expression to study DA and NA, the TH “tool” was used to investigate  
553 catecholamines in both species, zebrafish (Baronio *et al.*, 2021) and *Astyanax* (*this paper*)  
554 because immunohistochemistry against DA is technically challenging. Nevertheless, the  
555 definitive proof for an absence (or not) of major change in DA or NA signaling after *mao*  
556 knockout in fish will need further investigation, using HPLC for example.

557

558 ***mao* knockout and locomotion in larval fish**

559

560 In zebrafish, modulation of the serotonergic system (pharmacological treatment with  
561 serotonin, serotonin agonists, serotonin reuptake or synthesis inhibitors) from the fourth day  
562 of development onwards induces changes in locomotion (*Airhart et al., 2007; Airhart et al.,*  
563 *2012; Brusteina and Drapeau, 2005; Brusteina et al., 2003a; Brusteina et al., 2003b; Drapeau et*  
564 *al., 2002; Sallinen et al., 2009*). Moreover, zebrafish *mao* knockouts are hypoactive at 6dpf  
565 (*Baronio et al., 2021*). In *Astyanax* too, we found that *mao* KO individuals swim shorter  
566 distances than controls between 7 and 10 dpf. However, our time-course analysis revealed  
567 that at 11 and 12 dpf locomotor activity was back to values similar to controls. Strikingly, this  
568 variation is parallel to the changes we observed for serotonin levels in the hypothalamus, with  
569 a slight delay (**Fig. 8**). Indeed, the stages at which serotonin levels are lowest in PVO are  
570 between 5 and 8 dpf, and then they increase again at 10 and 12 dpf. It is therefore tempting  
571 to speculate that the two phenomena are related. Furthermore, if the causal relationship  
572 between hypothalamic 5-HT levels and locomotor activity were true, it would mean that the  
573 locomotion in fish larvae is controlled by the serotonergic neurons in the hypothalamus, not  
574 those of the raphe.

575

576 We also performed locomotion assays in 2 months old juveniles, and observed hyperactivity  
577 of the heterozygotes compared to the WT. These results are different from those obtained in  
578 adult zebrafish *mao* knockouts, where no difference was found (*Baronio et al., 2021*). In adult  
579 fish, treatment with a serotonin reuptake inhibitor (citalopram, fluoxetine) or directly with  
580 serotonin shows an inhibitory effect of serotonin on locomotion (*Fingerman, 1976; Gabriel et*  
581 *al., 2009; Perreault et al., 2003; Winberg et al., 1993*). Other studies, however, show no effect

582 of serotonin on locomotion (*Winberg and Thörnqvist, 2016*), or even an activating effect  
583 (*Clements et al., 2003*). In mammals as well, the effects of 5-HT signaling on locomotion are  
584 variable among studies. For example, treatment with a serotonin reuptake inhibitor  
585 (citalopram) reduces locomotion in neonatal mice (*Dunbar et al., 2010*). On the contrary, the  
586 double *MAO-A/B* KO mice are hyperactive (*Chen et al., 2004*). Thus, the contribution of  
587 serotonin in the control of locomotion appears complex, and probably depends on both the  
588 developmental stage (adult, juvenile, larva) and the model studied. The effect of serotonin in  
589 controlling locomotion is probably also modulated by other factors.

590

#### 591 ***mao* knockout affects neural progenitor proliferation and cell cycle kinetics**

592

593 Three pieces of evidence prompted us to investigate a potential influence of MAO function on  
594 embryonic and larval brain proliferation: (1) the anatomical correlation between the 5-HT  
595 clusters and their projections with highly proliferative areas of fish brain (*Grandel et al., 2006*);  
596 (2) a pioneer study suggesting a functional link between 5-HT signaling and proliferation in the  
597 hypothalamic ventricular region (*Perez et al., 2013*); (3) the recent report that in the  
598 embryonic ferret and fetal human neocortex, 5-HT stimulates the proliferation of basal  
599 progenitors, and notably of radial glial cells, through HTR2A receptor signaling (*Xing et al.,*  
600 *2020*). Thus, in the *Astyanax mao* KO model, we hypothesized that MAO loss-of-function and  
601 associated neuronal 5-HT level variations may influence the proliferation or, more generally,  
602 the biology of brain progenitors.

603

604 In line with our hypothesis, we discovered a dramatic loss in the number of proliferating cells  
605 in both the hypothalamus and the telencephalon, associated to an increase in cell cycle length

606 (hence a slowdown of the cell cycle) in *mao* knockout larvae, from 8dpf onwards. We do not  
607 know whether these two effects are linked or independent. The *mao* KO larvae have fewer  
608 EdU-positive cells that reach the M phase, which suggests that cells remain longer in G2 phase  
609 and therefore enter mitosis less quickly. We can only speculate that slowing down of the cell  
610 cycle progression may in part contribute to a decrease in the re-entry of progenitors into the  
611 cell cycle.

612  
613 Importantly, our results demonstrate that MAO function on monoamine homeostasis controls  
614 larval fish brain proliferation. This process is probably mediated by 5-HT signaling (see above).  
615 As for locomotion, the deregulation of serotonin levels and proliferation dynamics are slightly  
616 shifted in time but parallel (**Fig. 8**). Thus, there would be a delay between 5-HT signaling and  
617 its influence on proliferative cells. The proliferative phenotype observed from 10 dpf in *mao*  
618 KO larvae would therefore be the result of the decrease in hypothalamic serotonin level  
619 observed at 6 and 8 dpf. This result is consistent with the idea that 5-HT signaling stimulates  
620 progenitor proliferation in the hypothalamus, as proposed in zebrafish, in which acute  
621 pharmacological inhibition of serotonin production induces a decrease in proliferation in the  
622 adult PVO (*Pérez et al., 2013*).

623  
624 The results we observed in the telencephalon are more surprising. Indeed, our experiments  
625 show a reduction in the number of proliferating cells in the telencephalon that is comparable  
626 to that observed in the hypothalamus. Yet, 5-HT signaling in the telencephalon mostly  
627 originates from the raphe (*Lillesaar, 2011*), a cluster that shows very high 5-HT  
628 immunoreactivity in the *mao* KO throughout development. Could drastic 5-HT deregulation,  
629 whether positive or negative, induce the same type of phenotype? These seemingly

630 inconsistent results could be more easily explained by the specific expression of distinct  
631 serotonin receptors in the telencephalon and hypothalamus, which would have an opposite  
632 effect on proliferation.

633

634 The serotonin receptors HTR2A that stimulates the proliferation of radial glia in the ferret and  
635 human embryonic cortex is not expressed in the lissencephalic mouse embryonic neocortex;  
636 consequently, 5-HT does not modulate mouse basal progenitor proliferation (*Xing et al, 2020*).

637 This study illustrates an evolutionarily relevant function of 5-HT in the control of brain  
638 proliferation. This interspecies difference seems to be related to a different mode of  
639 progenitor division. In mice, basal progenitors undergo consumptive division to give rise to  
640 two neurons. In humans, basal radial glia are more abundant and capable of giving rise to  
641 more basal progenitors. In the zebrafish telencephalon, the mode of division of radial glia  
642 progenitors is more similar to that of humans than to that of mice (*Than-Trong et al, 2018*).

643 Thus, from an evolutionary point of view, it will be very interesting to explore the effect of  
644 MAO and serotonin on progenitor and stem cell biology in fish. There are 23 serotonin  
645 receptors in the zebrafish genome, and their specific spatiotemporal expression patterns  
646 description is fragmentary (*Zfin database*). It will be important to determine exactly where  
647 these receptors are expressed and how they are involved in the control of proliferation in fish  
648 brains.

649

650 **Conclusion**

651

652 Altogether, our study shows that the loss of MAO function strongly affects the homeostasis of  
653 serotonergic neurons, the locomotor activity and the proliferation of brain progenitors in  
654 *Astyanax* surface fish larvae (**Fig. 8**). The lethality of *mao* knockout at larval stages in the two  
655 fish species where it has been achieved so far (zebrafish and *Astyanax*) renders impossible the  
656 study of older animals and the further deciphering of MAO functions in mature brains.  
657 Interestingly, some populations of cavefish morphs of the *Astyanax* species carry a natural,  
658 partial loss-of-function mutation in *mao* that should allow investigating, probably with more  
659 subtle and physiologically-relevant effects than the complete gene inactivation, the outcomes  
660 of compromised MAO function on neural progenitors homeostasis as well as their  
661 consequences on behavior.

662

### 663 **Authors' contributions**

664 Conceptualization: AA, SR; Methodology: AA, CP, J T-P, NC, AL, MP, T E-P, ML, MB.

665 Writing - original draft: AA, SR; Funding acquisition: SR.

666

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672

673 **Competing interests**

674 The authors declare that they have no competing interests.



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852 **Legends**853 **Figure 1:** Generation of a *mao* KO *Astyanax mexicanus* surface fish line with CRISPR-Cas9.854 A, Genotyping by Labchip on F1 fish (progeny of Fo zero injected fish). Red asterisks indicate  
855 heterozygote fish with an indel in exon 4, visible by the presence of several bands  
856 corresponding to heteroduplexes of PCR fragments. The black arrow indicates the expected  
857 band size.858 B, Chromatograms showing the sequence of *mao* exon 4 in WT, KO and heterozygotes (het).  
859 The 11 nucleotides indel is indicated. The shift in the reading frame is visible on the het  
860 chromatogram.861 CC', Protein alignment and functional domain comparison. WT MAO is a 531 aminoacid  
862 protein. TheKO form the protein is affected after amino acid 106, which results in the almost  
863 complete truncation of the amino-oxidase functional domain.864 D, MAO enzymatic activity in the heads of 6dpf larvae that are WT (grey) or KO (yellow) for  
865 *mao*. Each sample (n=1) was formed with 15 heads. P-value after Mann-Whitney test.

866 E, Morphological aspect of 6dpf larvae from the 3 genotypes, as indicated.

867 **Figure 2:** Anatomy of TH-positive cells in the brain of 6dpf larvae.868 A-B, Whole brain confocal projections showing a general view of TH-immunopositive cells  
869 (cyan) in WT (A) and KO (B) fish. Eyes were removed. Scale bar: 100µm.870 C, Quantification of the number of TH<sup>+</sup> cells in the preoptic anterior parvocellular nucleus  
871 (Papc), the suprachiasmatic nucleus (Sup), the posterior tuberculum (Pt), the posterior tuberal  
872 nucleus (Ptn), the olfactory bulbs and subpallium (OB/Sp), the locus coeruleus (LC) and the  
873 medulla oblongata (MO) of WT (grey), het (orange) and KO (yellow) fish. The number of brains  
874 analysed is indicated. All pairwise Mann-Whitney comparisons retrieved p-values > 0.05,  
875 except one in the Sup.

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877 **Figure 3:** Anatomy of 5-HT-positive cells in the brain during the development of 5-HT-positive  
878 cells in the brain during the development.879 A-D, ventral view of serotonin immunostaining in WT and KO, 36 and 48 hpf embryos. The  
880 serotonergic neurons are present in the hypothalamus (hyp) and in the raphe (ra). Scale bar:  
881 40 µm.882 E-P, ventral view of serotonin immunostaining in WT and KO dissected brains (4dpf-12dpf).  
883 The three regions of the hypothalamus where the serotonergic neurons are present are  
884 indicated: the anterior paraventricular nucleus (pva), the intermediate paraventricular nucleus  
885 (pvi) and the posterior paraventricular nucleus (pvp). The serotonergic neurons are also  
886 present in the raphe (ra) indicated on the picture. Scale bar: 40 µm.887 Q, Schematic representation illustrating variations of serotonin immunostaining during brain  
888 development in *mao* KO *Astyanax mexicanus*.

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890 **Figure 4:** Behavioral experiments.

891 A, quantification of distance covered in 30 minutes at the seven stages studied and for the  
892 three genotypes.  $N = 11$  (WT) ;  $N = 25$  (het) ;  $N = 14$  (KO).

893 B-C, tracking the trajectories of fish according to their genotype. Two representative  
894 examples are shown for each genotype (7dpf and 10 dpf).

895 D, quantification of distance moved in 10 minutes in 2 months old fish (WT and  
896 heterozygotes). N is indicated on the graph.

897 E, quantification of attacks in 3 months old fish (WT and heterozygotes). N is indicated on the  
898 graph.

899

900 **Figure 5:** PH3 immunostaining in the telencephalon.

901 A, ventral view of confocal optical plane (3  $\mu$ m) of WT telencephalon at 6, 8 and 10 dpf stained  
902 for the proliferative marker PH3 (green). DAPI is in blue. Scale bar: 20 $\mu$ m.

903 B, ventral view of confocal optical plane (3  $\mu$ m) of KO telencephalon at 6, 8 and 10 dpf stained  
904 for the proliferative marker PH3 (green). DAPI is in blue.

905 C, quantification of PH3-positive cells in the three genotypes at 6, 8 and 10 dpf. N is indicated  
906 on the graph. \* $P_{adj} < 0.05$ , \*\* $P_{adj} < 0.005$ .

907

908 **Figure 6:** PH3 immunostaining in the hypothalamus.

909 A, ventral view of confocal optical plane (3  $\mu$ m) of WT hypothalamus at 6, 8 and 10 dpf stained  
910 for the proliferative marker PH3 (green). DAPI is in blue. Scale bar: 20 $\mu$ m.

911 B, ventral view of confocal stacks (3  $\mu$ m) of KO hypothalamus at 6, 8 and 10 dpf stained for the  
912 proliferative marker PH3 (green). DAPI is in blue.

913 C, quantification of PH3-positive cells in the three genotypes at 6, 8 and 10 dpf. N is indicated  
914 on the graph. \* $P_{adj} < 0.05$ , \*\* $P_{adj} < 0.005$ , \*\*\*\* $P_{adj} < 0.00005$ .

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916 **Figure 7:** Proliferation kinetics.

917 A, ventral view of confocal optical plane (3  $\mu$ m) of KO telencephalon at 8 dpf stained for the  
918 EdU (magenta), phase M marker PH3 (green) and general marker of the cell cycle PCNA (blue).  
919 DAPI is in gray. Scale bar: 20 $\mu$ m.

920 B, quantification of EdU-positive cells, PH3 positive cells and PCNA positive cells in the three  
921 genotypes at 8 dpf in the telencephalon. N is indicated on the graph. \* $P < 0.05$ , \*\* $P < 0.005$ .

922 C, quantification of the number of PH3-EdU-positive cells on the EdU total number of positive  
923 cells in the three genotypes at 8 dpf in the telencephalon. N is indicated on the graph.  $P_{adj} < 0.1$

924 D, ventral view of confocal optical plane (3  $\mu$ m) of KO hypothalamus at 8 dpf stained for the  
925 EdU (magenta), phase M marker PH3 (green) and general marker of the cell cycle PCNA (blue).  
926 DAPI is in gray. Scale bar: 20 $\mu$ m.

927 E, quantification of EdU-positive cells, PH3 positive cells and PCNA positive cells in the three  
928 genotypes at 8 dpf in the hypothalamus. N is indicated on the graph. \* $P_{adj} < 0.05$ , \*\* $P_{adj} < 0.005$ .

929 F, quantification of the number of PH3-EdU-positive cells on the EdU total number of positive  
930 cells in the three genotypes at 8 dpf in the hypothalamus. N is indicated on the graph.

931 . \* $P_{adj} < 0.05$ .

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**Figure 8: Summary**

Graphical and schematic representations of the variations we have observed on various phenotypes in *mao* KO larvae across developmental stages (indicated in dpf). WT are in grey and KO are in yellow.

A, serotonin in the raphe.

B, serotonin in the hypothalamus.

C, locomotion.

D, proliferation.

**Supplemental Figure 1.** Lack of effect of *mao* KO on gut motility and heart beat rhythm at 8dpf.

A-D, Examples of kymographs from WT (A and B) and KO fish (C and D). The ordinate represents time, the abscissa the position along the digestive tract from the stomach to the rectum. Waves of peristalsis are well visible on images A and C. Fish of both genotypes (WT and KO) have phases where peristalsis is intense (A and C), and other phases where it is less intense (B and D).

E, Quantification of heart beat in WT, het and KO fish at 8dpf.

**Supplemental Figure2:**

A, ventral view of confocal optical plane (3  $\mu$ m) of WT and KO embryos at 36hpf stained for the proliferative marker PH3 (green). DAPI is in blue. Scale bar: 40  $\mu$ m.

B, ventral view of confocal optical plane (3  $\mu$ m) of WT and KO embryos at 48hpf stained for the proliferative marker PH3 (green). DAPI is in blue.

C, quantification of PH3-positive cells in the three genotypes at 36hpf and 48hpf in the total brain, in the telencephalon and in the hypothalamus.

D, ventral view of confocal optical plane (3  $\mu$ m) of WT and KO embryos at 36hpf stained for the activated caspase (green). DAPI is in blue. Scale bar: 40  $\mu$ m.

E, ventral view of confocal optical plane (3  $\mu$ m) of WT and KO embryos at 36hpf stained for the activated caspase (green). DAPI is in blue.

F, quantification of caspase-positive cells in the three genotypes at 36hpf and 48hpf in the total brain, in the telencephalon and in the hypothalamus.

G, *in situ* hybridization for *tph1a* at 36 hpf in WT and KO. *Tph1a* is express in the hypothalamus. In high magnification, we can see that it is express in a few number of cells. Scale bar: 40  $\mu$ m.

H, *in situ* hybridization for *tph2* at 36 hpf in WT and KO. *Tp2* is express in the raphe. In high magnification, we can see that it is express in a two areas.