1   **Bixafen, a succinate dehydrogenase inhibitor fungicide, causes microcephaly and**

2   **motor neuron axon defects during development**

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4   Alexandre Brenet#, Rahma Hassan-Abdi# and Nadia Soussi-Yanicostas*

5   Université de Paris, NeuroDiderot, Inserm, F-75019 Paris, France

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8   (#) Co-first authors

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10   **(*)Correspondence:** nadia.soussi@inserm.fr; Tel.: +00-33140-03-1931 (France)

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16   **Running title:** Neurodevelopmental defects in bixafen-exposed embryos

17   **Abstract**

18   Succinate dehydrogenase inhibitors (SDHIs), the most widely used fungicides in

19   agriculture today, act by blocking succinate dehydrogenase (SDH), an essential and

20   evolutionarily conserved component of mitochondrial respiratory chain. Recent results

21   showed that several SDHIs used as fungicides not only inhibit the SDH activity of target

22   fungi but also block this activity in human cells in *in vitro* models, revealing a lack of

23   specificity and thus a possible health risk for exposed organisms, including humans.

24   Despite the frequent detection of SDHIs in the environment and on harvested products

25   and their increasing use in modern agriculture, their potential toxic effects *in vivo*,

26   especially on neurodevelopment, are still under-evaluated. Here we assessed the

27   neurotoxicity of bixafen, one of the latest-generation SDHIs, which had never been tested

28   during neurodevelopment. For this purpose, we used a well-known vertebrate model for

29   toxicity testing, namely zebrafish transparent embryos, and live imaging using transgenic

30   lines labelling the brain and spinal cord. Here we show that bixafen causes microcephaly

31   and defects on motor neuron axon outgrowth and their branching during development.

32   Our findings show that the central nervous system is highly sensitive to bixafen, thus

33   demonstrating *in vivo* that bixafen is neurotoxic in vertebrates and causes

34   neurodevelopmental defects. This work adds to our knowledge of the toxic effect of SDHIs

35   on neurodevelopment and may help us take appropriate precautions to ensure protection

36   against the neurotoxicity of these substances.

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Introduction

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42   Bixafen, a methyl-pyrazole carboxamide, is a fungicide widely used on cereal and

43   rapeseed crops. It was initially approved and marketed in 2011 by Bayer (Ravichandra

44   2018). Its high efficiency and rapid penetration resulted in a significant rise in usage in

45   Europe and the US. Currently, nearly 30 different products are sold on the French market

46   alone that contain bixafen as sole active substance or mixed with other fungicides. Bixafen

47   belongs to the succinate dehydrogenase inhibitor (SDHI) family, the fungicides most

48   widely used in agriculture to fight a broad range of fungal diseases (Lalève et al. 2014;

49   Zhang et al. 2019). Bixafen, which is derived from carboxin and categorized as a latest-

50   generation SDHIs, acts through inhibition of mitochondrial respiration chain complex II,

51   also known as succinate dehydrogenase (SDH). However, the inhibitory effect of SDHIs is

52   not restricted to fungi: they also block the mitochondrial respiration chain in many other

53   species, including human cells (Bénit et al. 2019). Additionally, the latest-generation

54   SDHIs have been shown to also inhibit mitochondrial respiration complex III (Bénit et al.

55   2019), suggesting more critical effects of these new fungicides. SDH activity is essential

56   for proper mitochondrial respiration and metabolism, and defects in its activity impair

57   the cellular metabolome and functions (Bénit et al. 2014). Many human diseases are

58   related to SDH deficiency, including neurodegenerative disorders, such as Leigh

59   syndrome, an early-onset neurodegenerative disease characterized by developmental

60   delay, ataxia and seizures (Birch-Machin et al. 2000; Bourgeron 2015; Van Coster et al.

61   2003; Finsterer 2008; Horváth et al. 2006; Parfait et al. 2000), and cancers, such as

62   familial paraganglioma syndrome, infantile leukoencephalopathy, and neuroblastoma

63   (Ghezzi et al. 2009; Martin et al. 2007; Perry et al. 2006; Ricketts et al. 2009; Stratakis and

64   Carney 2009; Timmers et al. 2009). The Norwegian scientific committee for food safety

65   (VKM) has assessed the health and environmental risk of Aviator Xpro EC225, a fungicide

66   containing bixafen, and concluded that the effects of bixafen in animals should be

67   considered relevant for humans (VKM Report 2014). Also, M44, a bixafen metabolite,

68   which has the potential to contaminate groundwater, causes abnormalities in the rabbit

69   foetus, suggesting that similar effects in humans cannot be excluded (VKM Report 2015).

70   However, despite the increasing use of SDHIs in modern agriculture and their frequent

71   detection in the environment and on harvested products (Abad-Fuentes et al. 2015;

72   Añasco, Koyama, and Uno 2010; Tanabe and Kawata 2009; Tsuda et al. 2009; Vu et al.

73   2016), the potential toxic effects of these substances, especially on neurodevelopment,

74   have so far been poorly evaluated.

75   To fill this gap and assess the neurotoxicity of these latest-generation SDHI fungicides

76   *in vivo,* we tested the potential toxic effect of bixafen *in vivo* on zebrafish embryos. We

77   observed that embryos exposed to low or medium bixafen concentrations, LOAEL and

78   LC30, developed a series of defects, including microcephaly and disorganized motor

79   neuron axons and branches. This study demonstrated the toxicity of bixafen on CNS

80   development and gives insight into the environmental risk of this SDHI on the nervous

81   system in vertebrates.

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83 Results

84   1.   Toxicity of bixafen *in vivo* in zebrafish embryos

85   To assess the potential toxicity of bixafen, an SDHI belonging to the pyrazole group of

86   fungicides, zebrafish embryos were exposed to increasing concentrations of bixafen

87   ranging from 0.1 μM to 4 μM, from 6 hours post-fertilization (hpf) onward up to 96 hpf

88   (supplementary Figure 1A). As no differences in survival rates or embryo morphology

89   could be detected between the 0.1% DMSO and fish water control groups (data not

90   shown), embryos incubated in 0.1% DMSO were used as controls. Exposure of zebrafish

91   embryos to 0.5 μM bixafen and above induced gross morphological defects and

92   significantly increased mortality, compared with that seen in controls (supplementary

93   Figure 1).   Moreover,   we   observed   a   dose-dependent   effect,   with   increasing

94   concentrations of bixafen inducing increased mortality rates and increased phenotypic

95   defects in surviving embryos (Supplementary Figure $1B_1$-K).

96   Results indicated that the median lethal concentration of bixafen was 2.7 μM for wild-

97   type zebrafish embryos (AB line) after 96 hours of incubation, i.e. 96 hpf LC50, and closely

98   similar values were observed with the two other zebrafish lines used in this study, the

99   transgenic Tg[*Olig2:eGFP*] and Tg [*HuC:RFP*] (Supplementary Figure 1K).

100   To investigate the potential toxicity of bixafen, and particularly the effect of this SDHI

101   on the morphology of the brain and spinal cord, we chose 0.2 μM, the lowest-observed-

102   adverse-effect-level (LOAEL), and 0.5 μM, the 96 hpf LC30, two concentrations that induce

103   mild and moderate phenotypic defects in 96 hpf embryos, respectively, except for small

104   heart oedema in embryos exposed to 0.2 μM bixafen (Figure 1C), and large heart oedema

105   and curved tail in embryos exposed to 0.5 μM bixafen (Figure 1D). We next carefully

106   examined the morphological parameters of embryos exposed to 0.2 and 0.5 μM bixafen,

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107   namely body length, head size, eye diameter and eye-otolith distance. Results indicated

108   that following exposure to either 0.2 or 0.5 μM bixafen, surviving embryos show reduced

109   body length and eye diameter, and decreased head size and eye-otolith distance,

110   compared with that observed in age-matched controls (Figure 1E-I).

111   **2. Brain defects in embryos exposed to bixafen**

112   Because several investigations have reported that exposure to SDHIs may induce

113   neurotoxicity (Wang et al. 2020; Yao et al. 2018), we took advantage of the

114   Tg[*elavl3:Gal4/5xUAS:RFP*] double transgenic line (Akerboom et al. 2012; Asakawa et al.

115   2008), hereafter referred to as Tg[*HuC:G/U:RFP*], a line in which all post-mitotic brain

116   neurons are labelled by the fluorescent RFP protein, to precisely examine the morphology

117   of the brain of 36 and 66 hpf embryos, following exposure to 0.2 and 0.5 μM bixafen. Live

118   imaging of the brain of 36 and 66 hpf embryos, clearly indicated that exposure to both 0.2

119   and 0.5 μM bixafen, induced smaller brain size (Figure 1J-O). Precise quantification of

120   brain volumes (Imaris software, Bitplane), indicated that 36 hpf embryos exposed to 0.2

121    $(3.07 \pm 0.13 \times 10^6 \ \mu m^3)$ or 0.5 μM bixafen $(1.60 \pm 0.13 \times 10^6 \ \mu m^3)$, displayed a significantly

122   smaller brain compared with that seen in age-matched controls $(3.57 \pm 0.07 \times 10^6 \ \mu m^3)$

123   (Figure 1P). Similarly, 66 hpf embryos exposed to 0.2 $(7.45 \pm 0.24 \times 10^6 \ \mu m^3)$ or 0.5 μM

124   bixafen $(3.64 \pm 0.15 \times 10^6 \ \mu m^3)$, also displayed small brains compared to age-matched

125   controls $(8.51 \pm 0.25 \times 10^6 \ \mu m^3)$ (Figure 1Q). Our data thus showed that exposure of

126   zebrafish embryos to low-dose bixafen corresponding to LOAEL induced microcephaly, a

127   phenotype exacerbated in individuals incubated in 0.5 μM bixafen.

128   **3. Impaired mobility and spinal motor neuron axon outgrowth defects in**

129   **embryos exposed to bixafen**

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130   The small size of the brain observed in embryos exposed to bixafen suggests a

131   neurotoxic effect of this SDHI. To further investigate this issue and precisely assess the

132   consequences of bixafen exposure on brain functioning, we studied and compared the

133   locomotor activity of 0.2 and 0.5 µM bixafen-exposed and control embryos by measuring

134   the distance swum by these individuals over a 25-min period, using a ZebraBox tracking

135   automaton (ViewPoint®). Results indicated that embryos treated with 0.2 or 0.5 µM

136   bixafen, displayed reduced mobility, as shown by the distance traveled over a 25 minute

137   period 77.3 ± 5.7 mm (N = 4, n = 72) and 52.7 ± 4.4 mm (N = 4, n = 77), respectively,

138   compared with the distance travelled by controls (97.5 ± 4.9 mm, N = 4, n = 85) (Figure

139   2B-C). Interestingly, the hypoactive behavior of embryos exposed to 0.2 µM bixafen was

140   markedly worsened in those exposed to 0.5 µM, which were almost motionless.

141   Because bixafen exposure induces brain defects, we next determined whether the

142   behavioral deficits observed in bixafen-exposed embryos was a consequence of defects in

143   spinal cord motor neurons or their axons. To address this issue, we next analyzed the

144   morphology of the spinal cord in 36 and 66 hpf embryos exposed to 0.2 and 0.5 µM

145   bixafen, using the Tg[*Olig2:eGFP*] transgenic line (Shin et al. 2003), in which all motor

146   neuron progenitors and their axons are labelled by eGFP. In particular, we focused on

147   caudal primary motor neurons (CaPMN) and their axons, which are the most accessible.

148   Live imaging indicated that in 36 hpf controls, primary motor neuron axons extended

149   ventrally to contact target muscles, forming a highly regular pattern (Figure 2D). By

150   contrast, motor neuron axons were markedly shortened in 36 hpf embryos treated with

151   0.2 bixafen (Figure 2E), a phenotype markedly exacerbated in age-matched embryos

152   exposed to 0.5 µM, bixafen (Figure 2F). Three-dimensional reconstruction of CaPMN and

153   their axons in 66 hpf control embryos revealed a highly stereotyped pattern with axons

154   extending ventrally and branches projecting dorsally and laterally along the dorsal

155 myoseptum and forming a regular pattern in the space between the notochord and the

156 myotome (Figure 2G, G'). By contrast, following exposure to 0.2 μM bixafen, 66 hpf

157 embryos displayed significantly shorter axons (Figure 2H, H'), a phenotype significantly

158 worsened in embryos exposed to 0.5 μM bixafen (Figure 2I, I'), which also showed a

159 marked disorganization of motor neuron axons and their branches, which extended in all

160 directions.

161 Measurements of the length of CaPMN axons confirmed that bixafen exposure induced a

162 marked shortening of these axons. Whereas in control embryos, the length of CaPMN

163 axons was 167.9 ± 2.4 μm and 368.3 ± 9.3 μm, at 36 and 66 hpf, respectively, in embryos

164 exposed to 0.2 μM, these values were reduced to 148.6 ± 3.1 μm at 36 hpf and 296.4 ± 9.0

165 μm at 66 hpf. Moreover, these defects were exacerbated in embryos exposed to 0.5 μM

166 bixafen, with a mean CaPMN axon length of 61.8 ± 3.7 μm at 36 hpf and 223.7 ± 9.6 μm at

167 66 hpf (Figure 2J, K). Thus bixafen is neurotoxic and impairs the outgrowth and patterning

168 of spinal motor axons and their branches.

169   **Discussion**

170   It is well-known that the developing CNS in vertebrates is especially sensitive and

171   vulnerable to toxic chemicals, including phytosanitary agents, which are increasingly

172   present in the environment. More specifically, in recent years, bixafen, a latest-generation

173   succinate dehydrogenase inhibitor (SDHI), has become one of the most widely-used

174   fungicides worldwide. However, the toxicity of bixafen has been under-appraised *in vivo*

175   in vertebrate models so far, and especially its neurotoxicity for the developing CNS, an

176   especially serious issue because of the complete lack of species-specificity of this latest-

177   generation SDHI (Bénit et al. 2019). In the present work, we used zebrafish embryos as

178   an *in vivo* model and demonstrate that bixafen exposure during embryogenesis induces a

179   series of CNS defects.

180   In close agreement with previous investigations (Li et al. 2020; Wu et al. 2018), we first

181   observed that zebrafish embryos exposed to bixafen showed clear developmental delays,

182   as shown by morphological examination of embryos exposed to 0.5 μM bixafen and above,

183   but also by a decrease in the eye-otolith distance and hypopigmentation, both observed

184   in embryos incubated in 0.2 and 0.5 μM bixafen, corresponding to LOAEL and 96hpf LC30,

185   respectively. Moreover, at the brain level, we observed that these two same

186   concentrations induced brain defects and microcephaly, an observation in close

187   agreement with the markedly reduced expression of the neuronal *neuroD* gene observed

188   in zebrafish embryos treated with bixafen (Li et al. 2020). Microcephaly has been

189   repeatedly observed in embryos exposed to many pesticides, including Maxim® XL

190   (Svartz, Meijide, and Pérez Coll 2016) and glyphosate (Paganelli et al. 2010), emphasizing

191   the high vulnerability of the brain to these chemicals, and our study shows that SDHI

192   bixafen also induces microcephaly, even at low doses.

193   In addition, using an automaton that quantifies the speed and distance traveled by

194   embryos, we also observed that embryos exposed to 0.2 and 0.5 M bixafen displayed

195   reduced motility and almost complete paralysis, respectively, at 96 hpf. The quantification

196   of swimming behavior clearly showed motor deficits in embryos exposed to 0.2 µM of bixafen

197   which worsens in embryos exposed to 0.5 µM of bixafen (Figure 2C), that likely results of

198   neuron abnormalities, not developmental delay. As it is known that the motor neuron is a

199   major cell type regulating swimming behavior in zebrafish during early life (Brustein et

200   al. 2003), the motor defects seen in bixafen-exposed embryos prompted us to investigate

201   the differentiation of motor neuron axons and branches in embryos exposed to 0.2 and

202   0.5 µM bixafen. In control embryos, motor neurons extend their axons in a highly

203   stereotyped manner during development. In particular, caudal primary motor neuron

204   (CaPMN) axons exit the spinal cord and extend toward the ventral muscle. Interestingly,

205   in embryos exposed to bixafen, we observed a reduction in axon outgrowth and defects

206   in their branches, even in those exposed to 0.2 µM bixafen corresponding to LOAEL,

207   indicating that the development of motor neuron axons is highly sensitive to bixafen.

208   Although the toxic effects of bixafen on the CNS are probably due at least in part to the

209   developmental delay, the disorganization of motor neuron axons and branches clearly

210   reveals a neurotoxicity of bixafen in zebrafish embryos.

211   Bixafen is a methyl-pyrazole, one of the SDHI fungicides used to manage plant diseases

212   and inhibit the respiration of pathogenic fungi by blocking succinate complex II in the

213   mitochondrial respiratory chain. SDH is an enzyme involved in both oxidative

214   phosphorylation and the tricarboxylic acid cycle, two processes that generate energy. This

215   enzyme has been shown to be irreplaceable in mitochondrial and cell metabolism and also

216   to be highly conserved among all fungi, plants and animal species (Rehfus et al. 2016;

217   Veloukas, Markoglou, and Karaoglanidis 2013; Yamashita and Fraaije 2018). Any adverse

218 change or inhibition of SDH activity can therefore lead to many diseases, including those

219 that have been linked to mitochondrial dysfunction (Birch-Machin et al. 2000; Bourgeron

220 2015; Van Coster et al. 2003; Finsterer 2008; Horváth et al. 2006; Parfait et al. 2000).

221   Several epidemiological studies published in the last two decades suggest harmful

222 effects of pesticides on human health (Merhi et al. 2007; Weichenthal, Moase, and Chan

223 2010). Pesticide poisoning is a serious health problem that disproportionately affects

224 infants and children (Rauh et al. 2012). Pesticides are known to cause millions of acute

225 poisoning cases per year (Bertolote et al. 2006). Human exposure to pesticides can occur

226 environmentally, through consumption in food and water (van den Berg et al. 2012).

227 Bixafen is considered to be highly persistent in the environment (EFSA 2012). The half-

228 lives of bixafen were 105 days in loam, 316 days in sandy loam, 1235 days in slit loam,

229 and 82 days in water (EFSA 2012). A number of studies show that prenatal and early

230 childhood exposure to some pesticides is associated with neurodevelopmental and

231 cognitive disorders (Muñoz-Quezada et al. 2013; Ross et al. 2013). Aside from SDH

232 inhibition, very little is known about the consequences of SDHI exposure on human

233 health, and it is not known how bixafen affects the development of the CNS. As it is the

234 case for other SDHIs, the toxic effect of bixafen observed in this study may be linked to the

235 basic disruption of mitochondrial respiration chain complex II activity and subsequent

236 accumulation of reactive oxygen species and oxidative DNA damage. Results showed that

237 a high concentration of bixafen induces reactive oxygen species production and

238 subsequent DNA damage in human cell lines (Graillot et al. 2012). However, a recent study

239 also demonstrated that bixafen exposure did not cause oxidative stress in zebrafish (Li et

240 al. 2020), suggesting that induction of oxidative stress may not be the main mechanism

241 underlying bixafen-induced neurotoxicity in zebrafish embryos. Thus the brain and spinal

242 cord defects observed in embryos exposed to low-medium bixafen concentrations may be

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243   associated with the metabolic abnormalities reported in SDHI-exposed animals (Graillot

244   et al. 2012; Qian et al. 2018; Wu et al. 2018; Yang et al. 2018).

245   In summary, our study provides new evidence of bixafen toxicity on

246   neurodevelopment in a vertebrate model, and may help us take appropriate precautions

247   to ensure protection against the neurotoxic effects of these substances.

Materials and Methods

1. Ethics statement

All the animal experiments described in the present study were conducted at the French National Institute of Health and Medical Research (INSERM) UMR 1141 in Paris in accordance with European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). They were approved by the Direction Départementale de la Protection des Populations de Paris and the French Animal Ethics Committee under reference No. 2012-15/676-0069

2. Zebrafish lines and maintenance

Zebrafish were maintained at 26.5 °C in 14 h light and 10 h dark cycles. Embryos were collected by natural spawning, and 0.003% 1-phenyl-2-thiourea (PTU) was added at 1 dpf (day post-fertilization) for embryos which were imaged *in vivo* to avoid pigmentation. The following transgenic lines were used: Tg[*elavl3:Gal4*]^{zf349} (Akerboom et al. 2012), T[*5xUAS:RFP*]^{nkuasrfp1a} (Asakawa et al. 2008), Tg[*Olig2:eGFP*] (Shin et al. 2003).

3. Chemicals

Dimethyl sulfoxide (DMSO) hybrid-max sterile (Sigma, D2650) was diluted in fish water to a final concentration of 0.1% (v/v). Bixafen, (*N*-(3′,4′-dichloro-5-fluorobiphenyl-2-yl)-3-(difluoromethyl)-1-methylpyrazole-4-carboxamide) (Bixafen, (Sigma-Aldrich, St. Louis, MO, USA, code: 32581, CAS-No: 581809-46-3, Lot# BCBS9732V, purity > 98%).) was dissolved at a stock concentration of 400 mM in pure DMSO (100%) and conserved at −20 °C in aliquots until use.

4. Toxicity assay

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270 Wild-type (AB line) and transgenic lines, Tg[*HuC:G/U:RFP*] or Tg[*Olig2:eGFP*] embryos

271 were collected after spawning and washed twice with clean fish water. Fertilized and

272 normal 6 hpf embryos were selected, distributed randomly in 6-well microplates, 30

273 embryos per well, following OECD TG 236 fish acute toxicity test (OECD 2013). Each well was

274 filled with 6 mL of solution for each condition, with 12 conditions in all: fish water, 0.1%

275 DMSO, 0.1 μM, 0.2 μM, 0.3 μM, 0.5 μM, 1 μM, 1.5 μM, 2 μM, 2.5 μM, 3 μM and 4 μM bixafen

276 in 0.1% DMSO. Each day, dead embryos were selected under OECD guidelines

277 (coagulation, no heartbeat or no somite) and discarded.

278 **5. Locomotor activity**

279 96 hpf larvae were distributed individually in a 96 well plate in 200 μl of fish water. The

280 plate was then placed in the recording chamber of the zebrabox system for 30 min

281 habituation before 25 min recording of larvae locomotion (Brenet et al. 2019). The results

282 indicate the distance swum by the larvae during the recording.

283 **6. Embryo imaging**

284 36 hpf or 66 hpf embryos were anesthetized with 112 μg/mL 3-aminobenzoic acid ethyl

285 ester (tricaine, Sigma), immobilized in 1% low melting-point agarose in the center of a

286 35 mm glass-bottomed dish (Corning), and covered with fish water containing 112 μg/mL

287 tricaine. For embryo morphology analysis, bright field images were captured using a

288 stereomicroscope (Zeiss). Live imaging of transgenic lines Tg[*HuC:G/U:RFP*] and

289 Tg[*Olig2:eGFP*] was done using a Leica SP8 confocal scanning laser microscope equipped

290 with a Leica 20x/0.75 multi-immersion objective.

291 **7. Image analysis**

292   Body length, head size, eye diameter and eye-otolith distance measurements from bright

293   field embryo images were made using the ruler tool in ImageJ. Brain volume and spinal

294   neuron CaPMN axon length three-dimensional reconstructions and quantifications were

295   analyzed using Imaris Measurement Pro (Bitplane Inc.).

296   ### 8. Statistics

297   All statistics were obtained on Prism5 (GraphPad) and assessed using a one-way ANOVA

298   test followed by a Tukey post-test. All data are represented as means ± SEM.

299   **Author Contributions:**

300   A.B. and R.H.A. performed the experiments and the analysis, designed the figures, and

301   wrote the original draft. N.S.Y. supervised the project and wrote the manuscript.

309

310   **Conflicts of Interest:** The authors declare that the research was conducted in the absence

311   of any commercial or financial relationships that could be construed as a potential conflict
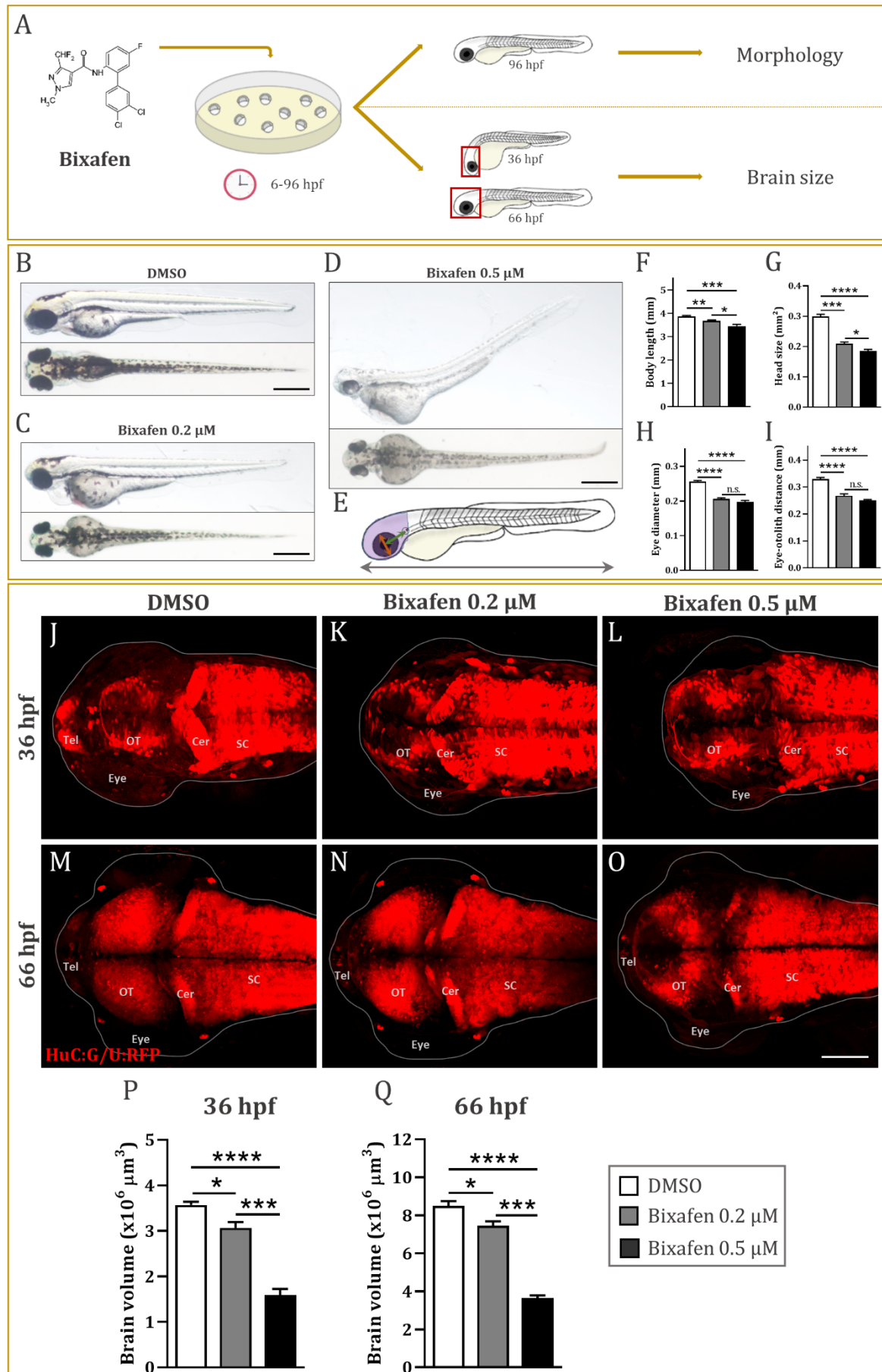
312   of interest.

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313

314 **Figure 1: Bixafen exposure causes microcephaly.**

315 (**A**) Experimental setup used to analyze the body morphological parameters and brain

316 size of zebrafish embryos exposed to 0.2 or 0.5 μM bixafen, from 6 hpf onward up to 36

317 or 66 hpf (brain analysis), or 96 hpf (morphological analysis). (**B-D**) Lateral and dorsal

318 views of 96 hpf larvae treated with 0.1% DMSO (**B**) or 0.2 μM (**C**) or 0.5 μM bixafen (**D**).

319 (**E**) Scheme depicting the different morphological parameters measured: body length

320 (black double-arrow), head size (purple area), eye diameter (red double-arrow) and eye-

321 otolith distance (green double-arrow). (**F-I**) Body length (**F**), head size (**G**), eye diameter

322 (**H**) and eye-otolith distance (**I**), of 96 hpf larvae exposed to 0.1% DMSO (n = 14), or 0.2

323 μM (n = 15) or 0.5 μM (n = 12) bixafen. (**J-O**) Dorsal views of the brain of 36 (**J-L**) or 66

324 hpf transgenic Tg[*huC:G/U:RFP*] embryos (**M-O**), treated with 0.1 % DMSO (**J, M**), or 0.2

325 (**K, N**) or 0.5 μM bixafen (**L, O**), and showing the microcephaly of embryos exposed to

326 low/medium doses bixafen. (**P-Q**) Measurements of brain volume of 36 (**P**) or 66 hpf (**Q**)

327 embryos exposed to 0.1% DMSO ($n_{36hpf} = 8$; $n_{66hpf} = 5$), or 0.2 μM ($n_{36hpf} = 6$; $n_{66hpf} = 5$) or

328 0.5 μM bixafen ($n_{36hpf} = 8$; $n_{66hpf} = 4$), further illustrate the microcephaly of embryos

329 exposed to bixafen at 96 hpf LC30 and LOAEL concentrations. ****, $p < 0.0001$; ***, $p <$

330 0.001; **, $p < 0.01$; *, $p < 0.05$; n.s., non-significant. Scale bar: (**B-D**) = 0.5 mm; (**J-O**) = 100

331 μm. Abbreviations: Tel, telencephalon; OT, optic tectum; Cer, cerebellum; SC, spinal cord.
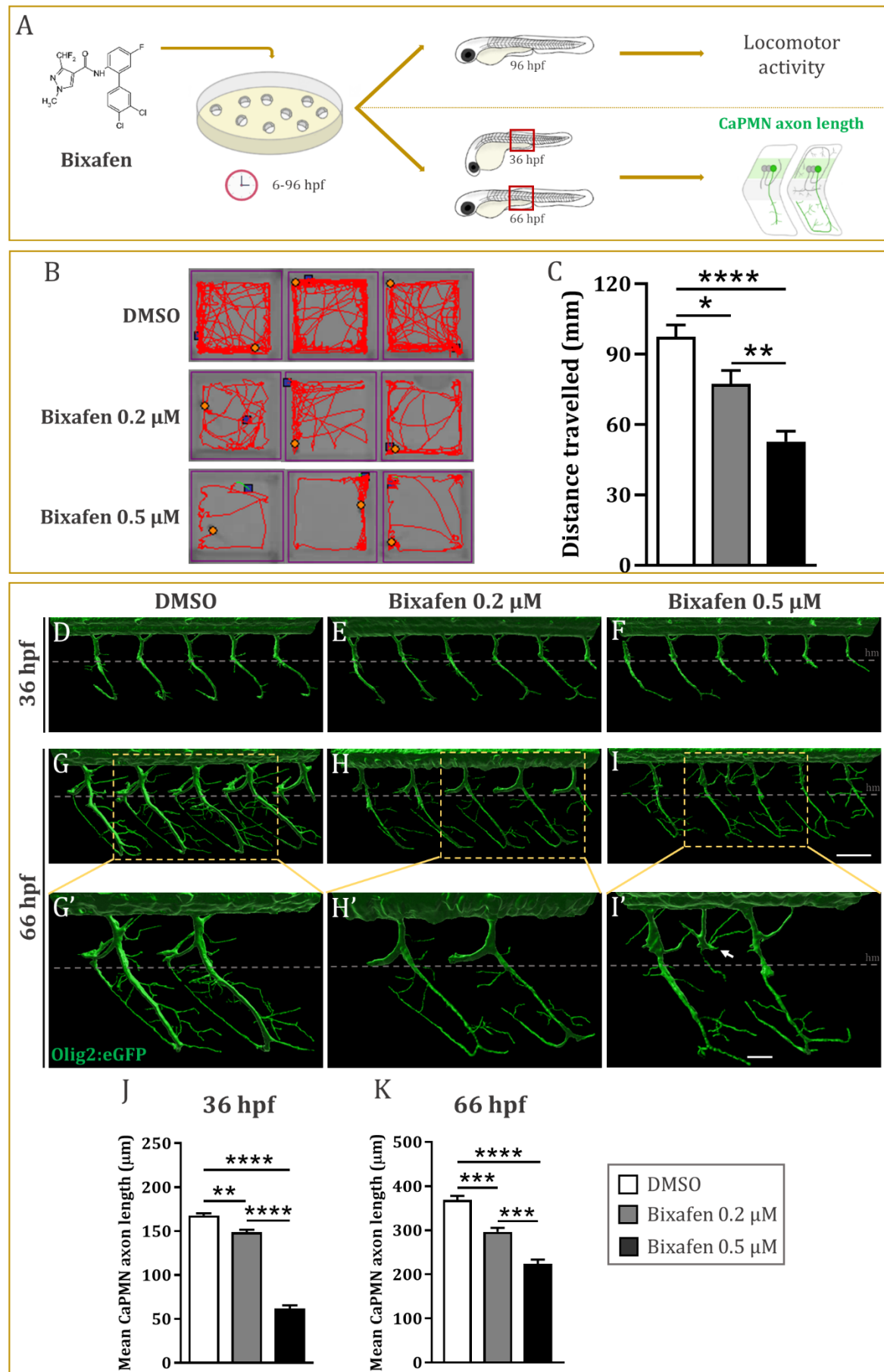
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333   **Figure 2: Bixafen exposure induces motor deficit and motor neuron axon defects.**

334   (**A**) Experimental setup used to analyze the motor activity and patterning of caudal

335   primary motor neuron (CaPMN) axons and branches, in zebrafish embryos exposed to 0.2

336   or 0.5 µM bixafen, from 6 hpf onward up to 36 or 66 hpf (analysis of CaPMN axon

337   morphology), or 96 hpf (analysis of motor activity). (**B**) Tracking plots illustrating the

338   distance traveled by 96 hpf larvae treated with 0.1% DMSO (n = 85), or 0.2 (n = 72) or 0.5

339   µM bixafen (n = 77), during a 25 min time period. (**C**) Quantification of the distance

340   traveled by 96 hpf larvae exposed to 0.1% DMSO, or 0.2 or 0.5 µM bixafen, during a 25

341   min time period. (**D-I**) Three-dimensional reconstruction of the organization of CaPMN

342   axons and branches in 36 (**D-F**) or 66 hpf transgenic Tg[Olig2:eGFP] embryos (**G-I'),**

343   exposed to 0.1% DMSO (**D**, **G**), 0.2 (**E**, **H**) or 0.5 µM bixafen (**F**, **I**), showing the

344   disorganization of CaPMN axons and branches, following exposure to 0.2 and, even more,

345   0.5 µM bixafen (white arrow). (**G'-I'**) Higher magnifications of the morphology of CaPMN

346   axons and branches, in embryos exposed to 0.1% DMSO (**G'**), or 0.2 (**H'**) or 0.5 µM bixafen

347   (**I'**). (**J**, **K**) Quantification of the length of the primary CaPMN axons in 36 (**J**) and 66 hpf

348   embryos (**K**), treated with DMSO (n_{36hpf} = 13; n_{66hpf} = 7), or 0.2 (n_{36hpf} = 8; n_{66hpf} = 6) or 0.5

349   µM bixafen (n_{36hpf}= 22; n_{66hpf} = 9), showing significantly shorter axons following exposure

350   to bixafen at LOAEL (0.2 µM) and, even more, 96hpf LC30 (0.5 µM). ****, $p < 0.0001$; ***,

351     $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. Scale bar: (**D-I**) = 100 µm; (**G'-I'**) = 50 µm.

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