Bixafen, a succinate dehydrogenase inhibitor fungicide, causes microcephaly and motor neuron axon defects during development

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Abstract

Succinate dehydrogenase inhibitors (SDHIs) are the most widely used fungicides against plant parasitic fungi. They act by blocking the enzyme succinate dehydrogenase (SDH), a key component of mitochondrial respiration, which is highly conserved throughout evolution. Recently, it has been reported that some SDHIs used in many fungicides do not only inhibit the SDH activity of target fungi but can also block several non-target human cells in *in vitro* models. This study reveals a lack of SDHI species specificity and so points to a major health risk for exposed organisms, including humans. Despite the frequent detection of SDHIs in the environment and on harvested products and their increasing use in modern agriculture, their potential toxic effects *in vivo*, especially on neurodevelopment, are still under-evaluated. Here, we assessed the neurotoxicity of bixafen, one of the latest-generation SDHIs, which had never been tested during neurodevelopment. For this purpose, we used a well-known vertebrate model for toxicity testing, namely zebrafish transparent embryos, and live imaging using transgenic lines labelling the brain and spinal cord. Here we show that bixafen causes microcephaly and defects on motor neuron axon outgrowth and their branching during development. Our findings show that the central nervous system is highly sensitive to bixafen, thus demonstrating for the first time *in vivo* that an SDHI, bixafen, widely used in agriculture, is neurotoxic in vertebrates and causes neurodevelopmental defects. This work adds to our knowledge of the toxic effect of SDHIs on neurodevelopment and may help us take the appropriate precautions to ensure protection against the neurotoxic effects of these substances

Introduction

Bixafen, a methyl-pyrazole carboxamide, is a fungicide widely used on cereal and rapeseed crops. It was initially approved and launched in the market in 2011 by Bayer (Ravichandra, 2018).Its high efficiency and fast penetration resulted in a significant rise in usage in Europe and the US. Currently, nearly thirty different products are authorized and present on the French market alone that contain bixafen as sole active substance or mixed with other fungicides. Bixafen belongs to the succinate dehydrogenase inhibitor (SDHI) family, the most widely used fungicides in agriculture as a treatment against a broad range of fungal diseases. It is categorized as one of the latest-generation SDHIs derived from carboxin. Bixafen, like all the latest generation SDHIs, acts in a similar way to carboxin, through inhibition of mitochondrial respiration chain complex II, also known as succinate dehydrogenase (SDH). The complex II targeted by SDHI is not specific to the targeted disease fungi but is also closely similar to that present in all plant and animals species, including humans. SDHI inhibitory action on mitochondrial activity has thus been shown to be effective in various models and human cells (Bénit et al., 2019). Additionally, these latest generation SDHIs have been shown to also inhibit mitochondrial respiration complex III (Bénit et al., 2019), suggesting more critical effects of these new fungicides than those expected. SDH activity has been shown to be irreplaceable in mitochondrial metabolism, and defects in its activity impair the cellular metabolome and functions (Bénit et al., 2014). A broad range of human diseases from neurodegeneration to cancer are related to SDH complex defects. Several studies have shown that SDH complex mutations are associated with several diseases, such as Leigh syndrome, an early-onset progressive neurodegenerative disease characterized by developmental delay, ataxia, seizures, and defects in the brain and spinal cord (Birch-Machin et al., 2000; Bourgeron, 2015; Finsterer, 2008; Horváth et al., 2006; Parfait et al., 2000; Van Coster et al., 2003).

74 SDH mutations have also been associated with several tumors, familial paraganglioma
75 syndrome, infantile leukoencephalopathy, thyroid and renal cancers, and neuroblastoma
76 (Ghezzi et al., 2009; Martin et al., 2007; Perry et al., 2006; Ricketts et al., 2009; Stratakis
77 and Carney, 2009; Timmers et al., 2009). One study showed that the SDHI fungicide
78 isopyrazam increased uterine tumor formation in rats (Yoshida et al., 2015). The
79 Norwegian scientific committee for food safety (VKM) has assessed the health and
80 environmental risk of Aviator Xpro EC225, a fungicide containing bixafen, and concluded
81 that the effects of bixafen in animals should be considered relevant for humans (VKM
82 Report, 2014). Also, M44, a bixafen metabolite, which has the potential to contaminate
83 groundwater, causes abnormalities in the rabbit fetus, suggesting that similar effects in
84 humans cannot be excluded (VKM Report, 2015).
85 Despite the frequent detection of SDHIs in the environment and on harvested products
86 (Abad-Fuentes et al., 2015; Añasco et al., 2010; Tanabe and Kawata, 2009; Tsuda et al.,
87 2009; Vu et al., 2016) and their increasing use in modern agriculture, their potential toxic
88 effects, especially on neurodevelopment, are still under-evaluated. Hence the need to
89 assess the neurotoxicity of these SDHI fungicides *in vivo* on neurodevelopment. In this
90 study, we tested the potential toxic effect of one of the latest-generation SDHIs, bixafen, in
91 zebrafish embryos. We observed that embryos exposed to bixafen developed a series of
92 defects during growth, including microcephaly and major defects in spinal cord motor
93 neuron axons and their branching, showing the toxicity of this SDHI on central nervous
94 system neurodevelopment. Our study gives insight into the environmental risk of bixafen
95 on neurodevelopment.
96

97 **Results**

98 **1. Toxicity of bixafen toward zebrafish embryos**

99 To assess the potential toxicity of bixafen, a succinate dehydrogenase inhibitor
100 belonging to the pyrazole group of fungicides, 6 hours post-fertilization (hpf) zebrafish
101 embryos were treated with increasing doses of bixafen from 0.1 μM to 1 μM. As there were
102 no differences in survival percentage or embryo morphology between the 0.1% DMSO
103 group and the fish water control group (data not shown), we used 0.1% DMSO for the
104 control group. Exposure of zebrafish embryos to bixafen resulted in significant
105 developmental abnormalities and mortality compared with the DMSO control. Mortality
106 showed a concentration-response effect, with an increasing mortality rate with bixafen
107 exposure concentration. The median lethal concentration (LC50) of bixafen for zebrafish
108 embryos, causing a 50% mortality rate at 4 days post-fertilization (dpf), was calculated at
109 0.5 μM. The surviving embryos treated with 0.5 μM of bixafen displayed a curved tail
110 (Figure 1E, red arrowhead) causing body shortening (Figure 1F, 33.8 ± 1.5 mm); and
111 edemas around the heart, eye and head (Figure 1E, yellow arrowheads) causing a swollen
112 head (Figure 1G, 38.1 ± 0.8 mm), compared to the DMSO control embryos (Figure 1C)
113 body (Figure 1F, 37.2 ± 0.6 mm) and head (Figure 1G, 5.8 ± 0.2 mm).

114 We then determined the lowest-observed-adverse-effect-level (LOAEL) concentration
115 of bixafen (0.3 μM), i.e. the lowest exposure level at which we observed the first noticeable
116 body deformity observed in most bixafen exposed embryos, which was a slight edema
117 around the heart. Embryos treated at the LOAEL (0.3 μM) displayed only a modest-size
118 pericardial edema (Figure 1D, yellow arrowhead) but no significant difference in body
119 size (Figure 1F, 35.7 ± 0.7 mm *vs*. 37.2 ± 0.6 mm) or head size (Figure 1G, 5.5 ± 0.4 mm *vs.*
120 5.8 ± 0.2 mm) after body and head measurements (Figure 1B). In this study, we used two

121 transgenic lines Tg(huC:G/U:RFP) and Tg(Olig2:eGFP) to analyze the embryos exposed to
122 LOAEL and LC50 concentrations and compare the resulting defects.
123

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

125 Several studies have reported that a number of SDHIs cause neurotoxicity (Wang et al.,
126 2020; Yao et al., 2018). We investigated the effect of bixafen exposure on the development
127 of the central nervous system. To assess the effect of bixafen on brain development
128 reliably under a fluorescence microscope, we used *elavl3:Gal4/5xUAS:RFP* double
129 transgenic embryos (hereafter called Tg(huC:G/U:RFP)), in which red fluorescence
130 protein (RFP) is expressed in postmitotic neurons, thus clearly delineating central
131 nervous system boundaries. We treated 6 hpf embryos from Tg(huC:G/U:RFP) transgenic
132 line with the determined LOAEL (0.3 μM) and LC50 (0.5 μM) concentrations, and imaged
133 live brain morphology at 30 hpf and 60 hpf (Figure 1A, N). Live imaging of embryo brains
134 treated at 30 hpf and 60 hpf showed a smaller brain structure (Figure 1H-M). These
135 observations were confirmed by quantifications of brain volume after three-dimensional
136 reconstruction of embryo brains based on RFP positive neurons (Imaris software,
137 Bitplane) at 30 hpf (Figure 1O; DMSO: 3.50 ± 0.18 x 10^6 μm^3 , Bixafen 0.3μM: 2.06 ± 0.17
138 x 10^6 μm^3, Bixafen 0.5μM: 1.43 ± 0.23 x 10^6 μm^3) and 60 hpf (Figure 1P; DMSO: 15.37 ±
139 0.44 x 10^6 μm^3 , Bixafen 0.3μM: 10.35 ± 0.52 x 10^6 μm^3, Bixafen 0.5μM: 5.66 ± 0.73 x 10^6
140 μm^3). We also observed an absence of photoreceptors in the retina of embryos exposed
141 to the LC50 (Figure 1J, M, asterisks). Brain volume measurements were significantly
142 reduced in embryos treated with bixafen at LOAEL (Figure 1 I, L). This defect was

143 worsened at LC50 (Figure 1 J, M) compared to controls (Figure 1 H, K), showing that the
144 toxicity of bixafen led to microcephaly.
145

146 **3. Defective spinal motor neuron axon outgrowth in embryos exposed to**
147 **bixafen**

148 We found that more than 80% of embryos treated with LC50 displayed behavior defects
149 in a touch-response test (data not shown) compared to DMSO controls. We therefore
150 investigated whether these motor defects were due to abnormal development of motor
151 neurons or impaired pathfinding of their axons. To determine whether the spinal motor
152 neuron axons were affected in bixafen-treated embryos, we used transgenic line
153 Tg(Olig2:eGFP), in which eGFP is expressed in motor neuron progenitor cells, labeling
154 motor axons. Live embryos treated or not with bixafen were imaged by confocal
155 microscopy at 30 hpf and 60 hpf. We specifically analyzed caudal primary motor neuron
156 (CaPMN) axons, which are the most accessible (Figure 2A, H). At 30 hpf, in control
157 embryos and LOAEL treated embryos, 100% of spinal motor neuron axons extended
158 ventrally to contact their muscle targets (Figures 2B, C). By contrast, in LC50 treated
159 embryos, at 30 hpf, the spinal neuron axons exited the spinal cord and showed a severe
160 delay in outgrowth (Figure 2D). Length measurements of three-dimensionally
161 reconstructed CaPMN axons confirmed that these axons were significantly shorter in
162 embryos treated with bixafen at LOAEL and that this effect was worsened in embryos
163 exposed to bixafen LC50 concentrations (Figure 2I). At 60 hpf, in control embryos, the
164 CaPMN axons followed a stereotyped pathway, extending ventrally in the space between
165 the notochord and the myotome (Figure 2E, E'). At the ventral edge of the musculature,
166 each axon turned dorsally and laterally to grow along the dorsal myoseptum. However, in
167 bixafen-treated embryos at 60 hpf, axons showed a defective phenotype as determined by

168 their branching pattern at the choice point and/or the horizontal myoseptum (Figures 2
169 F, G, F', G'). At LC50, CaPMN axons extended aberrant branches projecting in all directions
170 instead of extending ventrally within the myotome (Figure 2G'). Hence treatment with
171 bixafen disorganized motor axon growth at LOAEL concentration and this effect was
172 worsened at the LC50 concentration. Length measurements of three-dimensionally
173 reconstructed CaPMN axons confirmed this observation, with significantly shorter axons
174 in embryos treated with bixafen at 30 hpf (Figure 1I; DMSO: 172.2 ± 3.03 µm, Bixafen
175 0.3µM: 97.1 ± 3.56 µm, Bixafen 0.5µM: 46.2 ± 2.3 µm) and 60 hpf (Figure 1J; DMSO: 406.3
176 ± 7.65 µm, Bixafen 0.3µM: 272.9 ± 9.07 µm, Bixafen 0.5µM: 171.3 ± 11.52 µm). These data
177 show that exposure to bixafen has a drastic effect on the outgrowth of the spinal motor
178 axons.

179 **Discussion**

180 The developing central nervous system is especially vulnerable and sensitive to
181 exposure to toxic chemicals in the environment. Succinate dehydrogenase inhibitors
182 (SDHIs) are widely used as fungicides to control a broad range of fungal diseases, but it
183 was not known whether these SDHIs had a toxic effect on the developing central nervous
184 system. In this study we report evidence that exposure to an SDHI, bixafen, induces a
185 series of neurodevelopmental defects.

186 In this study we used the zebrafish, a valuable vertebrate system for environmental
187 health research. It enables real-time *in vivo* studies that address potential health hazards
188 to human embryos, and in particular studies investigating neurodevelopment in a
189 vertebrate model (Lein et al., 2005). Regarding brain development, we found that bixafen
190 exposure at the median lethal concentration (LC50) caused major malformation
191 phenotypes, including eye and brain defects leading to microcephaly. We also observed a
192 pericardial edema, indicating that bixafen exerts cardiac toxicity. We also found that
193 embryos treated with the lowest-observed-adverse-effect-level (LOAEL) concentration of
194 bixafen showed a very discreet morphological phenotype sufficient to induce significant
195 defects in brain size, indicating that the sublethal dose of bixafen could influence normal
196 development. Microcephaly has been found in embryos exposed to many fungicides,
197 including Maxim® XL (Svartz et al., 2016), and glyphosate (Paganelli et al., 2010).
198 Moreover, bixafen-exposed embryos displayed absence of photoreceptors in the retina.
199 All these findings suggest that the brain is very vulnerable to many toxic substances even
200 at low concentrations. A number of studies have shown that prenatal and early childhood
201 exposure to some pesticides is associated with neurodevelopmental and cognitive
202 disorders (Muñoz-Quezada et al., 2013). Perinatal exposure of rats to organophosphorus
203 pesticides alters brain morphometry (Veronesi and Pope, 1990). Similarly, our recent

204 studies have shown that embryos exposed to organophosphorus pesticides showed
205 neurodevelopmental disorders, including altered excitatory/inhibitory synapse balance
206 (Brenet et al., 2019).

207 Regarding the spinal cord, our data show that embryos exposed to bixafen show
208 deficits in touch-induced swimming behavior and display a typical low-activity swimming
209 behavior. As it is known that the motor neuron is a major cell type regulating swimming
210 behavior in zebrafish during early life (Brustein et al., 2003), we investigated the
211 development of the axons of the motor neurons in embryos exposed to bixafen. In the
212 zebrafish embryos, there are two different kinds of spinal motor neurons, called primary
213 and secondary motor neurons (Myers, 1985; Myers et al., 1986). The caudal primary
214 motor neurons (CaPMN) have a soma located in the middle of each spinal cord and axons
215 extending along a ventral pathway to innervate ventral axial muscle (Westerfield et al.,
216 1986). In this study, we focused on the CaPMN axons because of their easy observation
217 and distinct axon projections. In control embryos exposed to 0.1% DMSO, motor neurons
218 extend their axonal trajectory in a highly stereotyped manner during development.
219 CaPMN axons exit the spinal cord and extend toward the ventral muscle. However, in
220 embryos exposed to bixafen, we observed a reduction in axon outgrowth and later defects
221 in branching of motor axons. Similarly, we found that the low concentration of bixafen
222 (LOAEL) was sufficient to induce defects in axon outgrowth and branching of motor
223 neurons during development, indicating that the development of motor neuron axons is
224 highly sensitive to bixafen. This study reveals motor neuron axon defects in embryos
225 exposed to bixafen. It also provides data on the toxicity of bixafen fungicides to a
226 nontarget organism, zebrafish embryos. It has been shown that chlorpyrifos-oxon
227 pesticide disrupts motor neuron axons (Howard et al., 2005).

228 Bixafen is a methyl-pyrazole, one of the SDHI fungicides used to manage plant diseases
229 and inhibit the respiration of pathogenic fungi by blocking succinate complex II in the
230 mitochondrial respiratory chain. SDH is an enzyme involved in both oxidative
231 phosphorylation and the tricarboxylic acid cycle, two processes that generate energy. This
232 enzyme has been shown to be irreplaceable in mitochondrial and cell metabolism and also
233 to be highly conserved among all fungi, plants and animal species (Rehfus et al., 2016;
234 Veloukas et al., 2013; Yamashita and Fraaije, 2018). Therefore, any adverse change or
235 inhibition of SDH activity can lead to many diseases, including those that have been linked
236 to mitochondrial dysfunction (Birch-Machin et al., 2000; Bourgeron, 2015; Finsterer,
237 2008; Horváth et al., 2006; Parfait et al., 2000; Van Coster et al., 2003).
238 A recent study has questioned the specificity of these SDHIs, including bixafen. It showed
239 that the latest-generation SDHIs sold on the market could inhibit the SDH of several
240 species ranging from earthworms to human cells, evidence that these SDHIs are
241 nonspecific (Bénit et al., 2019). Several epidemiological studies published during the last
242 two decades suggest harmful effects of pesticides on human health (Merhi et al., 2007;
243 Weichenthal et al., 2010). Pesticide poisoning is a serious health problem that
244 disproportionately affects infants and children (Rauh et al., 2012). Pesticides are known
245 to cause millions of acute poisoning cases per year (Bertolote et al., 2006). Human
246 exposure to pesticides can occur environmentally, through consumption in food and
247 water (van den Berg et al., 2012). A number of studies show that prenatal and early
248 childhood exposure to some pesticides is associated with neurodevelopmental and
249 cognitive disorders (Muñoz-Quezada et al., 2013; Ross et al., 2013). Very little is known
250 about the consequences of exposure to SDHIs on human health. Currently, we know only
251 the consequences of the changes in their target, the SDH complex. Mutations in the SDH
252 complex are associated with several human diseases, including neurodegenerative

253 diseases and cancer (Ardissone et al., 2015; Birch-Machin et al., 2000; Bourgeron, 2015;
254 Finsterer, 2008; Horváth et al., 2006; Parfait et al., 2000; Van Coster et al., 2003; Martin et
255 al., 2007; Perry et al., 2006; Ricketts et al., 2009; Stratakis and Carney, 2009; Timmers et
256 al., 2009).
257 The fungicide bixafen exists as a pure compound or as an ingredient in 27 approved and
258 marketed fungicides. Our results will be used as basic data to study the complex mixtures
259 that contain bixafen. It is not known how bixafen can affect the development of the central
260 nervous system .Like for all SDHIs, the toxic effect of bixafen observed in this study may
261 be linked to the basic disruption of mitochondrial activity and accumulation of reactive
262 oxygen species caused on Complex II inhibition and subsequent oxidative DNA damage.
263 These severe brain and spinal cord malformations may also be related to the different
264 metabolic abnormalities reported in SDHI exposed animals (Graillot et al., 2012; Qian et
265 al., 2018; Wu et al., 2018; Yang et al., 2018). A recent work (Bénit et al., 2019) has shown
266 that bixafen also exerts an effect on mitochondrial respiration complex III, suggesting an
267 adverse effect of bixafen on cellular metabolism.
268
269 In summary, our study provides new evidence of bixafen toxicity on neurodevelopment
270 in a vertebrate model, and and may help us take the appropriate precautions to ensure
271 protection against the neurotoxic effects of these substances.
272
273

274 **Materials and Methods**

275 **1. Ethics statement**

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

282

283 **2. Zebrafish lines and maintenance**

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

289 **3. Chemicals**

290 Dimethyl sulfoxide (DMSO) hybrid-max sterile (Sigma, D2650) was diluted in fish water
291 to a final concentration of 0.1% (v/v). Bixafen, (*N*-(3′,4′-dichloro-5-fluorobiphenyl-2-yl)-
292 3-(difluoromethyl)-1-methylpyrazole-4-carboxamide) (Sigma, 32581) was dissolved at a
293 stock concentration of 100 µM in pure DMSO (100%) and conserved at −20 °C in aliquots
294 until use. 100 µM stocks were diluted in a 0.1% DMSO solution in fish water to final
295 concentrations of 0.1 µM, 0.2 µM, 0.3 µM, 0.4 µM, 0.5 µM, 0.6 µM, 0.7 µM, 0.8 µM, 0.9 µM
296 and 1.0 µM to determine lethality and deduce chemical toxicity values.

297 **4. Toxicity assay**

298 Transgenic lines, Tg(huC:G/U:RFP) or Tg (Olig2:eGFP) embryos were collected after
299 spawning and washed twice with clean fish water. Fertilized and normal 6 hpf embryos
300 were selected, distributed randomly in 24-well microplates, 20 embryos per well,
301 following OECD zebrafish embryo toxicological assay directives. Each well was filled with
302 2 mL of solution for each condition, with 12 conditions in all: fish water, DMSO 0.1%,
303 Bixafen diluted in DMSO 0.1% at final concentrations of 0.1 μM, 0.2 μM, 0.3 μM, 0.4 μM,
304 0.5 μM, 0.6 μM, 0.7 μM, 0.8 μM, 0.9 μM and 1.0 μM. Each day, dead embryos were selected
305 under OECD guidelines (coagulation, no heartbeat or no somite) and discarded.

306 **5. Embryo imaging**

307 30 hpf or 60 hpf embryos were anesthetized with 112 μg/mL 3-aminobenzoic acid ethyl
308 ester (tricaine, Sigma), immobilized in 1% low melting-point agarose in the center of a
309 35 mm glass-bottomed dish (Corning), and covered with fish water containing 112 μg/mL
310 tricaine. For embryo morphology analysis, bright field images were captured using a
311 stereomicroscope (Zeiss). Live imaging of transgenic lines Tg(huC:G/U:RFP) and
312 Tg(Olig2:eGFP) was done using a Leica SP8 confocal scanning laser microscope equipped
313 with a Leica 20x/0.75 multi-immersion objective.

314 **6. Image analysis**

315 Body and head measurements from bright field embryo images were made using the ruler
316 tool in ImageJ. Brain volume and spinal neuron CaPMN axon length three-dimensional
317 reconstructions and quantifications were analyzed using Imaris Measurement Pro
318 (Bitplane Inc.).

319 **7. Statistics**

320 All statistics were obtained on Prism5 (GraphPad) and assessed using a Kruskal-Wallis
321 test followed by a Dunn post-test. All data are represented as means ± SEM.
322
323 **Author Contributions:**
324 R.H.A. performed the experiments and the analysis, designed the figures, and wrote the
325 original draft. A.B. performed data analysis. N.S.Y. supervised the project and wrote the
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327
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338 of any commercial or financial relationships that could be construed as a potential conflict
339 of interest.
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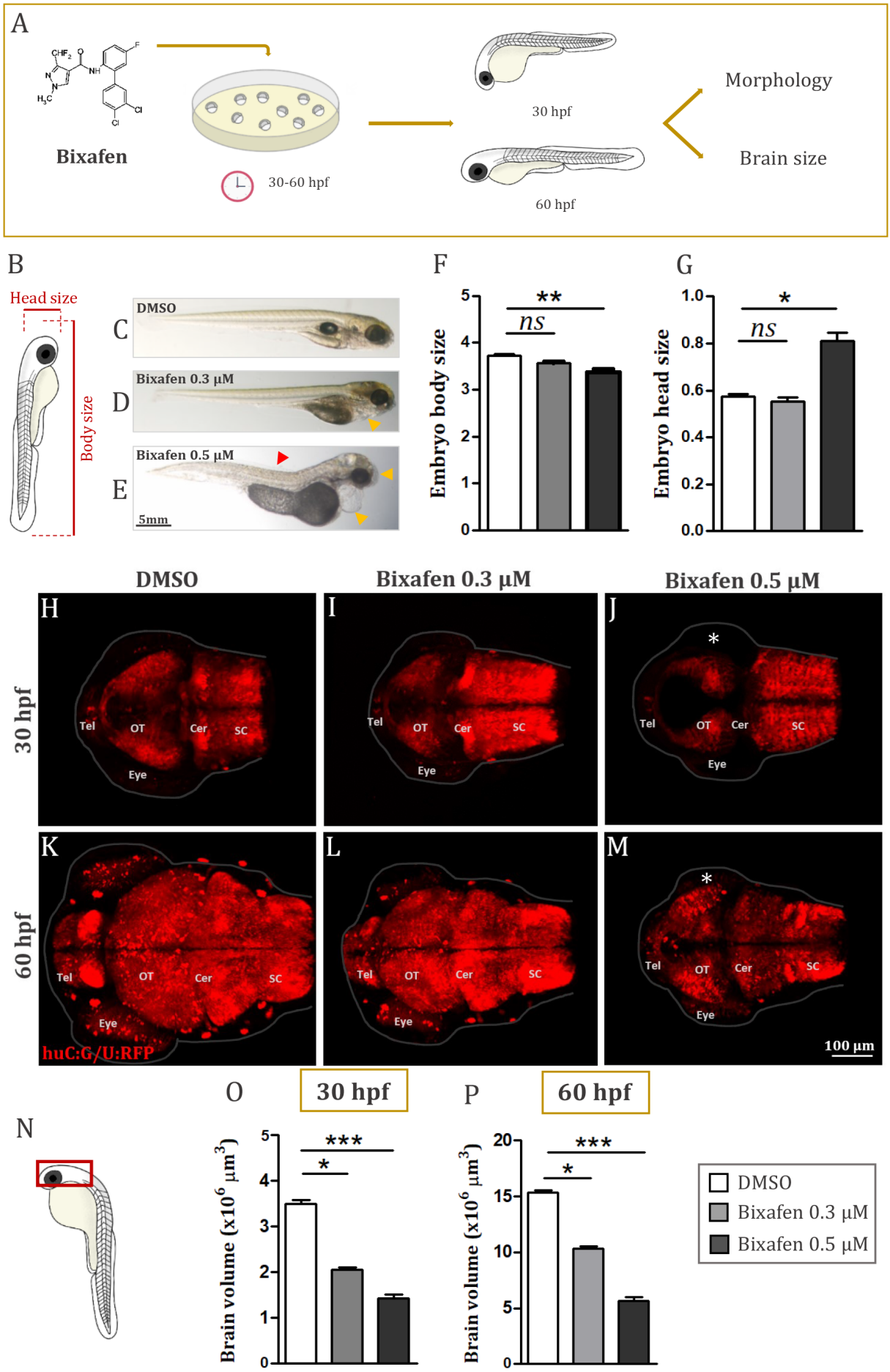
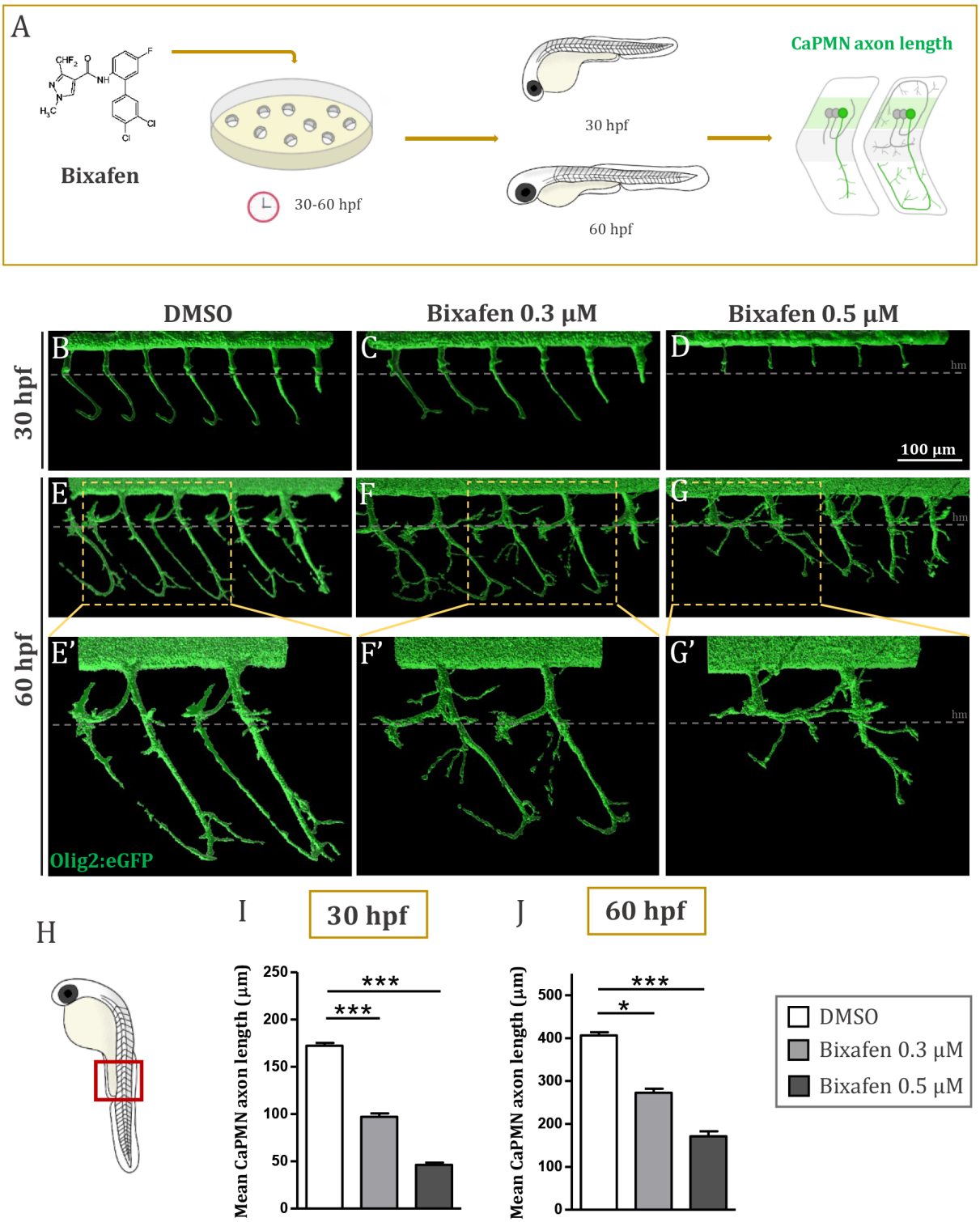

A
Bixafen
30-60 hpf
30 hpf
60 hpf
Morphology
Brain size
B
Head size
Body size
C
DMSO
D
Bixafen 0.3 µM
E
Bixafen 0.5 µM
5mm
F
Embryo body size
**
ns
G
Embryo head size
*
ns
DMSO
Bixafen 0.3 µM
Bixafen 0.5 µM
30 hpf
60 hpf
H
I
J
K
L
M
Tel
OT
Cer
SC
Eye
huC:G/U:RFP
100 µm
N
O
30 hpf
P
60 hpf
Brain volume (x10^6 µm^3)
***
*
DMSO
Bixafen 0.3 µM
Bixafen 0.5 µM

344

345 **Figure 1: Acute exposure to bixafen causes microcephaly.**

346 (**A**) Illustration of the experimental setup used to analyze body morphology and brain size
347 on acute exposure to bixafen. (**B**) Illustration of the measurements used to assess body
348 size and brain size of embryos. (**C-E**) Images of 4 dpf embryos treated with DMSO (**C**), 0.3
349 µM (**D**) or 0.5 µM bixafen (**E**). (**D**) Image showing a small pericardial edema at the LOAEL
350 (0.3 µM, **D**, yellow arrowhead) or severe pericardial and periocular edema at the LC50
351 (0.5 µM, **E**, yellow arrowheads) and a curved tail (**E**, red arrowhead). (**F**, **G**) Measurements
352 of body (**F**) and head (**G**) size of 4 dpf embryos treated with DMSO (N=4), 0.3 µM (N=4) or
353 0.5 µM (N=4) bixafen. (**H-M**) Dorsal view images of Tg(huC:G/U:RFP) embryos at 30 hpf
354 (**H-J**) or 60 hpf (**K-M**), treated with DMSO (**H**, **K**), 0.3 µM bixafen (**I**, **L**) or 0.5 µM bixafen
355 (**J**, **M**), showing a microcephaly in both stages on exposure to bixafen, with an increase in
356 severity at 0.5 µM. (**N**) Illustration of a zebrafish embryo; the red line marks the depth at
357 which confocal imaging was performed on (**H-M**). (**O**, **P**) Quantifications of the brain size
358 of embryos at 30 hpf (**O**) or 60 hpf (**P**), treated with DMSO (N(30hpf)=5; N(60hpf)=5), 0.3
359 µM bixafen (N(30hpf)=13; N(60hpf)=12) or 0.5 µM bixafen (N(30hpf)=6; N(60hpf)=5),
360 showing significantly smaller brains in embryos exposed to bixafen at LOAEL
361 concentration and worsened with increase in concentration LC50, confirming the
362 observations in (**H-M**). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Scale bar : (**C-E**) = 5 mm ; (**H-**
363 **M**) = 100 µm.



364

365 **Figure 2: Acute exposure to bixafen alters motor neuron axon development.**

366 (**A**) Illustration of the experimental setup used to analyze caudal primary motor neuron

367 (CaPMN) axons length on acute exposure to bixafen. (**B-G**) Three-dimensional

368 reconstructions of the lateral view of a section of Tg(Olig2:eGFP) embryo tails at 30 hpf
369 (**B**-**D**) or 60 hpf (**E**-**G**) treated with DMSO (vehicle, **B**, **E**), 0.3 μM bixafen (**C**, **F**) or 0.5 μM
370 bixafen (**D**, **G**), showing developmental defects of the CaPMN axon on exposure to bixafen
371 at 0.3 μM concentration that is worsened with the concentration increase (0.5 μM). (**E'**-
372 **G'**) Close-ups extracted from respectively **E**, **F** and **G**, showing that bixafen causes
373 developmental defects of CaPMN axons, that is worsened by increasing concentrations.
374 (**H**) Illustration of a zebrafish embryo; the red line marks the depth at which confocal
375 imaging was performed on (**B**-**G**). (**I**, **J**) Quantifications of the CaPMN axon length of
376 embryos at 30 hpf (**I**) or 60 hpf (**J**), treated with DMSO (N(30hpf)=8; N(60hpf)=8), 0.3 μM
377 bixafen (N(30hpf)=17; N(60hpf)=17) or 0.5 μM bixafen (N(30hpf)=16; N(60hpf)=13),
378 showing significantly shorter axons in embryos exposed to bixafen at LOAEL (0.3 μM) and
379 worsened with increase in concentration (LC50 0.5 μM), confirming the observations in
380 (**B-G**). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Scale bar : (**B-G**) = 100 μm.
381

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