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

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Running title: Neurodevelopmental defects in bixafen-exposed embryos

Keywords: Succinate dehydrogenase inhibitors, SDHI, Fungicide, Zebrafish, Neurodevelopment, *In vivo* imaging

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

Results

1. Toxicity of bixafen toward zebrafish embryos

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

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

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

2. Brain defects in embryos exposed to bixafen

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

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

Discussion

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

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

Bixafen is a methyl-pyrazole, one of the SDHI fungicides used to manage plant diseases and inhibit the respiration of pathogenic fungi by blocking succinate complex II in the mitochondrial respiratory chain. SDH is an enzyme involved in both oxidative phosphorylation and the tricarboxylic acid cycle, two processes that generate energy. This enzyme has been shown to be irreplaceable in mitochondrial and cell metabolism and also to be highly conserved among all fungi, plants and animal species (Rehfus et al., 2016; Veloukas et al., 2013; Yamashita and Fraaije, 2018). Therefore, any adverse change or inhibition of SDH activity can lead to many diseases, including those that have been linked to mitochondrial dysfunction (Birch-Machin et al., 2000; Bourgeron, 2015; Finsterer, 2008; Horváth et al., 2006; Parfait et al., 2000; Van Coster et al., 2003).

A recent study has questioned the specificity of these SDHIs, including bixafen. It showed that the latest-generation SDHIs sold on the market could inhibit the SDH of several species ranging from earthworms to human cells, evidence that these SDHIs are nonspecific (Bénit et al., 2019). Several epidemiological studies published during the last two decades suggest harmful effects of pesticides on human health (Merhi et al., 2007; Weichenthal et al., 2010). Pesticide poisoning is a serious health problem that disproportionately affects infants and children (Rauh et al., 2012). Pesticides are known to cause millions of acute poisoning cases per year (Bertolote et al., 2006). Human exposure to pesticides can occur environmentally, through consumption in food and water (van den Berg et al., 2012). A number of studies show that prenatal and early childhood exposure to some pesticides is associated with neurodevelopmental and cognitive disorders (Muñoz-Quezada et al., 2013; Ross et al., 2013). Very little is known about the consequences of exposure to SDHIs on human health. Currently, we know only the consequences of the changes in their target, the SDH complex. Mutations in the SDH complex are associated with several human diseases, including neurodegenerative

diseases and cancer (Ardissone et al., 2015; Birch-Machin et al., 2000; Bourgeron, 2015; Finsterer, 2008; Horváth et al., 2006; Parfait et al., 2000; Van Coster et al., 2003; Martin et al., 2007; Perry et al., 2006; Ricketts et al., 2009; Stratakis and Carney, 2009; Timmers et al., 2009).

The fungicide bixafen exists as a pure compound or as an ingredient in 27 approved and marketed fungicides. Our results will be used as basic data to study the complex mixtures that contain bixafen. It is not known how bixafen can affect the development of the central nervous system .Like for all SDHIs, the toxic effect of bixafen observed in this study may be linked to the basic disruption of mitochondrial activity and accumulation of reactive oxygen species caused on Complex II inhibition and subsequent oxidative DNA damage. These severe brain and spinal cord malformations may also be related to the different metabolic abnormalities reported in SDHI exposed animals (Graillot et al., 2012; Qian et al., 2018; Wu et al., 2018; Yang et al., 2018). A recent work (Bénit et al., 2019) has shown that bixafen also exerts an effect on mitochondrial respiration complex III, suggesting an adverse effect of bixafen on cellular metabolism.

In summary, our study provides new evidence of bixafen toxicity on neurodevelopment in a vertebrate model, and and may help us take the appropriate precautions 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 to avoid pigmentation, 0.003% 1-phenyl-2-thiourea (PTU) was added at 1 dpf (day post-fertilization). The following transgenic lines were used: Tg(elavl3:Gal4)^{zf349} (Akerboom et al., 2012), Tg(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) (Sigma, 32581) was dissolved at a stock concentration of 100 µM in pure DMSO (100%) and conserved at −20 °C in aliquots until use. 100 µM stocks were diluted in a 0.1% DMSO solution in fish water to final 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 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**

All statistics were obtained on Prism5 (GraphPad) and assessed using a Kruskal-Wallis test followed by a Dunn post-test. All data are represented as means ± SEM.

Author Contributions:

R.H.A. performed the experiments and the analysis, designed the figures, and wrote the original draft. A.B. performed data analysis. N.S.Y. supervised the project and wrote the manuscript.

Funding: This work was supported by Institut National de la Santé et la Recherche Médicale (INSERM), the National Center for Scientific Research (CNRS), and the French National Research Agency (ANR-16-CE18-0010). Funding sources had no involvement in study design, collection, analysis or interpretation of data, or decision to publish.

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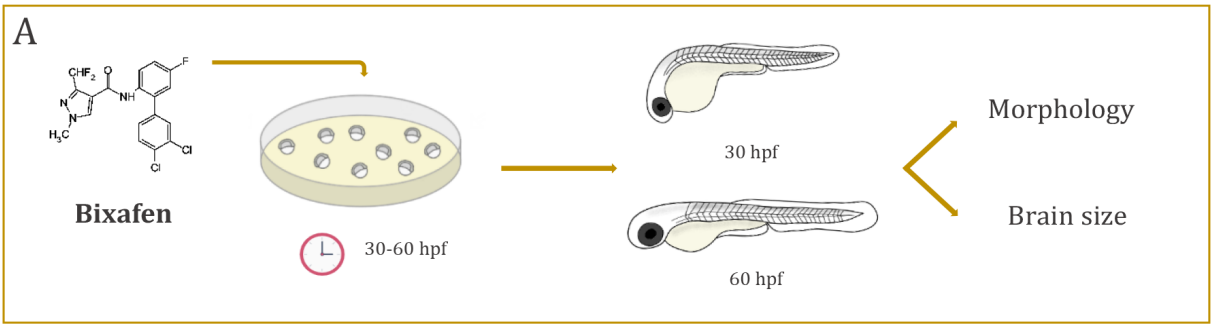
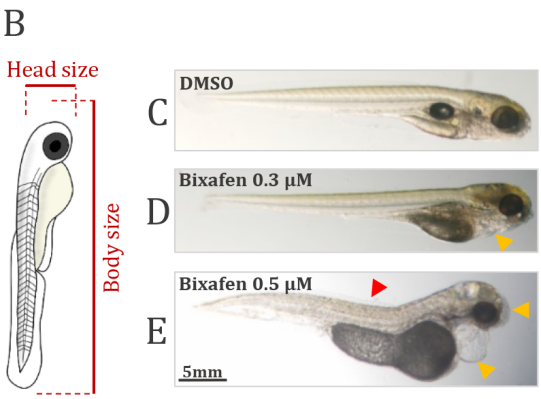
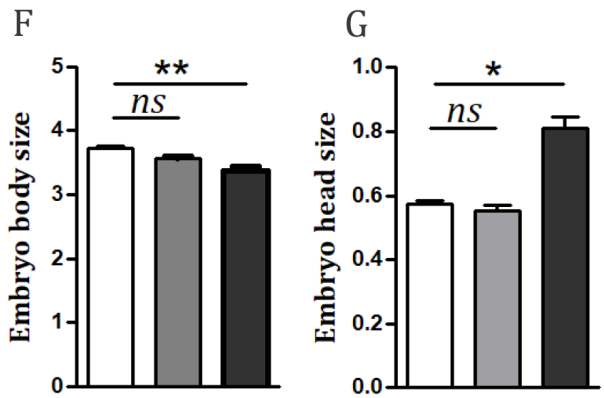
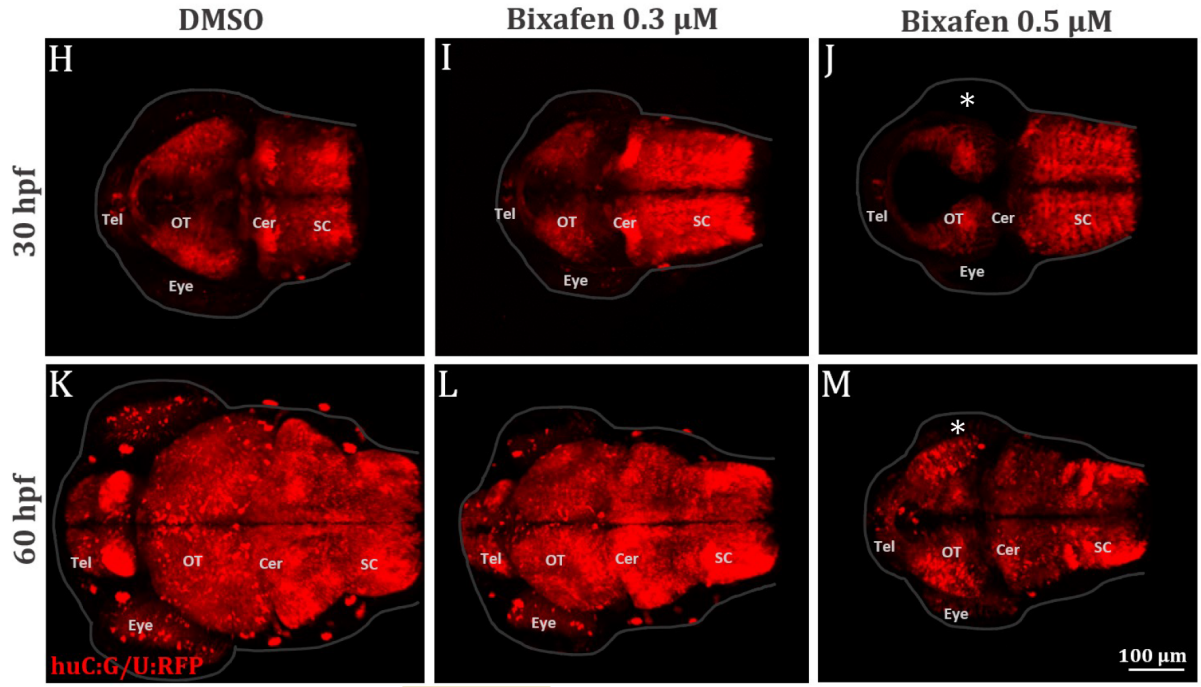
Acknowledgments: We thank Christiane Romain (Inserm UMR 1141), Olivier Bar (Inserm UMR 1141) for their technical assistance.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.









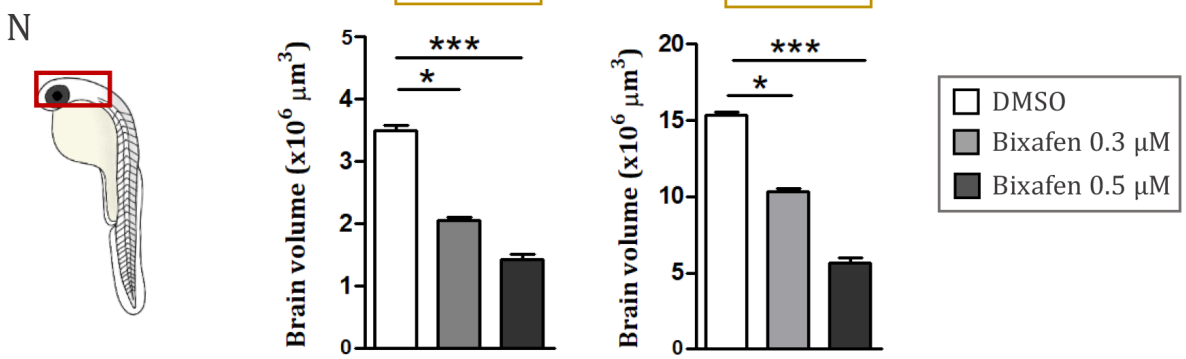


Figure 1: Acute exposure to bixafen causes microcephaly.

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

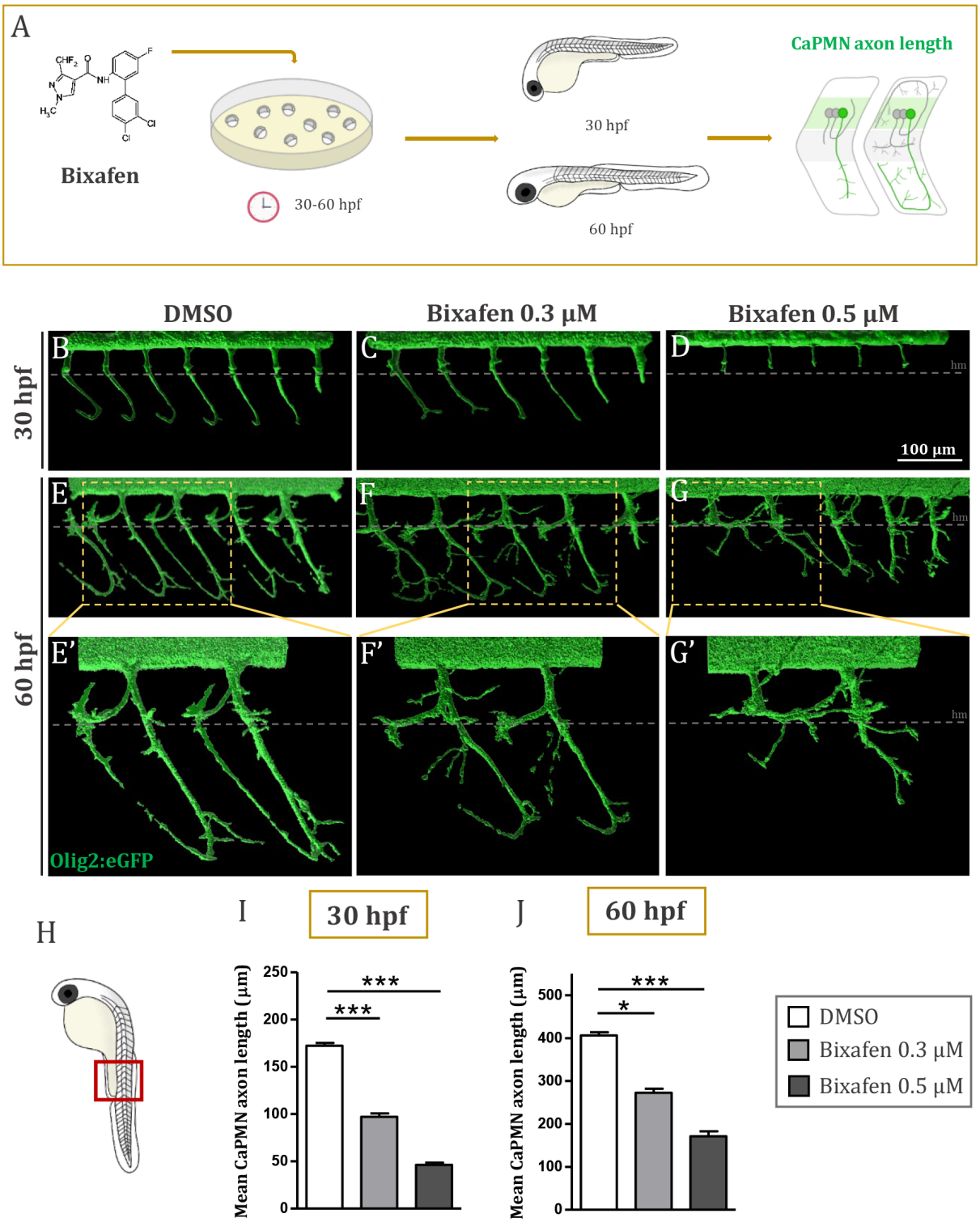


Figure 2: Acute exposure to bixafen alters motor neuron axon development.

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