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A proof of concept study demonstrating that environmental levels of carbamazepine impair early stages of chick embryonic development

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ABSTRACT

Carbamazepine (CBZ) is an anticonvulsant drug used for epilepsy and other disorders. Prescription of CBZ during pregnancy increases the risk for congenital malformations. CBZ is ubiquitous in effluents and persistent during wastewater treatment. Thus, it is re-introduced into agricultural ecosystems upon irrigation with reclaimed wastewater. People consuming produce irrigated with reclaimed wastewater were found to be exposed to CBZ. However, environmental concentrations of CBZ ($\mu g\ L^{-1}$) are magnitudes lower than its therapeutic levels ($\mu g\ ml^{-1}$), raising the question of whether and how environmental levels of CBZ affect embryonic development.

The chick embryo is a powerful and highly sensitive amniotic model system that enables to assess environmental contaminants in the living organism. Since the chick embryonic development is highly similar to mammalians, yet, it develops in an egg, toxic effects can be directly analyzed in a well-controlled system without maternal influences. This research utilized the chick embryo to test whether CBZ is embryo-toxic by using morphological, cellular, molecular and imaging strategies. Three key embryonic stages were monitored: after blastulation (st.1HH), gastrulation/neurulation (st.8HH) and organogenesis (st.15HH).

Here we demonstrate that environmental relevant concentrations of CBZ impair morphogenesis in a dose- and stage- dependent manner. Effects on gastrulation, neural tube closure, differentiation and proliferation were exhibited in early stages by exposing embryos to CBZ dose as low as 0.1 $\mu g\ L^{-1}$. Quantification of developmental progression revealed a significant difference in the total score obtained by CBZ-treated embryos compared to controls (up to 5-fold difference, $p < 0.05$). Yet, defects were unnoticed as embryos passed gastrulation/neurulation.

This study provides the first evidence for teratogenic effect of environmental-relevant concentrations of CBZ in amniotic embryos that impair early but not late stages of development. These findings call for in-depth risk analysis to ensure that the environmental presence of CBZ and other drugs is not causing irreversible ecological and public-health damages.

1. Introduction

Carbamazepine (CBZ) is an anticonvulsant drug used for epilepsy and psychiatric conditions. It acts as a channel blocker and modulator of neurotransmitter activity (Ambrósio et al., 2002; Grunze et al., 1999). Prescribed dosages to most patients range between 400 and 1200 mg day^{-1}, resulting in CBZ plasma levels of 4–12 $\mu g\ ml^{-1}$ (Neels et al., 2004). Congenital malformations were reported to occur upon exposure of fetuses to CBZ during pregnancy. These include neural-tube (NT) defects as well as craniofacial, cardiac and urinary system anomalies. Growth retardation and various cognitive deficits were also described (Afshar et al., 2010; Diav-citrin et al., 2011; Matalon et al., 2002; Ornoy and Cohen, 1996; Pearse et al., 1992). Yet, available data is somewhat conflicting since type, severity and frequency of defects vary between studies (Matlow and Koren, 2012; Vajda et al., 2016; Vorhees et al., 1990). Similar to humans, rodents that were exposed to clinical doses of CBZ during gestation developed birth-defects (Afshar et al., 2010; Finnell et al., 1986; Piersma et al., 1998).

In spite of these findings, it is less clear whether CBZ affects initial stages of embryogenesis, since young embryos are difficult to monitor and pregnancies may be terminated unrecognized. Nevertheless, when rodent/human embryonic stem cells (isolated from blastocysts prior to

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implantation) were added with therapeutic CBZ doses, their growth, differentiation and gene expression patterns were affected (Murabe et al., 2007; Schulpen et al., 2015; Shaikh Qureshi et al., 2014). Likewise, fish embryos grown in water containing clinical CBZ concentrations were smaller and perturbed in their morphology, gene expression and metabolism (Beker van Woudenberg et al., 2014; Lee et al., 2013; Nassef et al., 2010; van den Brandhof and Montforts, 2010; Weigt et al., 2011). These studies indicate that clinical concentrations of CBZ may impair early embryonic development.

Due to constant consumption and low removal efficiency during wastewater treatment, CBZ are commonly detected in effluents of wastewater treatment plants (Ferrari et al., 2003; Verlicchi et al., 2012). Low levels (ng L^{-1}) of CBZ were also reported in rivers and surface water and even in drinking water (Pomati et al., 2006; Qiang et al., 2016; Reemtsma et al., 2006; Verlicchi et al., 2012). These concentrations are 10^4–10^6 fold lower than clinical doses. Introduction of CBZ to agricultural soils via irrigation with reclaimed wastewater results in soil accumulation and uptake by plants (Czajkowski, 2010; Goldstein et al., 2014; Grossberger et al., 2014). Moreover, we recently showed that healthy individuals who consumed produce irrigated with reclaimed wastewater excrete CBZ in their urine (Paltiel et al., 2016). This study suggested that the population in water scare regions may be exposed to CBZ (and other pharmaceutics) via the food chain. Yet, risks associated with consuming such produce are currently unknown (Czajkowski, 2010; Prosser and Sibley, 2015). It is also unclear whether embryos exposed to environmental-relevant concentration of CBZ are affected.

CBZ can cross the intestinal and placental barriers of pregnant mice that consumed water spiked with low CBZ concentrations (Kaushik et al., 2016), implicating that mouse embryos can be exposed to low CBZ levels through their mothers. However, development of these embryos was not tested. Fish embryos exposed to similar low CBZ levels (0.5 μg L^{-1}) exhibited altered hatching time and body length, small increase in mortality rate and modified behavior (Galus et al., 2013a, 2013b; Qiang et al., 2016). These studies imply that environmental-relevant doses of CBZ affect fish embryonic development. Yet, whether environmental CBZ levels can also impact amniotic embryos is not known.

The chicken embryo (*Gallus gallus domesticus*) is a powerful model in developmental biology (Kain et al., 2014; Rashidi and Sottile, 2009; Stern, 2018). Its well-study anatomy, easy accessibility, availability of genomic databases and molecular markers, as well as feasible visualization, makes it an excellent platform to analyze developmental toxicity in vivo (Romanoff, 1972). As amniotic, its development is closer to mammals than teleost, and thus its relevance to toxicological and biomedical research is substantial (Alexander and Tuan, 2003; Beedie et al., 2016; Davey et al., 2018; Drake et al., 2006; Schock et al., 2016; Smith et al., 2012). Although mammalian embryos are fundamental for toxicological research, the high experimental costs, long gestation period, poor embryonic accessibly as well as ethical guidelines, limit their extensive applications. The chick embryo can compensate for these limitations as it develops in an egg and thus, large scale experiments can be performed to directly analyze teratogenic effects in a close system without maternal influences. Indeed, many studies utilized the chick embryo to investigate environmental contaminants such as various chemicals, drugs, heavy metals and other substances, in which different developmental defects and pathologies were found (Carro et al., 2013; Nordén et al., 2016; Peng et al., 2016; Roig et al., 2014; Salvaggio et al., 2018; Szabó et al., 2017; Veeriah et al., 2017).

Here, we utilized the chick embryo to determine whether CBZ at environmental-relevant and sub-therapeutic concentrations impact development. Three embryonic stages were chosen to cover key periods of early development. Combining morphological, cellular and molecular analyses with advanced imaging technology, we demonstrate that environmental levels of CBZ impair embryogenesis in a well-defined dose- and stage- dependent manner.

2. Materials and methods

2.1. In vivo experiments

Fertile Lohmann eggs (Gil-Guy Farm, Orot, Israel) were incubated for 5, 25 and 45 h in 38 °C until reaching Hamburger Hamilton (HH) stages of 1, 8, 15 (Hamburger and Hamilton, 1993), respectively (Fig. S1A). Eggs were windowed (Kohl et al., 2013) and CBZ (dissolved in double distilled water (DDW)) or DDW (control solution), were dripped over the embryos. Embryo, at different stages was exposed to the same amount of CBZ. For that, before the experiments were executed, embryos of the three stages were cut-out of the egg, measured and weighted. Based on embryonic weight, CBZ solution was added at quantitates of 0.02, 0.2, 2, 20, and 200 pg mg^{-1} embryonic weight. These CBZ concentrations correspond to concentrations of 0.01, 0.1, 1, 10, 100 μg L^{-1}, respectively, which are reported in treated wastewater. Embryos were exposed to CBZ every 8 h at 3 sequential additions (0, 8 and 16 h; Fig. S1B). Following each application, eggs were sealed and re-incubated. Embryos were incubated for 48 h, then fixed in 4% paraformaldehyde (Sigma-Aldrich, Rehovot, Israel) for 24 h at 4 °C prior to further analyses.

2.2. Ex-vivo experiments

Easy Culture method was applied to grow embryos ex-vivo, as describe elsewhere (Chapman et al., 2001). Briefly, a Whatman paper ring was placed around the embryo. The vitelline membrane was cut around the ring such that embryo was pulled away and placed onto agar-albumen dish with ventral side up. 200 μL of DMEM/F12 media (Biological Industries, Beit Haeemek, Israel) was added on top of embryos with/without 0.1 μg L^{-1} CBZ that were then incubated at 38 °C. Following 8 h of incubation, embryos were treated again with CBZ or DDW and taken for time lapse imaging analysis.

2.3. Morphological scoring

To measure changes in gross morphology, multiple anatomical criteria were selected (Table S1). Numerical scores were provided for each anatomical parameter, according to subsequent developmental stages of a typical embryo. Control or CBZ-exposed embryos were scored for each parameter. Sum of all criteria provided a numerical value of the overall developmental status.

2.4. Immunofluorescence

Immunostaining of whole-embryos was performed as previously described (Peretz et al., 2016), using rabbit-anti Sox2 antibody (1:400, Merck Millipore, MA, USA), and secondary anti-mouse Alexa 488 antibody (1:400, Thermo Fisher Scientific, MA, USA). Immunostaining of frozen section was performed as described elsewhere (Kohl et al., 2015). Briefly, embryos were incubated in 30% sucrose/PBS and embedded in optimal cutting temperature solution (Sakura Finetek, CA, USA). Blocks were cut to 12 μm-thick transverse slices using CM1860 cryostat (Leica Biosystems, Nußloch, Germany), and incubated with: rabbit anti PhH3 (1:300, Santa Cruz Biotechnology, TX, USA), mouse-anti 3A10 (1:100, Developmental Studies Hybridoma Bank, IW, USA), rabbit-anti cleaved Caspase 3 (Casp3, 1:500, Thermo Fisher Scientific, MA, USA), rabbit-anti Sox2 and rabbit-anti Laminin (1:300, Merck Millipore, MA, USA). Slides were added with secondary antibodies as mentioned above, washed and incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1 mg mL^{-1} stock solution,1:500, Sigma-Aldrich, Rehovot, Israel). In some cases, sections were stained with Phalloidin (1300, Thermo Fisher Scientific, MA, USA) (Roth et al., 2017). Embryos or sections were washed and mounted for microscope analysis.

2.5. Flow cytometry

Embryos were dissected, dissociated into single cells and prepared for analysis as recently described (Peretz et al., 2018). Cells were stained with PhH3 and Casp3 antibodies (1:300 each), incubated with Alexa-Fluor secondary antibodies (1:500) and analyzed with Accuri C6 Flow Cytometer (BD Biosciences, CA, USA). Data analysis was performed using BD Accuri C6 software.

2.6. Microscopy

Whole-mounted embryos were imaged under epi-fluorescent stereomicroscope attached to CCD camera (Discovery V20 Stereo and AxioCam, Zeiss, NY, USA). Sections were imaged under epi-fluorescent upright microscope (E400, NIKON, NY, USA) with a CCD camera (DP70, Olympus, MA, USA). Time lapse imaging was performed using the Delta-Vision Elite system (Applied Precision, Ontario, Canada), on an Olympus IX71 inverted microscope, running softWoRx 6.0 by a CoolSnap HQ2 CCD camera (Roper Scientific, USA). Embryos were captured for 30 h at 20 min intervals at x4 magnification. Captured images were analyzed by ImageJ software (Zamir et al., 2017). For high resolution episcopic microscopy (HREM), embryos were dehydrated and embedded as previously described (Pokhrel et al., 2017; Weninger and Mohun, 2007). Embryos were mounted in a vertical orientation and sliced to 1.5 μm-thick sections. Images of 1500 < sections were captured, stacked and processed using Fiji software (Schindelin et al., 2012). 3D reconstruction was performed using Amira software (FEI, Oregon, USA).

2.7. Cell counts and statistical analysis

Quantification of cells stained for PhH3/Casp3 was conducted by counting fluorescent-labeled cells out of total $DAPI^+$ cells in one side of the neural tube. Percentages represent a mean of five different sections from three different embryos. In all experiments, statistical analysis was performed using Microsoft Excel software (version 2013), SPSS 22.0 and WinPepi 11.43 statistical software. Comparisons of developmental scores or of stained cells in treated and untreated embryos were analyzed using one-sided student's *t*-test or Welch's test according to the equality of variances (Levene's test) and normality of distribution (Shapiro-Wilk's test). Associations were considered significant when $p < 0.05$. Error bars represent standard deviation.

3. Results and discussion

3.1. Dose and stage-dependent effects of CBZ

To address whether environmental-relevant and sub therapeutic levels of CBZ affect early development, chick embryos at stages 1, 8, 15 HH were exposed (Fig. S1A). These embryonic stages represent the following key developmental periods; (*i*) after blastulation/prior gastrulation, (*ii*) during gastrulation/early neurulation, (*iii*) during organogenesis. These stages correspond to 7–30 day-old human embryos (Carnegie stages 4–13), stages that are highly prone to teratogenicity (Sadler, 2012). The embryos were exposed to CBZ concentrations which are typically found in reclaimed wastewater and irrigation water (Malchi et al., 2014; Paltiel et al., 2016; Paz et al., 2016).

3.1.1. St.1HH (after blastulation/prior gastrulation)

Examination of control embryos (added with DDW) revealed that 50% developed normally, 25% died and 25% were vital but growth retarded or morphologically abnormal (Fig. 1A). The high proportion of affected embryos is a result of their vulnerability to any external manipulation at this stage. Yet, when embryos were exposed to CBZ none of them developed normally (Fig. 1A). Moreover, while at low CBZ dosages (0.02–0.2 pg mg^{-1}) ~50% were malformed or growth-retarded and the rest died, exposure to 2–200 pg mg^{-1} CBZ led to a gradual increase in lethality (from 50 to 90%) and decrease in malformed embryos (from 50 to only 10%).

3.1.2. St. 8HH (gastrulation/early neurulation)

Analysis of control embryos revealed that 75% developed normally, 12% died and 13% were growth retarded or malformed (Fig. 1D). The higher number of viable embryos in comparison to st.1HH embryos (Fig. 1A) is a result of their higher resistance to manipulation when they grow further. Exposure to 0.02 mg pg^{-1} CBZ led to only minimal alternations in development, similar to controls (63% developed normally, 25% died and 12% were perturbed). Yet, exposure to 0.2 mg pg^{-1} CBZ resulted in a marked increase in abnormal or dead embryos (~50% and 25%, respectively), which was accompanied by 25% decrease in normal embryos. Higher CBZ concentration (2–200 pg mg^{-1}) resulted in a dose-dependent increase in embryonic mortality (from 25 to 65%) and decrease in malformed or growth-delayed embryos (from 50 to 30%). Moreover, the presence of unaffected embryos gradually decreased from 65 to 0%, respectively (Fig. 1D).

3.1.3. St. 15HH (organogenesis)

Exposure of embryos to CBZ did not hamper their development (Fig. 1G). In any CBZ concentration, 90–100% of embryos survived and developed normally, similar to controls. The low percentage of dead or malformed embryos did not correlate with increasing CBZ concentrations and represents natural variations.

Altogether, these results demonstrate that environmental-relevant dosages of CBZ impair embryonic development in a stage- and dose-dependent manner; Embryos at pre-gastrula stages are the most sensitive to very low CBZ doses. Embryos undergoing gastrulation/neurolation are also highly susceptible to CBZ, but mortality rates are lower than earlier stages. Embryos undergoing organogenesis are largely unaffected by CBZ. These data are the first indication that environmental concentrations of CBZ can impair embryonic development in amniotes. Similar dose response effects of CBZ were reported in fish, in which chronic exposure to environmental relevant levels of CBZ impacted multiple organs (Galus et al., 2013a, 2013b). For example, exposure to CBZ led to a dose-dependent decline in reproduction and increase in internal organ pathologies in adult fish. Behavioral tests also demonstrated altered responses of fish to environmental stimuli that were correlated with gradual increase in environmental CBZ dosages (Qiang et al., 2016; Thomas, 2012). Moreover, gradual appearance of edemas and pre-hatching mortality were evident in zebrafish embryos added with increasing dosages of CBZ (Pohl et al., 2019). Yet, the dose-dependent effects found in chick embryos are more prominent than in fish, as 3 orders of magnitude higher CBZ concentrations were required to induce large rate of mortality and deformation in zebrafish embryos in comparison to chick (Hermsen et al., 2011, 2013). In line with these differences, a recent comparative study screened multiple analogs of thalidomide in chicken and zebrafish embryos and revealed that different compound concertation is required to elicit similar defect in each type of embryo (Beedie et al., 2016).

The finding that CBZ affects chick development in a stage dependent manner is also in agreement with recent studies in fish in which age-dependent differences in sensitivity to environmental contaminants were shown (Corrales et al., 2017; Jeffries et al., 2014; Kristofco et al., 2018; Steele et al., 2018). For example, exposure of zebrafish to chemicals causing oxidative stress or to the antihistamine diphenhydramine, led to varied modifications in gene expression, fish behavior and mortality, according to certain embryonic or larvae stages (Hahn et al., 2014; Kristofco et al., 2016, 2018). Such varied sensitivities were suggested to occur by age-dependent alterations in toxicant uptake and/or metabolism or due to differential expression of target genes at different stages (Corrales et al., 2017; Jeffries et al., 2014; Kristofco et al., 2018; Steele et al., 2018). Yet, in these studies, the effects of the contaminants increased along with progression in development,

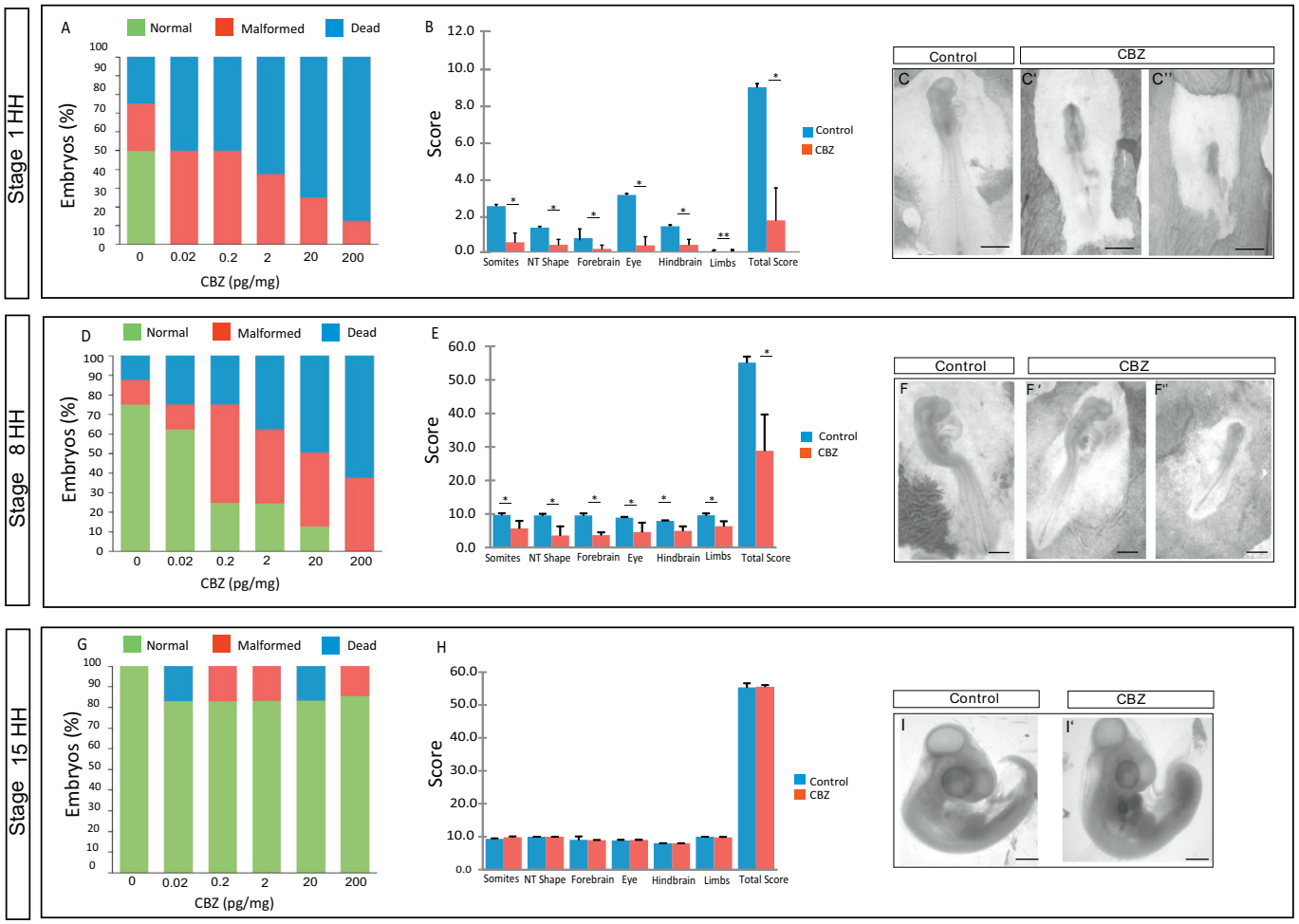

Fig. 1. CBZ at environmental concentrations perturbs embryonic development in a dose and stage dependent manner.
(A,D,G): Dose dependent effect of CBZ (0–200 pg mg^{-1}) on embryonic survival and morphology at different stages (1HH n = 8; 8HH n = 12; 15HH n = 10 for each concentration).
(B,E,H): Graphs of morphological scoring of embryos at different stages after exposure to 0.2 pg mg^{-1} CBZ. Bars represent scoring of individual morphological parameters. Left bar represent the sum of all scores (1HH n = 27; 8HH n = 35; 15HH n = 42–43), *$p < 0.05$. (C, F, I): Bright field images of representative embryo of different stages after exposure to 0.2 pg mg^{-1} CBZ. Scale bar-100 um. In all panels stages and CBZ concentrations are indicated. Control embryos were added with DDW.

whereas in our findings CBZ effects were more profound at early rather than late stages. This discrepancy may result from differences in the model organisms, type of drug and its possible toxicodynamic modifications at different stages.

3.2. Morphological scoring of CBZ effects

To quantify the type and severity of defects induced by CBZ, an unbiased scoring method was established, based on anatomical landmarks that define precise developmental stages. Parameters included somite number, NT flexures, size and shape of forebrain, hindbrain, eyes and limb-buds (Table S1). Each parameter received a numerical score which increased with developmental progression. Embryos from all examined stages were added with 0.2 pg mg^{-1} CBZ, the lowest concentration that impaired morphology and viability in both st.1HH and 8HH (Fig. 1A,D). Measurements were performed only in surviving embryos.

3.2.1. St.1HH (after blastulation/prior gastrulation)

CBZ induced multiple morphological defects in all tissues. Hence, low score was gained for each parameter compared to control embryos (Fig. 1B). Summing up all scores showed ~ 5-fold decrease in the overall grade of CBZ-treated embryos compared to controls. This result corroborates the dose-dependent assay (Fig. 1A), where embryogenesis was largely impaired at very low CBZ dosages. Examples of affected embryos demonstrate abnormal cranial morphology, heart hyperplasia, fewer and compressed somites, abnormal NT and shortened body-length (Fig. 1C′,C″). These phenotypes were not observed in controls (Fig. 1C).

3.2.2. St. 8HH (gastrulation/early neurulation)

Exposure to CBZ led to lower score for each parameter in comparison to controls (Fig. 1E). Nevertheless, scores gained for ectodermal tissues (i.e., NT, forebrain, eyes) were lower than those received for mesodermal tissues (i.e., somites, limb-buds), implicating for a stronger effect of CBZ on neural derivatives. The overall grade of CBZ-added embryos was ~2-fold lower than that of controls, further supporting its harmful effect at this embryonic stage. Noteworthy is that the score-difference between control and treated embryos of st.8HH was lower than of st.1HH. This corresponds to the dose-response analysis (Fig. 1A,D), where effects were severer in pre-gastrulating embryos than in gastrulating stages. Representative examples of CBZ-treated embryos demonstrate either mild growth-retardation (Fig. 1F′), or severe deformities (body-size reduction, fewer somites, impaired NT

closure, abnormal head and eye vesicles, hyperplastic heart) (Fig. 1F″). Control embryos displayed normal morphology (Fig. 1F″).

3.2.3. St.15HH (organogenesis)

Control or CBZ-added embryos yielded similar high scores in all parameters without significant difference between the groups (Fig. 1H). Examples of embryos with normal morphology are provided (Fig. 1I,I′). This is consistent with the dose-dependent results (Fig. 1G), where exposure to CBZ did not impact embryonic development.

Together, this analysis further indicates that sensitivity to environmentally-relevant levels of CBZ correlates with developmental stages; the younger the embryos is, the more profound the effect is. Yet, malformations were found at both pre-gastrula and gastrula stages. In contrast, CBZ did not impact development in older embryos. These observations are compatible with data from humans, where the impact of exposure to teratogens frequently depends on embryonic age (Hertz-Picciotto et al., 1996; Sadler, 2012). For instance, during the first 2 weeks of gestation many teratogens cause mortality rather than birth-defects. This time-window is comparable with stage 1 HH chick embryos, where CBZ was the most toxic. At later stages of human embryogenesis, each organ has a critical period during which its development is disrupted. For example, the central nervous system is the most sensitive to perturbation at 6–14 gestation weeks (Bell and Gosden, 1978; Edwards and Hui, 2018). This is compatible with our data where neural tissues were more affected then mesodermal tissues at embryos of st.8 HH, and later neither tissue was sensitive to CBZ.

Notwithstanding, CBZ undergoes metabolism in the body. The main pathway of CBZ is epoxidation by cytochrome P-450 to the reactive and

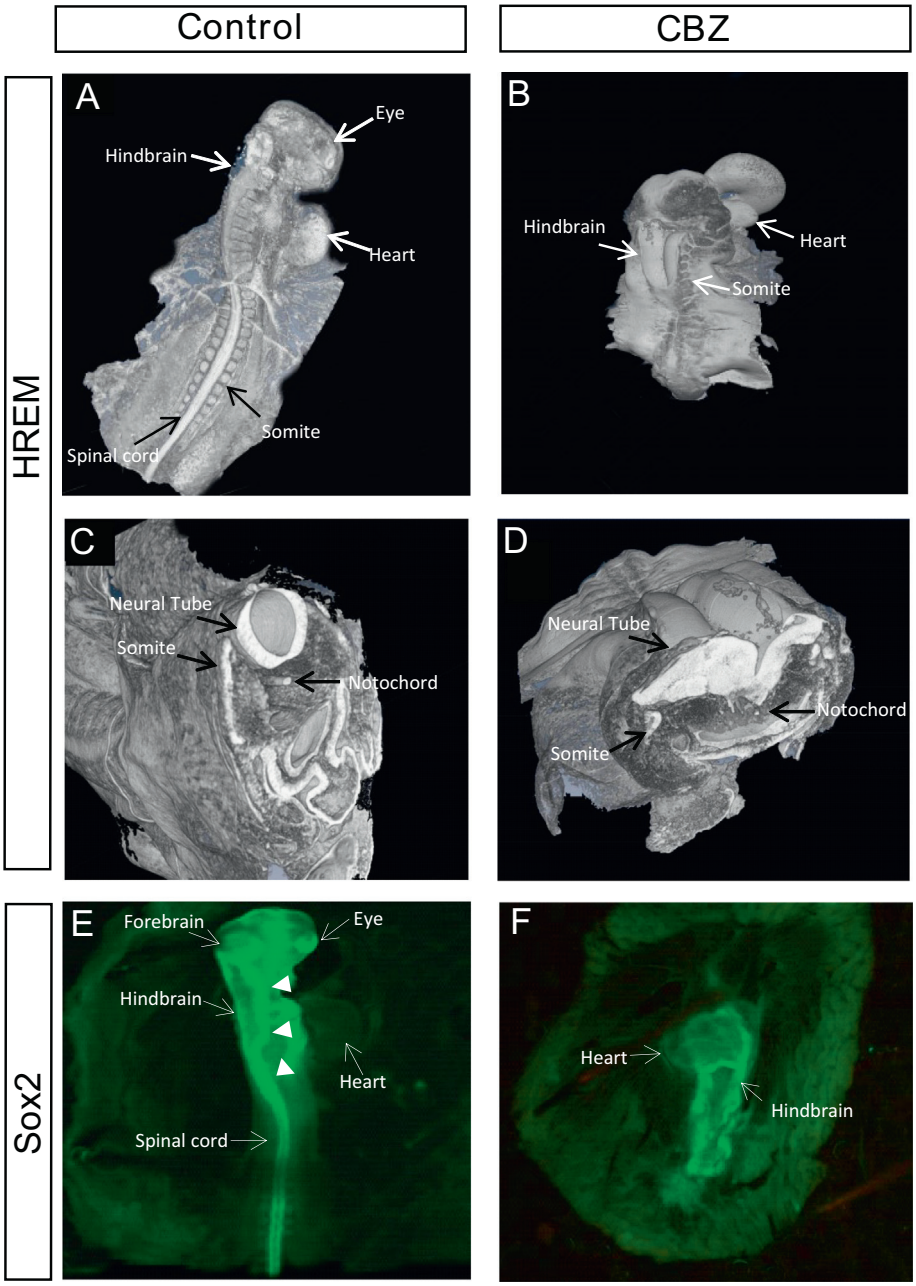

Fig. 2. HREM and marker expression analyses of embryos of stage 1HH.
(A–D) HREM analysis of control or CBZ-added embryos. 3D views are shown in whole mounts (A, B) or in transverse sections taken from the cervical level (C, D) (n = 3 for each group). (E,F) whole-mount Immunofluorescence staining of Sox2 in control or CBZ-added embryos (n = 3 for each group). In all panels, CBZ concentration is 0.2 pg mg^{-1} and anatomical landmarks are indicated. Control embryos were added with DDW.

pharmacologically active metabolite 10,11-epoxycarbamazepine (epoxy-CBZ) (Bernus et al., 1996; Bu et al., 2005; Sankar and Lerner, 2008). In clinical concentrations, epoxy-CBZ is known to cause teratogenic effects in murine (Bennett et al., 1996; Hill et al., 2010). In zebrafish embryos, increased concentrations of epoxy-CBZ were detected upon continuous exposure to CBZ (Park, 2016), suggesting that epoxy-CBZ may be more significant in causing embryonic/larval defects (Pohl et al., 2019). Whether in young chick embryos CBZ undergoes similar metabolism to produce epoxy-CBZ (or other metabolites), that in turn mediates the embryo-toxicity, awaits further research.

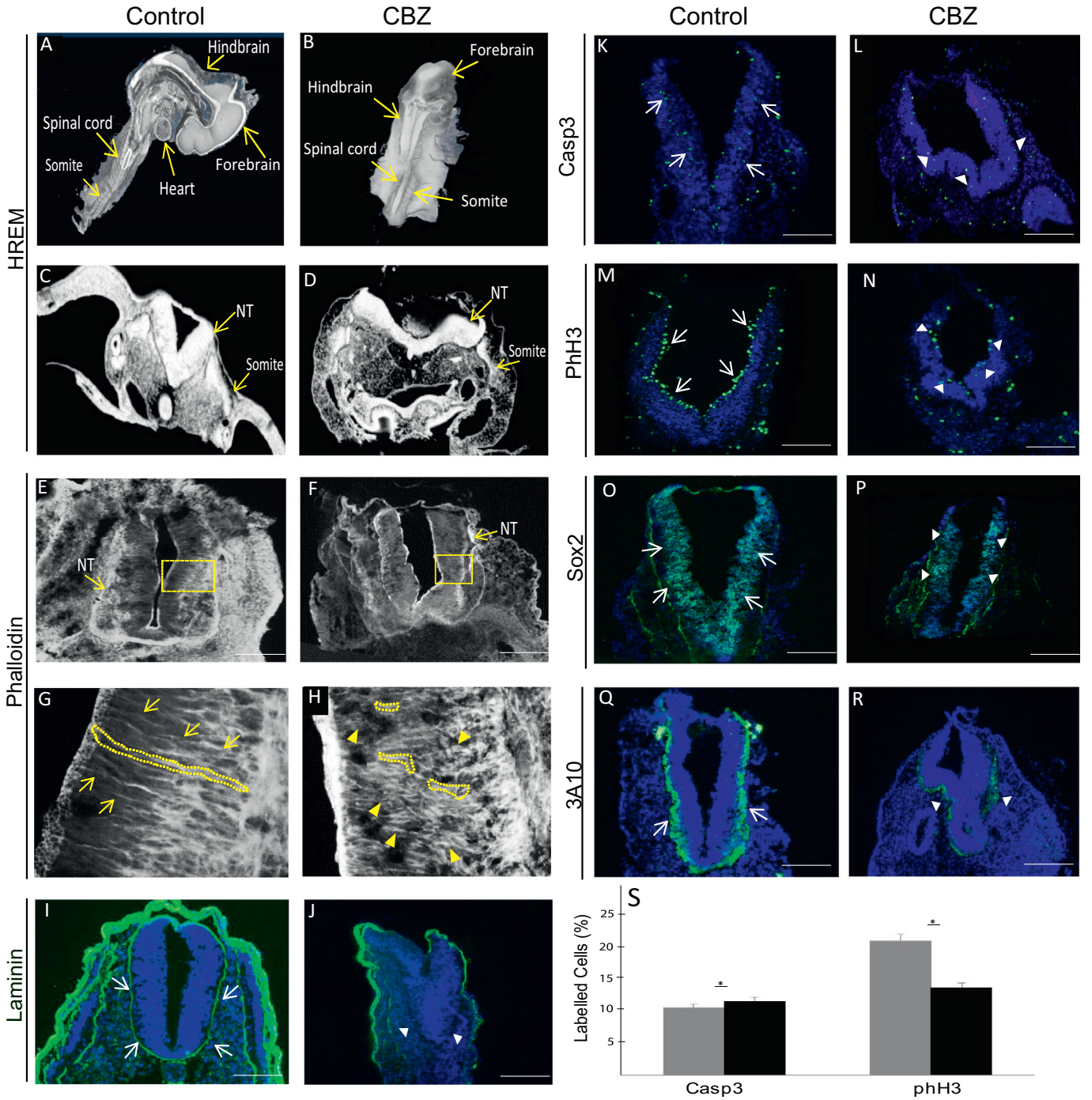

Fig. 3. HREM and marker expression analyses of embryos of stage 8HH.
(A–D): HREM analysis of control or CBZ-exposed embryos (n = 3 for each group). 3D views are shown in whole mounts (A, B) or in transverse sections taken from the hindbrain level (C,D). (E–H): Images of control or CBZ- exposed embryos stained for phalloidin (n = 5 for each group). Sections were taken from hindbrain level. Boxed areas in E and F are shown as enlargements in panels G, H, respectively. (I-R): Images of control or CBZ- exposed embryos immunostained for multiple markers (green, n = 6 for each marker). Cell nuclei are marked by DAPI. Arrows and arrowheads indicate marker expression in control or CBZ treated embryos, respectively. (S): Graphical representation of cells expressing Casp3 and PhH3 markers in control or CBZ treated embryos. *$p < 0.05$. In all panels, CBZ concentration is 0.2 pg mg^{-1}, anatomical landmarks are indicated and control embryos were added with DDW. Scale bar-50um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Microscopic, cellular and molecular analyses

To further determine the precise effect of CBZ on embryonic morphology and expression of cell markers, a series of analyses were performed on embryos following treatment with 0.2 pg mg^{-1} CBZ. These included: (*i*) HREM imaging to reveal morphological features in a high 3D resolution (Pokhrel et al., 2017; Weninger and Mohun, 2007), (*ii*) immunofluorescence staining, (*iii*) time-lapse analysis.

3.3.1. St.1HH (after blastulation/prior gastrulation)

HREM analysis of whole control embryo showed typical morphology (as expected at the time of fixation) such as 25 aligned somites, looped heart and elongated closed NT with right-turned head (Fig. 2A, and Movie S1). 3D reconstruction of sections revealed defined neural, mesodermal and endodermal layers, closed NT, characteristic notochord and somites, distinct endodermal tube and closed body wall (Fig. 2C and Movie S2). In contrast, CBZ-exposed embryo demonstrated truncated and shortened body axis, reduced brain, opened NT, inflated heart tube and fewer compressed somites (Fig. 2B, Movie S3). Analysis of 3D section reconstruction revealed a marked NT closure defect, irregular and asymmetrical neural tissue, abnormal somite structures, and expanded gut tube (Fig. 2D, Movie S4).

As the neural tissue was highly distorted, we examined whether neurulation, the process that follows gastrulation and generates the NT, is affected. Embryos were stained for Sox2, a landmark gene that labels all neural precursor cells in early embryonic stages (Ellis et al., 2004; Uchikawa et al., 2011). In control embryos Sox2 expression was evident along the entire NT and optic vesicles (Fig. 2E). Yet, it was significantly reduced in CBZ-exposed embryos (Fig. 2F). The reduced Sox2 expression corresponds to the lack of normally formed neural plate as found in the HREM analysis, which is likely to result from aberrant gastrulation that failed to progress further. Overall, these data suggest that exposure of post-blastula/pre-gastrula embryos to environmental levels of CBZ perturbs gastrulation/neurulation, leading to high rates of defects and mortality.

3.3.2. St. 8HH (gastrulation/early neurulation)

HREM analysis of control embryo revealed typical morphology of cranial and cervical flexures, distinct brain vesicles, elongated body axis with proper somites as well as normal heart shape, as expected at the time of analysis (Fig. 3A, Movie S5). A representative control section shows a properly aligned and curved NT with distinct roof-plate as well as typical mesodermal layer with properly-developed dermomyotome, sclerotome, notochord and aorta (Fig. 3C, Movie S6). Exposure to CBZ led to major malformations such as much shortened body axis, abnormal NT flexures, under-developed brain vesicles, inflated heart and compressed somites (Fig. 3B, Movie S7). Analysis of transverse sections confirmed that the NT is wiggled and fails to close and that the dermomyotome, sclerotome and notochord are abnormal in size and shape (Fig. 3D, Movie S8). This analysis verified that exposure to CBZ during gastrulation/neurulation results in various neuronal and mesodermal tissue defects as well as in growth retardation. Nevertheless, embryos were largely viable as opposed to the younger-stage embryos.

Notably, similar types of defects were reported in zebrafish embryos exposed to CBZ, which presented increased mortality and defects in gastrulation, somite formation and tail structure, malformation of head and heart, abnormal trunk flexure and reduced hatching success (van den Brandhof and Montforts, 2010). Moreover, exposure of post-implanted rat embryos to CBZ in vitro showed similar types of NT defects and growth retardation (Piersma et al., 1998). However, in both reports, CBZ concentrations were ~1000 higher than those used in our study.

To trace the onset of morphological deformities, live embryos were monitored. Embryos were grown ex-vivo and imaged every 20 min for 30 h (Fig. S2A–B″, Movie S9). While controls displayed proper body elongation, closure of the NT, division of the forebrain into two vesicles and formation of > 20 somites, CBZ-exposed embryos failed to demonstrate such orchestrated development. Rather, their body axis remained shortened and twisted, NT remained open in the cervical level and the forebrain did not divide into two vesicles. Moreover, only 15 somites were formed and had abnormal shape. Differences between the embryos were initially noticed after 10 h of exposure to CBZ. The gap between the time of CBZ application and the initial appearance of aberrations argues against the possibility that CBZ induces a non-specific toxic effect on the entire embryo such as a rapid induction of cell death, and supports the option of a more specific effect on CBZ on designated cellular processes.

The early NT is composed of pseudostratified neuroepithelium where each cell anchors to the apical and basal surfaces of the NT and its dorsal folds merge to form a tube shape. This structure depends on dynamic modulations of multiple cytoskeletal proteins, including F-actin (Letort et al., 2015). As we found that CBZ greatly affects NT shape and closure (Figs. 1–3), we analyzed its cellular organization by labelling actin fibers with Phalloidin. Control embryos demonstrated typical actin staining where cells displayed columnar organization and attached the ventricular and basal NT surfaces (Fig. 3E,G). Moreover, NT folds were merged, both NT sides were aligned and symmetric and actin-rich rings were observed in the cell's apical end-foot. Addition of CBZ led to a drastic distortion in NT architecture (Fig. 3F,H). Cells were not aligned but adopted an irregular shape without reaching the apico-basal surfaces of the NT and actin was deposited along the ventricular surface of the NT. The NT dorsal folds remained open, the floor plate was under-developed and the width of the NT was reduced and both sides were not symmetrical. These deformities resemble early phenotype of NT defects (NTDs), where apico-basal polarity of the NT is lost together with neuropore closure failure (Bahri et al., 2010; Eom et al., 2011).

Notably, similar phenotype was observed in fish embryos that grew in water containing a mixture of environmentally-relevant pharmaceuticals, including CBZ. However, low concentrations of CBZ alone did not cause similar NTDs in fish (Galus et al., 2013a, 2013b), although higher CBZ doses led to NTDs in zebrafish, human and murine embryos. Testing of another antiepileptic drug, valproic acid (VPA) was previously performed in the chick embryonic model (Hsieh et al., 2012; Whitsel et al., 2002). Increased mortality, growth delay, NTDs and another anomalies were demonstrated, similar to those seen in human fetuses exposed to VPA. VPA was shown to downregulate the levels of superoxide dismutase, histone deacetylase and folate, all suggested to be linked to the appearance of NTDs. As some of the malformations observed by VPA resemble the effects observed by CBZ, it will be interesting to examine whether CBZ induces change in the expression of these (or other genes), leading to NTDs and growth retardation in the chick embryo.

The basement membrane (BM) is located at the border between the NT, ectoderm and paraxial mesoderm. It is essential for NT integrity by providing a substratum for cell attachment and by instructing cell migration/differentiation decisions (Amenta et al., 1983; Hynes et al., 1986; Rutka et al., 1988). Since NT cells were distorted upon CBZ, we analyzed the integrity of the BM by staining for Laminin, a main BM protein (Segal et al., 2010; Tuckett and Morriss-Kay, 1986). Control embryos showed typical Laminin deposition around the entire NT, notochord, somite and ectoderm (Fig. 3I). In CBZ-treated embryos Laminin expression was largely reduced from the NT surroundings (Fig. 3J), although it persisted in the ectoderm. This finding excludes the possibility of a non-specific toxicity that leads to an overall loss in Laminin gene expression. The absence of Laminin from the BM around the NT is concomitant with its bent shape and loss of apico-basal anchoring of cells (Fig. 3F,H). Interestingly, Laminin expression was reported to decrease in rat embryos upon exposure of their pregnant mothers to the CBZ derivative oxcarbazepine (OXC) (Gürgen et al., 2012). As cell differentiation and tissue architecture were also distorted in the OXC-exposed rat embryos, the effect of OXC on BM proteins was

suggested to impair embryonic implantation that may result in early pregnancy ending.

Due to the drastic effects of CBZ on tissue morphology, we next assess whether CBZ induces cell death. Embryos were immunostained for Caspase3 (Casp3), an enzyme known to be upregulated during apoptosis (Elmore, 2007; Shi, 2004). Low and similar occurrence of cells undergoing apoptosis was found in control and CBZ-added embryos (Fig. 3K,L). In particular, even though the NT shape was distorted upon CBZ, no elevated cell-death was found in the NT. Cell counting revealed that ~10% of cells were stained for Casp3 in both groups (Fig. 3S). Apoptotic cell death was also analyzed by flow cytometry method (Fig. S2C–E). This analysis revealed 10.2% or 11.4% of control or CBZ-treated cells, respectively, that expressed Casp3. The minute increase in apoptosis upon CBZ cannot explain the major deformities in the exposed embryos. Hence, the possibility that CBZ triggers cell death that leads to embryonic malformations is less favorable. Similar conclusion was suggested in zebrafish exposed to environmental concentrations of CBZ. Although treated animals developed significant kidney and liver histopathology, no elevated cell-death was found in these tissues (Galus et al., 2013b).

Changes in the cell cycle are another mechanism that may lead to developmental defects. Embryos were stained for Phospho-histone H3 (PhH3) to label dividing cells during metaphase (Kim et al., 2017; Sawicka and Seiser, 2012). Sections obtained from CBZ-treated embryos demonstrated marked decrease in PhH3 expression, together with distorted NT shape, compared to controls (Fig. 3M,N). Cell counting revealed ~50% decrease in dividing cells upon exposure to CBZ (Fig. 3S). The proportion of dividing cells was also assessed by flow cytometry which showed > 2-fold decrease in the proliferating cells in CBZ-added embryos versus controls (Fig. S2F–H). These results suggest that effects induced by low concentrations CBZ are associated with decrease in cell division, which corresponds with the growth retardation that was morphologically observed (Figs. 1–3).

Notably, experiments in rat and zebrafish embryos, as well as in cell lines of human, primates and murine origin, described similar growth retardation and/or anti-proliferating effects of CBZ (Ahmed and El-Gareib, 2017; Jos et al., 2003; Pérez Martín et al., 2008; Shaikh Qureshi et al., 2014; Tittle and Schaumann, 1992; van den Brandhof and Montforts, 2010; Weigt et al., 2011). These CBZ effects were shown to be mediated by alterations in cell-cycle genes (Hermsen et al., 2013; Song et al., 2011), inhibition of hormonal secretion (Ahmed et al., 2008) or impairing of centrosome separation (Pérez Martín et al., 2008). However, these studies tested clinical/sub-clinical doses of CBZ. Interestingly, exposure of zebrafish to environmental-relevant doses of CBZ revealed conflicting results; it was either reported to increase mortality (Galus et al., 2013a, 2013b); to accelerate embryonic growth (Qiang et al., 2016); or not to affect development (Martinez et al., 2018). Hence, research is needed to elucidate whether the proliferation effects in chick embryo result from its higher sensitivity to CBZ compared to teleost, and whether similar effect can be found in mammalian embryos. Moreover, the mechanism through which CBZ decreases proliferation in chick embryos awaits further investigation.

During neurogenesis, neural progenitors undergo differentiation, migrate and begin to extend axons (Ayala et al., 2007; Nornes and Das, 1972). As CBZ altered NT organization (Fig. 3), we tested whether it also affects neural differentiation. Embryos were stained for Sox2 and for the neurofilament associated antigen 3A10, to label neural progenitor cells and axons of differentiated neurons, respectively (Lumsden and Keynes, 1989; Storey et al., 1992). Control embryos displayed typical Sox2 expression in the ventricular part of the NT, along its dorsal-ventral axis (Fig. 3O). Sox2 expression was also observed in CBZ-added embryos (Fig. 3P), but in somewhat lower levels. Reduced expression was more prominent in the ventral and dorsal-most subdomains of the NT. Concomitantly, typical distribution of 3A10-labeled axons was found throughout the mantle layer of the NT in controls, as expected from mature neurons (Fig. 3Q). Axonal fibers were also observed in CBZ-treated embryos but to a lesser extent (Fig. 3R). As expression of progenitor and differentiation cell markers was decreased but not lost upon exposure to CBZ, this data suggest that neurogenesis was not blocked, in spite of the perturbed NT morphology.

Another cell lineage that derives from the NT is the neural crest. Neural crest cells (NCCs) originate at the dorsal NT folds and migrate throughout the embryo to give rise to peripheral neurons, Schwann cells and cranial skeleton (Dupin and Sommer, 2012; Le Douarin, 1999). Due to the distorted NT morphology, we also assessed the development of NCCs using the specific marker HNK1 (Giovannone et al., 2015; Luider et al., 1992). Control embryos displayed typical migration patterns of NCCs toward the heart, branchial arches and paraxial mesoderm (Fig. S2I). stereotypic NCC streams were also evident upon CBZ treatment, although embryos were much smaller (Fig. S2J). This implicates that in spite of the growth retardation induced by CBZ, the overall development of NCC is maintained, further arguing against the option that CBZ affects non-specifically all embryonic cells.

Altogether, the results observed in st. 8HH embryos further establish that low levels of CBZ affect NT morphology, closure, cell proliferation and differentiation. While these diverse phenotypes are probably developmentally linked, the cascade of events leading to these outcomes is not known. Noticeably, growing of fish embryos and juveniles in environmental levels of CBZ led to aberrant expression of neural genes (Qiang et al., 2016; Thomas, 2012), and to altered behavior of the animals, probably due to abnormal neural development. Negative effects on neural differentiation were also shown in embryonic stem cells that were treated with clinical CBZ concentrations, raising the possibility that these CBZ effects may result in neuronal defects similar to those reported in human fetuses (Murabe et al., 2007; Schulpen et al., 2015).

3.3.3. St.15HH (organogenesis)

HREM analysis revealed similar 3D morphology in control and CBZ-treated embryos. Both groups displayed typical body shape and length with normal somites, brain vesicles, limb buds, etc. (Fig. 4A,B, Movies S10, S11). Transverse sections showed symmetrical and closed NT, as well as typical notochord, dermomyotome and ectodermal layer (Fig. 4C,D, Movies S12, S13). To fully confirm the lack of CBZ impact, embryos were examined for cell proliferation, apoptosis and neural differentiation. Both groups displayed similar distribution of dividing cells, very low rate of apoptotic cells and typical axonal patterns (Fig. 4E–K). These data are consistent with the dose-dependent and morphological scoring assays (Figs. 1, 2), indicating that embryos of this stage are not susceptible to environmental CBZ levels. Hence, sensitivity of embryos to environmental levels of CBZ is stage-specific, as embryos beyond gastrulation are unaffected by its application.

Comparative studies in teleost such as zebrafish, Japanese Medaka and Fathead Minnow, revealed that exposure to environmental contaminants (i.e, drugs and metals) affected differently the different species. These observations were suggested to occur due to species-specific differences in physiological and anatomical parameters such as in the chorion membrane which serves as a molecular barrier that can slow down or prevent chemicals from reaching the embryo (Ali et al., 2011; Jeffries et al., 2015; Laban et al., 2010) As in our experiments the amniotic sac in not yet developed at st.1/8HH but is present at st.15HH, further experiments are required to test whether the resistance to CBZ at the later stage is associated with the existence of the amniotic sac. In addition, as birds are routinely exposed to environmental contaminations (Hill and Hoffman, 1984), it would be interesting to compare the sensitivity to CBZ of chick to that of other avian species such as quail, wild duck and zebra finch.

3.4. Environmental and embryo-toxic implications

This study emphasizes the multiple advantages of using the chick embryonic model to study embryo-toxicity in-vivo: (1) the embryo

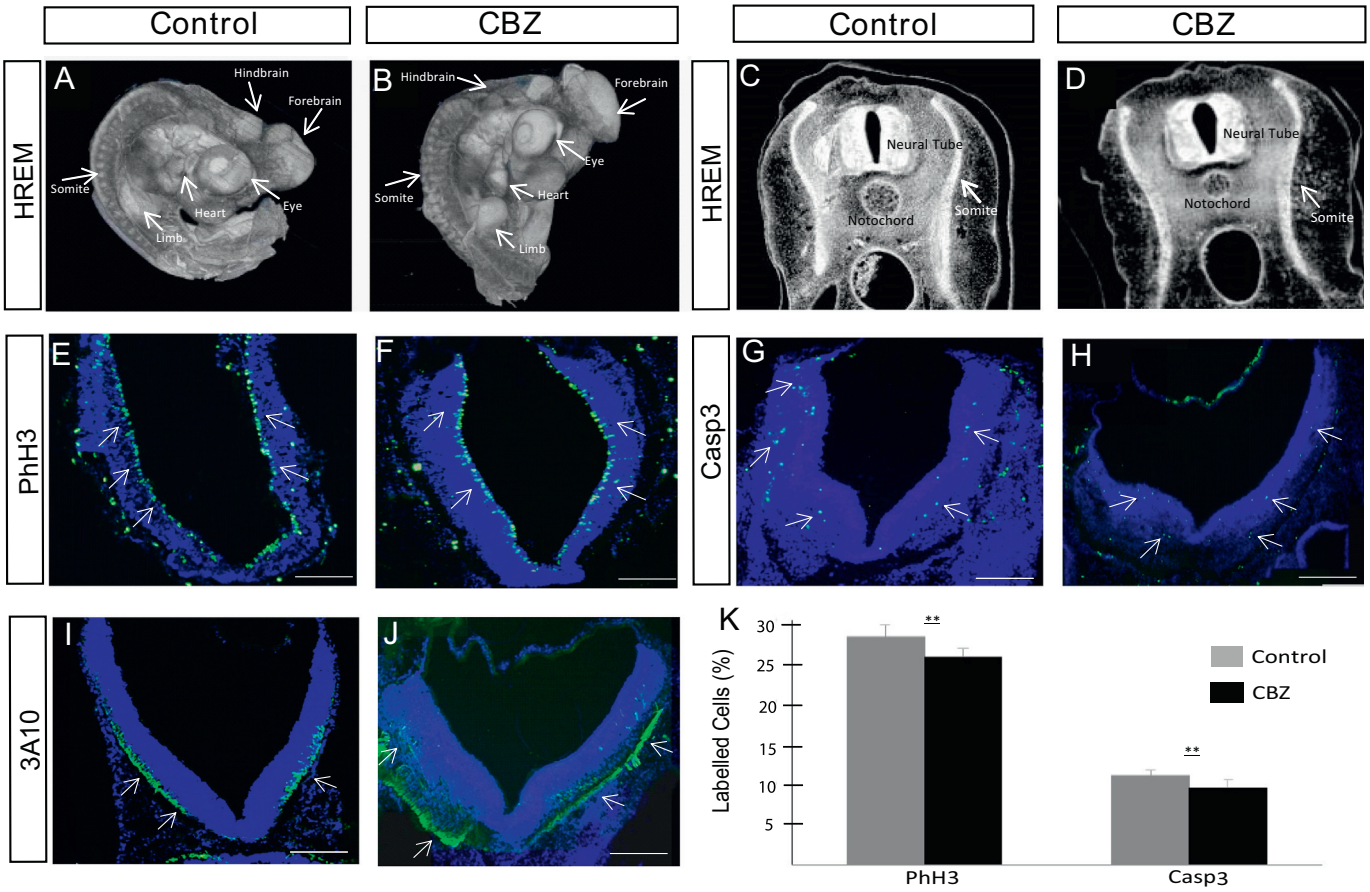

Fig. 4. HREM and marker expression analyses of embryos of stage 15HH.
(A–D): HREM analysis of control or CBZ-added embryos (n = 3 for each group). 3D views are shown in whole mounts (A,B) or in transverse sections taken from the spinal cord level (C,D). (E–J): Images of control or CBZ-added embryos immunostained for multiple markers (green, n = 6 for each marker). Cell nuclei are marked by DAPI. Arrowheads indicate marker expression. (K): Graphical representation of cells expressing Casp3 and PhH3 markers in control or CBZ treated embryos. $^{**}p > 0.05$. In all panels, CBZ concentration is 0.2 pg mg^{-1}, anatomical landmarks are indicated and control embryos were added with DDW. Scale bar-50um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

develops rapidly, is easily accessible and can be readily treated by dropping chemical solutions on to it, (2) each egg is a close system not affected by external conditions, enabling to test toxicants in desired stages using large scale experimental platforms, (3) as amniotes, avian and mammalian embryos (including human) share similar developmental mechanisms. However, since the usage of mammalians for embryo-toxic studies is limited, the chick model system is a great alternative, (4) multiple molecular markers and genomic resources are available for chick studies. Hence, we suggest that the chick embryo is an exciting model system that should be further used to examine environmental contaminants.

CBZ is a recalcitrant environmental chemical; it is persistent in biologically active ecosystems such as agricultural soils and in wastewater treatment facilities. As such, CBZ is detected in water resources, the food-chain and even in human biological matrices (Paltiel et al., 2016). Yet, risks associated with chronical exposure to CBZ are currently unknown. Moreover, it is also unclear whether embryos exposed to environmental-relevant concentration of CBZ can be affected. Combining morphological, cellular and molecular analyses with advanced imaging technology this study demonstrates that environmental relevant levels of CBZ impair morphogenesis is dose- and stage-dependent manner. Effects were exhibited in early embryonic stages by exposing embryos to ~100 ng L^{-1} CBZ. This study provides the first evidence for teratogenic effect of environmental-relevant concentrations of CBZ in amniotes that impairs their early stages of development. Hence, our data calls for in-depth risk analysis to ensure that the environmental presence of CBZ is not causing irreversible ecological and functional damages.

Supporting information includes: Numerical scoring of defined morphological features according to succeeding embryonic stages (Table S1); Views of selected embryonic stages and experimental procedure used in this study (Fig. S1); Data from st. 8 HH embryos during in-vivo time lapse analysis, flow cytometry assay and immunofluorescence staining (Fig. S2); HREM movies of st.1 HH embryos (Movies S1–S4), st. 8HH embryos (Movies S5–S8) and st. 15 HH embryos (Movies S10–S13); Time lapse movie of st. 8HH embryos (Movie S9).

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