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Microcystin-LR acute exposure increases AChE activity via transcriptional *ache* activation in zebrafish (*Danio rerio*) brain

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ABSTRACT

Microcystins (MCs) constitute a family of cyanobacterial toxins, with more than 80 variants. These toxins are able to induce hepatotoxicity in several organisms mainly through the inhibition of protein phosphatases PP1 and PP2A and oxidative stress generation. Since recent evidence shows that MCs can either accumulate in brain or alter behavior patterns of fish species, in this study we tested the *in vitro* and *in vivo* effects of MC-LR at different concentrations on acetylcholinesterase (AChE) activity in zebrafish brain. *In vivo* studies showed that 100 µg/L MC-LR led to a significant increase in the AChE activity (27%) when zebrafish were exposed to the toxin dissolved in water, but did not cause any significant changes when injected intraperitoneally. In addition, semiquantitative RT-PCR analysis demonstrated that 100 µg/L MC-LR exposure also increased *ache* mRNA levels in zebrafish brain. The *in vitro* assays did not reveal any significant changes in AChE activity. These findings provide the first evidence that brain AChE is another potential target for MCs and suggest that the observed increases in AChE enzymatic activity and in *ache* transcript levels after MC-LR exposure depend, at least partially, on branchial uptake or ingestion.

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1. Introduction

Microcystins (MCs) are potent hepatotoxins produced by the cyanobacteria of the genera *Planktothrix*, *Microcystis*, *Aphanizomenon*, *Nostoc* and *Anabaena* (Dai et al., 2008). They are among the most frequently detected toxins in fresh waters, and produce potent hepatotoxic effects on terrestrial animals (for example, Puschner et al., 1998) and fish (Malbrouck and Kestemont, 2006). There are over 80 different variants of this molecule, including amino acid variations and modifications, among which microcystin-LR (MC-LR) is one of the most common and toxic forms, and which occurs more often in cyanobacterial blooms (Dawson, 1998). MCs

inhibit phosphatases PP1 and PP2A and they appear to interact with the mitochondria of animal cells triggering oxidative stress and apoptosis (for review see Campos and Vasconcelos, 2010). This pathway is considered to be one of the main mechanisms of MCs toxicity. Nevertheless, MCs toxicity is a multi-pathway process and, regardless of recent achievements, the molecular mechanisms underlying MCs toxicity are still not completely understood (Campos and Vasconcelos, 2010).

Studies have demonstrated that MCs accumulate in several fish tissues, such as liver (Williams et al., 1995; Sipia et al., 2001; Malbrouck et al., 2003; Mohamed et al., 2003), intestine (Williams et al., 1995; Mohamed et al., 2003; Xie et al., 2004, 2005), gills (Carbis et al., 1997; Cazenave et al., 2005), kidney (Williams et al., 1995; Mohamed et al., 2003), muscle (Bury et al., 1998; Magalhaes et al., 2001, 2003; Xie et al., 2004), gallbladder (Sahin et al., 1996), and blood (Tencalla and Dietrich, 1997; Xie et al., 2004, 2005). Studies have shown that MCs can also accumulate in fish brain. Fischer and Dietrich (2000) detected MC-LR in *Cyprinus carpio* brain after gavage with the toxin, and Cazenave et al. (2005) reported the presence of microcystin-RR

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(MC-RR) in the brain of *Jenynsia multidentata* exposed to the toxin dissolved in water for 24 h. In addition, changes in the spontaneous locomotor behavior of zebrafish (*Danio rerio*) and *Leucaspilus delinatus* after MC-LR exposure (Baganz et al., 2004), in the swimming activity and in the glutathione S-transferase activity of *J. multidentata* fed with MC-RR (Cazenave et al., 2008), and the induction of lipid peroxidation in the brain of *Corydoras paleatus* after MC-RR exposure (Cazenave et al., 2006) indicate probable neurotoxic effects of MCs. More recently, Wang et al. (2010) using proteomic analysis revealed that MC-LR neurotoxicity induced oxidative stress and a dysfunction of cytoskeleton assembly and macromolecule metabolism. These findings suggest that MC-LR toxicity to the brain is complex and diverse.

The zebrafish has long been considered a powerful animal model because of its tractable genetics and embryology, but it has more recently become a model of choice in environmental studies, pharmaceutical screening, and physiologic analysis (Hernández and Allende, 2008). Its greatest assets are its small body size, sensitivity to drugs, and the ability to rapidly absorb chemicals from the water and then to accumulate them in several tissues (Goldsmith, 2004; Hill et al., 2005).

The cholinergic system is one of the most important modulatory neurotransmitter systems and has long been recognized to play key roles in many functions in the central nervous system (CNS), including the control of locomotor activity, emotional behavior, and cognitive processes (Pepeu and Giovannini, 2004; Martins-Silva et al., 2011). In cholinergic neurotransmission, choline acetyltransferase (ChAT) is responsible for the synthesis of acetylcholine (ACh) in the presynaptic neuron. After its release into the synaptic cleft, cholinesterases rapidly cleave ACh into choline and acetate. Two different types of cholinesterases are able to carry out this hydrolysis: acetylcholinesterase (AChE) (E.C.3.1.1.7) and butyrylcholinesterase (BuChE) (E.C.3.1.1.8) (Soreq and Seidman, 2001). It has been demonstrated that BuChE is not encoded in the zebrafish genome, but AChE is encoded by a single gene that has already been cloned, sequenced and functionally detected in zebrafish brain (Bertrand et al., 2001). AChE enzymes in vertebrates exist as soluble monomers (G1), dimers (G2), and tetramers (G4). The tetramers may be associated with structural subunits, a collagenic tail in neuromuscular junctions, or a membrane protein in brain (Massoulié et al., 1998, 2008). Moreover, zebrafish AChE shows a transition, at the time of hatching, from the globular G4 form to an asymmetric form containing a collagenic subunit that becomes prominent in adults (Bertrand et al., 2001).

AChE activity has been widely used as a bioindicator of environmental exposure. For example, the inhibition of AChE as a biomarker for assessment of the exposure of organisms to organophosphate and carbamate insecticides is well known (Weiss, 1958; O'Brien, 1976; for review see Van Dyk and Pletschke, 2011), including in fish species (Bretaud et al., 2000; Roex et al., 2003). The inhibition of zebrafish brain AChE activity by toxic substances such as methanol (Rico et al., 2006) and the heavy metals mercury and lead (Richetti et al., 2011) also has been well established. On the other hand, AChE activation has also been demonstrated as a consequence of exposure to neurotoxic compounds such as aluminum (Kaizer et al., 2010; Senger et al., 2011) and ethanol (Rico et al., 2007).

Considering that: (i) MCs synthesized by cyanobacteria can either accumulate in brain and change locomotor behavior and swimming activity in fish; (ii) ACh is known to play a major role in the regulation of locomotor control; (iii) measurement of AChE activity in organisms is used worldwide as a biomarker of environmental contamination and that (iv) the zebrafish is a model organism to study drug mechanisms and toxicology, the aim of this study was to evaluate the *in vivo* and *in vitro* effects of different concentrations of MC-LR on AChE activity in zebrafish brain. Furthermore, when alterations in kinetics occurred after MC-LR exposure, we also determined the *ache* gene expression level in zebrafish brain.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish (*Danio rerio*, Cyprinidae) of both sexes (3–6 months old) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept at a density of up to five animals per liter in 50 L housing tanks with tap water that was previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O₂/L) at 26 ± 2 °C, pH 7.0, under a 14/10 h light/dark controlled photoperiod. Animals were acclimated for at least two weeks before the experiments and were fed three times a day with TetraMin Tropical Flake fish food®. The fish were maintained healthy and free of any signs of disease and were used according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health. All procedures in the present study were approved by the Animal Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (PUCRS), protocol number 10/00142-CEUA.

2.2. Chemicals

MC-LR (purity >95%) isolated from *Microcystis aeruginosa* (strain RST9501) were produced by Unidade de Pesquisas em Cianobactérias (Universidade Federal de Rio Grande — FURG, BR). The amount of MC-LR (strain RST9501) was detected by a Quantitative Antibody Immunoassay (Elisa) provided by Enviroligix (Portland, USA), within a range of detection from 0.05 to 2.5 µg/L MCs. A suitable dilution was applied to the sample to provide detection within the range. Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis(beta amino ethylether)-N,N,N',N'-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA). TRIzol® reagent, SuperScript™ III First-Strand Synthesis SuperMix, Platinum® Taq DNA Polymerase and GelRed® were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents used were of analytical grade.

2.3. *In vivo* treatment

The *in vivo* exposures were performed in 1-L aquariums (10 fish per aquarium). Fish were exposed to different MC-LR concentrations (50 and 100 µg/L) dissolved in water for 24 h and, immediately after the exposure, they were euthanized by decapitation. For the control group the animals were exposed only to water for 24 h, after which they were euthanized by decapitation. The MC-LR concentrations and the *in vivo* time of exposure were chosen based on a previous study using *J. multidentata* (Cazenave et al., 2005). In addition, such concentrations are commonly encountered in cyanobacterial bloom events (Oberholster et al., 2009; Backer et al., 2010).

2.4. *In vitro* treatment

In vitro assays were performed as previously described (Seibt et al., 2009; Siebel et al., 2010). Briefly, MC-LR was added to the reaction medium before the preincubation with the enzyme-containing lysate from zebrafish brain homogenate and maintained during the enzyme assays. MC-LR was tested at a final concentration of 10, 25, 50, 100, 500, 1000, and 5000 µg/L. For the control group, the enzyme assay was performed in the absence of MC-LR (i.e. no toxin was added to the reaction medium).

2.5. Intraperitoneal injection

Intraperitoneal injections were conducted using a 3/10-mL U-100 BD Ultra-Fine™ Short Insulin Syringe 8 mm (5/16") × 31 G Short Needle (Becton Dickinson and Company, New Jersey, USA) according to the protocol established by Phelps et al. (2009). Briefly, each fish was weighed prior to the intraperitoneal injection and the volume injected (10 µL) into the animal was adjusted to achieve a dose of 50 and 100 µg/kg of MC-LR, based on previously studies (Carbis et al., 1996; Fournie and Courtney, 2002). Anesthesia of the animals prior to the injection was obtained by its immersion in a solution of benzocaine (1 mM in MeOH 1%) until the animal showed a lack of motor coordination and reduced respiratory rate. The anesthetized animal was gently placed in a water-soaked gauze-wrapped hemostat with the abdomen facing up and the head of the fish positioned at the hinge of the hemostat (the pectoral fins were used as a landmark on the abdomen). The needle was inserted parallel to the spine in the midline of the abdomen posterior to the pectoral fins. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 10 s out of the water. After the injection the animals were placed in a separate tank with highly aerated unchlorinated tap water (26 ± 2 °C) to facilitate recovery from the anesthesia. Saline solution was used as control. All the animals that recovered within 2–3 min following the injection continued in the experiment, while animals that did not recover during this period were discarded. Twenty-four hours after the injection the animals were euthanized by decapitation and AChE activity was determined.

2.6. Determination of AChE activity (EC 3.1.1.7)

Zebrafish were euthanized and their whole brains were removed by dissection. The brains (two whole brains for each sample) were homogenized on ice in 60 vol. (v/w) of Tris-citrate buffer (50 mM Tris, 2 mM EDTA, and 2 mM EGTA, pH 7.4, adjusted with citric acid), in a glass-Teflon homogenizer. The rate of acetylthiocholine hydrolysis (ACSch, 0.88 mM) was assessed in a final volume of 300 µL with 11 mM phosphate buffer, pH 7.5, and 0.22 mM DTNB using a method previously described (Ellman et al., 1961). Before the addition of substrate, samples containing protein (5 µg) and the reaction medium described above were preincubated for 10 min at 25 °C. The hydrolysis of substrate was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) in a microplate reader. Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. The linearity of absorbance against time and protein concentration was previously determined. The AChE activity was expressed as micromoles of thiocholine (SCh) released per h per mg of protein. All enzyme assays were evaluated in triplicate and at least four independent experiments were performed.

2.7. Protein determination

Protein was measured by the Coomassie blue method (Bradford, 1976) using bovine serum albumin as standard.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

Forward (5'-CCAAAAGAATAGAGATGCCATGGACG-3') and reverse (5'-TGTGATGTTAAGCAGACGAGGCAGG-3') *ache* primers and the optimal conditions for RT-PCR were used according to Rico et al. (2006). The β -actin forward (5'-GTCCCTGTACGCCTCTGGTTCG-3') and reverse (5'-GCCGGACTCATCGTACTCCTG-3') primers were used as described previously (Chen et al., 2004).

Immediately after *in vivo* exposure to MC-LR (described above), the animals were euthanized by decapitation. For each sample, a pool of two zebrafish whole brains was used. Total RNA from zebrafish brain

was isolated using the TRIzol® reagent (Invitrogen) in accordance with the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm and its integrity was confirmed by electrophoresis through a 1.0% agarose gel. Afterwards, all samples were adjusted to 160 ng/µL and cDNA species were synthesized using SuperScript III™ First-Strand Synthesis SuperMix Kit (Invitrogen, USA), following the supplier's instructions. One microliter of RT reaction mix was used as a template for each PCR. For *ache*, the reaction was performed in a total volume of 25 µL using 0.08 µM of each primer, 0.2 µM dNTP, 2 mM MgCl₂ and 1 U Taq DNA polymerase (Invitrogen). PCR for the β -actin gene was performed in a total volume of 20 µL using 0.1 µM of each primer, 0.2 µM dNTP, 2 mM MgCl₂ and 0.5 U Taq DNA polymerase (Invitrogen). PCR parameters, including MgCl₂, cDNA template concentration and PCR cycles (25–45 cycles), were first optimized (data not shown) and reactions were performed using optimal conditions that allowed product detection within the linear phase of band densitometry analyzed. PCRs were conducted for 1 min at 94 °C, 1 min at 60 °C (*ache*) or at 54 °C (β -actin), and 1 min at 72 °C for 35 cycles. A post-extension step was performed for 10 min at 72 °C. For each PCR set, a negative control was included. PCR products were analyzed on a 1% agarose gel, containing GelRed® and visualized with ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker and samples were normalized by employing β -actin as a constitutive gene. The band intensities were measured by optical densitometry analysis and the enzyme/ β -actin mRNA ratios were established for each treatment using the freeware Image J 1.37. Each experiment was repeated at least four times, using RNA isolated from independent extractions.

2.9. Statistical analysis

AChE activity were expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA). Post-hoc comparisons were made using Tukey's test considering $p \leq 0.05$ as statistical significance. Molecular data were expressed as means ± S.E.M. and analyzed by Student's *t*-test considering $p \leq 0.05$ as statistical significance.

3. Results

The effect of different MC-LR concentrations on brain AChE activity was demonstrated by performing *in vivo* (50 and 100 µg/L) and *in vitro* (10, 25, 50, 100, 500, 1000 and 5000 µg/L) experiments using adult zebrafish. The *in vivo* analysis demonstrated that animals treated with the concentration of 100 µg/L for 24 h presented a significant increase in AChE activity (31.14 ± 1.39; 27%; $p < 0.05$) when compared to the control group (24.50 ± 1.80) (Fig. 1). On the other hand, MC-LR intraperitoneal injection did not alter the brain AChE activity (data not shown).

The up-regulation of brain AChE activity after exposure to 100 µg/L MC-LR could be a consequence of transcriptional control and/or post-translational modifications. In order to determine if transcriptional regulation of *ache* has occurred, semiquantitative RT-PCR analysis was performed. The results demonstrated that the relative levels of *ache* mRNA were significantly increased (1.07 ± 0.01; 17%; $p < 0.05$) after exposure to 100 µg/L MC-LR (Fig. 2).

To verify whether the toxin might have a direct effect on the enzyme, we tested the *in vitro* effect of MC-LR on AChE activity in zebrafish brain. The results showed that MC-LR did not bring about any alteration in AChE activity ($p > 0.05$; Fig. 3).

4. Discussion

The results presented herein demonstrated the influence of MC-LR on AChE activity and *ache* gene expression in zebrafish brain. We

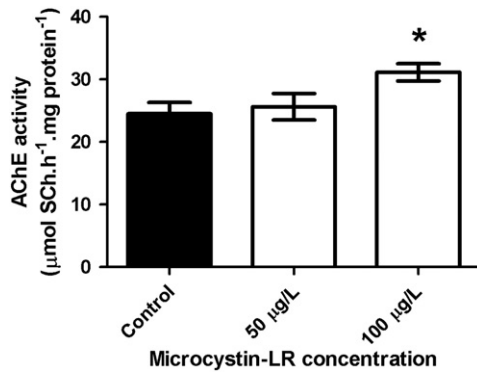


Fig. 1. *In vivo* AChE activity in zebrafish brain after 24 h of microcystin-LR exposure at distinct concentrations (50 and 100 µg/L). Data were expressed as means ± S.E.M. of four independent experiments, each one performed in triplicate. The specific enzyme activity is reported as micromoles of thiocholine released per hour per milligram of protein. The asterisk (*) indicates a significant difference when compared to the control group (one way ANOVA, followed by Tukey's test as post-hoc, $p < 0.05$).

found that AChE activity was significantly enhanced when zebrafish were exposed to 100 µg/L of MC-LR dissolved in water but not when intraperitoneal injected. The *ache* mRNA levels were significantly increased after MC-LR exposure, suggesting that, MC-LR could also modulate *ache* gene expression. The results also showed that none of the MC-LR concentrations tested caused significant changes in the AChE activity in zebrafish brain when added directly to the enzyme assays, as in the *in vitro* experiments, suggesting that MC-LR did not act directly on the enzyme. These findings provide further evidence for toxic effects on the brain caused by exposure to MCs.

The first evidence that MC-LR can accumulate in fish brain was reported by Fischer and Dietrich (2000), who detected the toxin by Western Blot 48 h after bolus dosing of freeze dried algae equivalent to 400 µg/kg of *C. carpio*. Later, Cazenave et al. (2005) showed the presence of MC-RR in the brain of *J. multidentata* exposed to the toxin. Fish were exposed for 24 h to 50 µg/L of MC-RR dissolved in water and were then analyzed by high performance liquid chromatography (HPLC). Recently, Papadimitriou et al. (2010) used ELISA to show toxin accumulation in brain tissue of *Carassius gibelio* collected from Greek Lakes.

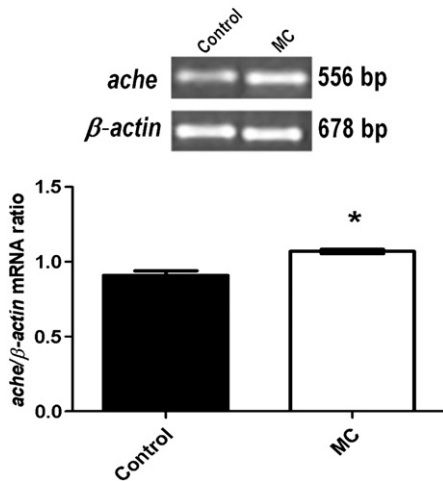


Fig. 2. Effect of exposure to 100 µg/L of microcystin-LR for 24 h on *ache* mRNA transcripts from zebrafish brain. The PCR products were subjected to electrophoresis on a 1% agarose gel, using β -actin as a constitutive gene. The figure shows a representative gel and the *ache*/ β -actin mRNA ratio (expressed as arbitrary units) obtained by optical densitometry analysis of three independent experiments, with entirely consistent results. Data were expressed as means ± S.E.M. The asterisk (*) indicates a significant difference when compared to the control group (Student's *t* test, $p < 0.05$).

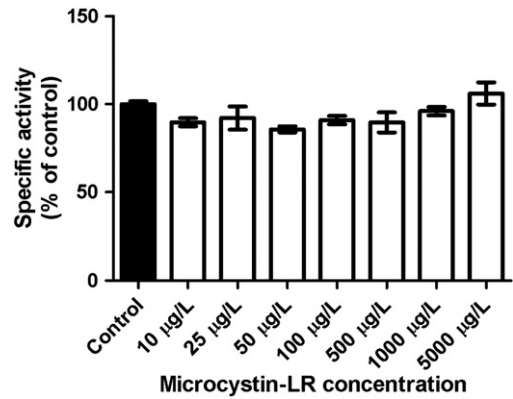


Fig. 3. *In vitro* effect of different concentrations of microcystin-LR (10–5,000 µg/L) on ACh hydrolysis in zebrafish brain. Data were expressed as means ± S.E.M. of four independent experiments, each one performed in triplicate. The control acetylcholinesterase activity was 26.19 ± 1.69 µmol SCh h⁻¹ mg protein⁻¹.

There is a large body of evidence in the literature associating changes in normal behavioral patterns with neurotoxic effects of exposure to pollutants. In this sense, although the effects of MCs on the behavior of fish are still mostly unknown some aspects have already been addressed. For example, changes in the spontaneous locomotor behavior of *D. rerio* and *L. delinatus* were observed after exposure to MC-LR at concentrations of 0.5, 5, and 15 µg/L for 17 days and 50 µg/L for six days. During the daytime, the motility of *D. rerio* as well as *L. delinatus* was significantly increased by exposure to the lowest concentrations, whereas higher concentrations led to significantly decreased motility. Furthermore, under the influence of MC-LR, the period of swimming activity in *L. delinatus* was reversed, going from a predominantly diurnal activity to a nocturnal one; *D. rerio* remained active during the daytime (Baganz et al. 2004). Additionally, Cazenave et al. (2008) reported changes in the swimming activity of *J. multidentata* fed with contaminated food pellets containing MC-RR. Low levels (0.01 µg/g) increased the swimming activity, while the highest dose (1 µg/g) produced significant changes with respect to the control group only after approximately 20 h of exposure, when the swimming activity was decreased.

As seen by other authors, the hepatotoxicity of MC depends on the route of uptake (for review see Malbrouck and Kestemont, 2006). Thus, different exposure routes have been used to examine the toxic effects of MCs on fishes including intraperitoneal injection, oral ingestion and immersion in water containing the dissolved toxin (Carbis et al., 1996; Landsberg, 2002). In general, injected MC has stronger effects than fed toxin whereas exposure via immersion causes the mildest effects in liver (Malbrouck and Kestemont, 2006). Therefore, an alternative route of exposure was subsequently tested to determine whether it also lead to a change in brain AChE activity as occurred when zebrafish were exposed to the toxin dissolved in water. Interestingly, when MC-LR (50 or 100 µg/kg) was injected intraperitoneally there was no change in AChE activity suggesting that the observed increases in brain AChE depend, at least partially, on branchial uptake or ingestion. It is important to highlight that doses used in immersion and intraperitoneal MC-LR exposures are not equivalents. These findings appear to be particularly relevant to better understanding of the molecular mechanisms of MC toxicity in brain since the toxins are synthesized during the growth phase of the cyanobacteria, and large quantities of MC are released directly into the water during the collapse of a bloom (Berg et al., 1987) or from actively growing cyanobacterial populations (Sivonen et al., 1990).

AChE has been well studied for its classical functions in terminating neurotransmission at cholinergic synapses and neuromuscular junctions (Taylor and Radic, 1994). In this context, AChE is a primary target of organophosphorus and carbamate insecticides, which are structural

analogues of acetylcholine. Upon binding of these insecticides to the enzyme, AChE is inactivated due to phosphorylation or carbamylation of the serine residue in the active site. As a result, the post-synaptic membrane remains depolarized and synaptic transmission fails (Matsumura, 1985).

However, more recently AChE has been redefined as an important regulator of apoptosis, because it can be induced by a variety of apoptotic stimuli (Zhang et al., 2002; for review see Jiang and Zhang, 2008). Thus, it has been demonstrated, for example, that over-expression of AChE is able to inhibit cell proliferation and promote apoptosis (Jin et al., 2004).

It is well known that apoptosis underlies the neurotoxic effects of various compounds. For instance, the apoptotic basis of the neurotoxic activity of β -amyloid and prion protein fragments was confirmed by ultrastructural examination of rat brain hippocampal neurons (Forloni et al., 1993). Moreover, brain AChE activation has also been demonstrated as a consequence of exposure to known neurotoxic compounds. For example, aluminum caused up-regulation of AChE activity in different mouse brain regions (Kaizer et al., 2005), in rat lymphocytes (Kaizer et al., 2010) and in zebrafish brain (Senger et al., 2011). In addition, a significant increase in zebrafish brain AChE activity was established after acute ethanol exposures (Rico et al., 2007).

MCs are strong inhibitors of the PP1 and PP2A and this is probably the main mechanism of action of these toxins in the liver (Campos and Vasconcelos, 2010). Another reasonable possibility is that MC-LR may influence brain AChE indirectly via inhibition of a serine/threonine phosphatase. However, to our knowledge, until now, the phosphatases PP1 and PP2A have not been described in zebrafish's brain. Calcineurin, a Ca^{2+} /calmodulin-activated phosphatase seems to be a good candidate once it has specifically been implicated in AChE regulation in mammalian cells (Pregelj et al., 2007) and it is present in the zebrafish brain (Yoshida et al., 2009).

In summary, the results presented in this article provide experimental evidence that brain AChE is another potential target of MCs, and that the observed increases in AChE enzymatic activity together with *ache* transcript levels after MC-LR exposure depend, at least partially, on branchial uptake or ingestion. It might also be speculated that the neurotoxicity resulting from MC-LR exposure is mediated by apoptosis. Accordingly, microcystin-induced apoptosis in a variety of mammalian non-brain cell types was already demonstrated (McDermott et al., 1998; Ding et al., 2000). Further studies must be performed in order to reinforce these findings.

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