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Distribution of carnosine-like peptides in the nervous system of developing and adult zebrafish (*Danio rerio*) and embryonic effects of chronic carnosine exposure

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Abstract

Carnosine-like peptides (carnosine-LP) are a family of histidine derivatives that are present in the nervous system of various species and that exhibit antioxidant, anti-matrix-metalloproteinase, anti-excitotoxic, and free-radical scavenging properties. They are also neuroprotective in animal models of cerebral ischemia. Although the function of carnosine-LP is largely unknown, the hypothesis has been advanced that they play a role in the developing nervous system. Since the zebrafish is an excellent vertebrate model for studying development and disease, we have examined the distribution pattern of carnosine-LP in the adult and developing zebrafish. In the adult, immunoreactivity for carnosine-LP is specifically concentrated in sensory neurons and non-sensory cells of the olfactory epithelium, the olfactory nerve, and the olfactory bulb. Robust staining has also been observed in the retinal outer nuclear layer and the corneal epithelium. Developmental studies have revealed immunostaining for carnosine-LP as early as 18 h, 24 h, and 7 days post-fertilization in, respectively, the olfactory, corneal, and retinal primordia. These data suggest that carnosine-LP are involved in olfactory and visual function. We have also investigated the effects of chronic (7 days) exposure to carnosine on embryonic development and show that 0.01 μ M to 10 mM concentrations of carnosine do not elicit significant deleterious effects.

Conversely, treatment with 100 mM carnosine results in developmental delay and compromised larval survival. These results indicate that, at lower concentrations, exogenously administered carnosine can be used to explore the role of carnosine in development and developmental disorders of the nervous system.

Keywords

Carnosine-like peptides; Nervous system; Development; Embryo toxicity; Zebrafish; *Danio rerio* (Teleostei)

Introduction

Amino-acyl histidine dipeptides are a family of structurally and functionally related peptides that include carnosine (β -alanyl-L-histidine), homocarnosine (γ -aminobutyryl-L-histidine), and anserine (β -alanyl-L-1-methyl-L-histidine; Gulewitsch and Amiradzibi 1900; Ackermann et al. 1929; Pisano et al. 1961). These naturally occurring peptides have since been identified in many classes of vertebrates, ranging from fish to humans (Clifford 1921; Crush 1970; Margolis and Grillo 1984; Bonfanti et al. 1999). They are found in a variety of tissues but are especially predominant in excitable tissues, such as skeletal muscle and the nervous system (Crush 1970; O'Dowd et al. 1990; Biffo et al. 1990; Bonfanti et al. 1999; De Marchis et al. 2000b; Lamas et al. 2007). In the central nervous system (CNS), biochemical and immunohistochemical studies of these carnosine-like peptides (carnosine-LP) have shown that their respective presence and cellular localization depends on the species considered and the tissue examined. For instance, anserine is present in the avian CNS but is mostly absent from the mammalian nervous system (Fisher et al. 1977; Biffo et al. 1990; Bonfanti et al. 1999). Furthermore, in the adult mammalian CNS, carnosine and homocarnosine are present in receptor neurons of the olfactory epithelium, but in the brain, they localize to glial and neural progenitor cells (Margolis 1974; Peretto et al. 1998; Bonfanti et al. 1999).

The evolutionary conservation of carnosine-LP suggests that they play an important role in the nervous system. However, the exact function that they play in the CNS remains unknown, although the hypothesis that they act as natural neuroprotectors against excitatory or stress-related cerebral insults has been advanced. This assumption is supported by numerous studies demonstrating that carnosine-related compounds display antioxidant and free radical scavenger properties, cytosolic buffering functions, an anti-glycating role, metal-ion chelating capabilities, and wound healing properties (Hipkiss et al. 1998; Abe 2000; Horning et al. 2000; Trombley et al. 2000). Carnosine and homocarnosine have been shown to protect neuronal cultures against glutamate-induced toxicity (Boldyrev et al. 1999, 2004; Horning et al. 2000; Trombley et al. 2000). Both dipeptides have also been reported to protect PC12 cells from oxygen glucose deprivation, because of their anti-oxidative properties (Tabakman et al. 2002). Interestingly, carnosine has been demonstrated to prevent β -amyloid aggregation in rat brain endothelial cells (Preston et al. 1998) and to rescue PC12 cells from Ab42-induced neurotoxicity through the regulation of glutamate release (Fu et al. 2008). A role as neuromodulator has also been proposed for carnosine in the olfactory epithelium/bulb and in the retina, where it is co-localized with glutamate (Margolis 1974; Sassoè-Pognetto et al. 1993; Panzanelli et al. 1997; Bonfanti et al. 1999).

A recent resurgence of interest in carnosine-LP has occurred because of their pharmacological properties, which make them attractive candidates for neuroprotective therapeutic strategies (Quinn et al. 1992; Hipkiss 2007). Thus, carnosine has been shown to display neuroprotective properties in animal models of global and cerebral ischemia (see

references in Rajanikant et al. 2007; Tang et al. 2007). Neurodegenerative diseases are disorders that can affect not only the adult CNS, but also the developing fetus (DuPlessis and Volpe 2002; Rees and Inder 2005). Consequently, the neuroprotective properties of carnosine-LP might also be beneficial during development. However, the role of carnosine-LP in the adult CNS may be different from its effects on the developing brain. Furthermore, the developing and neonatal CNS is remarkably more sensitive to exposure to a variety of agents than the adult brain. Exposure to drugs during development has been linked to neurological diseases, a feature that emphasizes the need to evaluate the role of carnosine-LP during brain ontogenesis.

The teleost fish *Danio rerio* (zebrafish) has emerged as a valuable system not only for exploring brain development and genetics, but also for modeling human diseases (Lieschke and Currie 2007). Unlike mammals, zebrafish, like many fish, exhibit external fertilization, which results in the production of hundreds of eggs/embryos. In addition, their transparency allows easy in vivo observation of organotypic development making them a useful model for toxicology studies (Hill et al. 2005). Although the presence of carnosine-LP has been extensively reported in the muscle of a variety of fish, including teleosts (Clifford 1921; Kutscher and Ackermann 1933; Bonfanti et al. 1999), little information is available about the CNS. A recent study has demonstrated the presence of the dipeptide carnosine in the neurons of the gray mullet (Lamas et al. 2007). However, to our knowledge, no information is available about the zebrafish. The peptide transporter known to deliver carnosine-LP into astrocytes (Xiang et al. 2006) has recently been cloned and analyzed in the zebrafish (Romano et al. 2006). Furthermore, the gene encoding serum carnosinase, one of the two degradation enzymes for carnosine-LP, has also been identified and sequenced in the zebrafish genome (Sanger Institute).

In this study, we establish the distribution pattern of carnosine-LP in the developing and adult zebrafish nervous system by using antisera directed against carnosine and anserine. Furthermore, since the zebrafish is a powerful system for assessing the toxicity of a variety of chemicals and drugs, we have also analyzed the effects that exposure to carnosine has on the survival and development of the zebrafish embryo/larva. Such data should provide a framework for future investigations on the therapeutic potentials of carnosine-LP for fetal brain disorders.

Materials and methods

Animals

Wild-type *Danio rerio* adult fish were maintained in our breeding colony and raised with a 14-h light/10-h dark cycle at a temperature of 28.0°C to 28.5°C. Embryos were produced by natural matings and developmentally staged in hours or days post-fertilization (hpf and dpf respectively) according to Kimmel et al. (1995). All experimental procedures were approved by the Institutional Animal Care & Use Committee of Michigan State University and conformed to NIH guidelines.

Tissue preparation

Embryos and larvae were anesthetized in 0.05% tricaine methane sulfonate (Sigma, St Louis, Mo.) and fixed by immersion *in toto* in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 4–6 h. Olfactory epithelia, brains, and eyes from adult fish were dissected out and fixed by immersion overnight at 4°C. Following fixation, samples were either cryoprotected in a gradient of sucrose (embryos/larvae; Barthel and Raymond 1990) or cryoprotected in 30% sucrose/PB, pH 7.4 (adult fish), before being embedded in O.C.T. mounting medium (Sakura Finetek USA, Torrance, Calif.). Coronal

serial sections (12 μ m thick) were obtained on a Bright cryostat (Hacker Instruments, Winnsboro, S.C.), collected on Super-frost/Plus slides (Fisher Scientific, Pittsburgh, Pa.), dried overnight at room temperature, and stored at -20°C until use.

Antibodies

The antibodies to carnosine-LP used in this study included a polyclonal rabbit serum raised against carnosine (1:400) and a rabbit polyclonal serum raised against anserine (1:500). These antibodies have been previously extensively characterized and used in brain and retina sections from various species (Margolis and Grillo 1984; Biffo et al. 1990; Panzanelli et al. 1997; Lamas et al. 2007). The anti-carnosine antibody was pre-absorbed with bovine serum albumin (BSA)-histidine and BSA-glycine to avoid non-specific reactions and also recognizes anserine and homocarnosine. The anti-anserine antibody was pre-absorbed with BSA-histidine, BSA-glycine, and BSA-carnosine and does not recognize carnosine. Western blots of zebrafish CNS protein extracts probed with the carnosine and anserine antisera, and an antiserum directed against BSA-coupled serotonin (Sigma-Aldrich, St Louis, Mo.) as control, indicated that no zebrafish proteins were specifically recognized by the anserine and carnosine antibodies.

Several other antibodies were also used for characterization: mouse Fret 11 (rod photoreceptor) and Fret 43 (double cone photoreceptor; 1:250; Zebrafish International Resource Center, Eugene, Ore.); mouse SV2 antibody (synaptic vesicles; 1:250; Developmental Studies Hybridoma Bank, Iowa City, Iowa); mouse anti-glial fibrillary acidic protein (GFAP; 1:500; Millipore, Billerica, Mass.); mouse anti-HuC/D (1:500; Invitrogen, Carlsbad, Calif.); mouse anti-zrf1 (glial marker; 1:250; Zebrafish International Resource Center); goat anti- β igH3 (1:500; Santa Cruz Biotechnology, Santa Cruz, Calif.); mouse anti-keratan sulfate proteoglycan (1:500; Millipore); mouse anti-acetylated tubulin (1:1000; Sigma).

Immunofluorescence

Immunostaining was performed according to previously published procedures (Senut et al. 2004). Briefly, primary antibodies were diluted in PB-saline (PBS) containing 1% donkey serum and 0.3% Triton X-100 [normal donkey serum Triton (NDST) 1%]. Cryostat sections were first rehydrated for 10 min in PBS, preincubated in NDST 3% for 30 min at room temperature, and then incubated in the primary antibodies overnight at 4°C . Sections were then rinsed and incubated with the corresponding secondary anti-mouse, anti-rabbit, or anti-goat antibodies conjugated to Alexa 488 (1:1000; Invitrogen) or cyanin 3 (1:250; Jackson Immunoresearch Laboratories, West Grove, Pa.). Sections were subsequently stained with 10 ng/ml 4, 6-diamidino-2-phenylindole (DAPI; Sigma) for nuclear staining and coverslipped in 100 mM TRIS pH 8.5, containing 25% glycerol, 10% polyvinyl alcohol and 2.5% 1,4 diazabicyclo-[2,2,2]-octane (Sigma). Omission of one of the reagents in the labeling protocol sequence did not result in any immunostaining.

Imaging

Fluorescence and immunofluorescence images were visualized, examined, and captured by using a Magnafire SP digital camera adapted onto an Olympus BX51 fluorescence microscope. Confocal microscopic images of fluorescent immunostaining were obtained on an Olympus Fluoview 1000 LSM confocal microscope. Images were collected sequentially by using appropriate filters. Collected micrographs and illustrations were composed and annotated with Adobe Photoshop 6.0 (San Jose, CA).

Exposure to exogenous carnosine and mannitol

Carnosine was obtained from Sigma-Aldrich. Stock solutions of 100 mM carnosine were prepared in egg water (Westerfield 2000), and other dose regimens were prepared by further diluting the stock in egg water.

Embryos at 4–6 hpf were collected and pooled from three to four independent matings, randomly assigned to wells of a 24-well plate, and maintained in 1 ml egg water. In a first paradigm, six concentrations of carnosine were tested: 0.01 μ M, 0.1 μ M, 1 μ M, 10 μ M, 100 μ M, and 1000 μ M. In a second paradigm aimed at determining the maximal tolerated concentration, four different concentrations of carnosine were tested: 0.1 mM, 1 mM, 10 mM, and 100 mM. In a third paradigm directed at identifying a possible osmotic effect of the highest concentration of carnosine, embryos were exposed to 100 mM carnosine (100 mosm/l), 100 mM mannitol (100 mosm/l), or 200 mM mannitol (200 mosm/l). The physiological osmolarity of zebrafish is about 250–300 mosm/l. Control groups consisted of embryos placed in egg water (1.8 mosm/l). Both treatment and control water were replaced daily. Experiments were performed in triplicate and repeated three times. Embryonic development was monitored regularly by using a Nikon Eclipse TE2000-S microscope. Parameters such as gastrulation, somite formation, hatching frequency, movement, and pigmentation were recorded daily. Survival rates were also determined daily for each experimental condition by counting dead embryos/larvae, which were subsequently removed from the wells. Experiments were conducted on chorionated embryos, since preliminary studies showed no significant differences in the response to carnosine between intact and dechorionated embryos.

Statistical analysis

The degree of statistical significance between groups was determined on the basis of one-way or two-Way ANOVA tests, followed by the post-hoc Fisher LSD test, and Kruskal-Wallis test by using SigmaStat 3.0 software (SYSTAT Software, Point Richmond, Calif., USA). Statistical significance was defined at P 0.05. All data are expressed as the mean $\pm$ SEM.

Results

Distribution of carnosine-like peptides in adult zebrafish nervous system

The tissue distribution of carnosine-like immunoreactivity (LI) and anserine-LI was first investigated in the adult zebrafish nervous system by using immunofluorescence staining. Both antibodies exhibited comparable labeling patterns, with the sole difference being that the anserine antiserum yielded a stronger intensity of immunostaining. Immunoreactivity was detected in both the peripheral nervous system and CNS but was by far the most abundant in the olfactory system. Since both antibodies gave similar results, data gathered with the anti-anserine antibody are mostly illustrated, unless specified otherwise.

Olfactory epithelium

The presence of carnosine-LP in the olfactory epithelium has been described in several species, including humans (Margolis 1974; Sakai et al. 1990; Artero et al. 1991). This prompted us to examine whether carnosine-LP were also present in the zebrafish olfactory epithelium.

In the adult zebrafish, the olfactory organ consists in a pair of cup-shaped rosettes that connect to the olfactory bulb via the olfactory nerve. The olfactory rosette is composed of several lamellae that are attached to a central raphe (Fig. 1a). Each lamella can further be subdivided into a sensory and a non-sensory epithelium. The sensory epithelium contains

sensory neurons that project their axons to olfactory bulb glomeruli and to basal cells (or stem cells) and support cells. The non-sensory epithelium is mostly composed of support cells (Hansen and Zeiske 1993; Byrd and Brunjes 1995).

High levels of carnosine-LI (Fig. 1b, c) and anserine-LI (Fig. 1d) were consistently found in the zebrafish olfactory epithelium. Intense immunostaining for carnosine-LI was observed throughout the olfactory epithelium, although the intensity of the labeling appeared to be stronger in the outer segments of the non-sensory epithelium and the middle portion of the sensory epithelium (Fig. 1b). Carnosine-LI labeling was also consistently observed in cell processes and fiber bundles clearly seen coursing through the raphe (Fig. 1b). As illustrated in Fig. 1d, a comparable pattern of immunoreactivity was observed for anserine-LI. Co-labeling with the cytoplasmic neuronal marker HuC/D indicated that a subset of carnosine-LI and anserine-LI cells were sensory neurons (Fig. 2a–c); however, not all HuC/D-positive cells were positive for carnosine-LP. In general, anserine-LI seemed to be associated with the cell membrane.

Brain

In the adult zebrafish brain, the majority of immunolabeling for carnosine-LI and anserine-LI was found in the olfactory bulb (Figs. 2, 3). In this structure, intense staining for carnosine-LI and anserine-LI was concentrated in both the olfactory nerve and the glomerular layers but was absent from the superficial internal layer, which includes the mitral cell/plexiform and the granule cell layers (Fuller et al. 2006; Figs. 2, 3). Co-staining with the vesicle synaptic marker SV2, which permitted the visualization of the glomerular layer (Sato et al. 2005), showed that most glomeruli were innervated with fibers positive for carnosine-LI or anserine-LI (Fig. 2d–f), suggesting that sensory olfactory neurons that contained carnosine-LP did not have any topographic preference. Fascicles of fibers positive for carnosine-LI or anserine-LI were also noticed in the medial olfactory tract and in the ventral medial telencephalic region (Fig. 3c). In addition to the olfactory bulb, chains of immunoreactive cells were consistently observed at the surface of the brain associated with the meninges (Fig. 3d). The occasional staining of blood vessels was also noticed (data not shown). No immunolabeling for anserine-LI or carnosine-LI could be detected in the rest of the brain or the spinal cord (data not shown).

Eye

Carnosine-LI and anserine-LI were examined in various tissues of the adult zebrafish eye, and both were found to be concentrated in the retina and the cornea.

The typical laminated cytoarchitecture of the adult zebrafish retina is illustrated in Fig. 4a. It consists of three nuclear and two plexiform layers (Malicki 1999). The outer nuclear layer contains the photoreceptors, the inner nuclear layer includes the bipolar, amacrine, interplexiform, and Müller glial cells, and the retinal ganglion cell layer is composed of retinal ganglion cells and displaced amacrine cells (Fig. 4a).

Immunofluorescent staining for carnosine-LI and anserine-LI was exclusively found in the outer nuclear layer of the retina where it exhibited a discrete punctate appearance (Fig. 4b, c). Co-staining with markers for double cone (zpr1, Fig. 4b) and rod (zpr3, Fig. 4c) photoreceptors revealed that the labeling for carnosine/anserine-LI outlined the base of the inner segments of double cones. No additional staining for carnosine-LP was detected in other retinal layers.

The zebrafish cornea is similar anatomically to that of other vertebrates, including humans. It shows a relatively complex stratified organization composed of an epithelium, a Bowman's layer, a stroma, a collagen-rich Descemet's membrane, and an endothelium

(Swamynathan et al. 2003; Soules and Link 2005; Zhao et al. 2006). Robust intensity of carnosine-LI and anserine-LI was found in the cornea where it concentrated in the epithelium but was mostly absent from all other strata (Fig. 4d, g). Within the corneal epithelium, staining varied from the apical to basal layers. Whereas little staining was found in the deeper layers, more robust and uniform cell-surrounding diffuse labeling was observed in the upper layers (Fig. 4d, g). Immunofluorescent stainings with the epithelial cell marker β igH3 and the stromal marker keratane sulfate proteoglycan confirmed that carnosine-like and anserine-like stainings were confined to the outer portions of the epithelial layer where they co-localized with β igH3 (Fig. 4d–f). In contrast, no staining was observed in the stroma, as demonstrated by the absence of association with keratan sulfate proteoglycan (Fig. 4g–i).

Distribution of carnosine-like peptides during development

In our attempt to elucidate the role that carnosine-related peptides play in the zebrafish nervous system, we next sought to elucidate the time of appearance of carnosine-LI and anserine-LI in the eye and the olfactory pathway of the developing zebrafish by following the evolution of the immunostaining from 18 hpf to 7 dpf. Since, as observed in the adult, carnosine and anserine antisera exhibited similar temporal distribution patterns of immunoreactivity, only data gathered with the anti-anserine antibody will be described.

Developing olfactory system—In the developing zebrafish brain, the olfactory placodes consist of a pair of structures rostrally located between the eyes and the forebrain. Around 16–18 hpf, olfactory placodes emerge from a thickening of the ectoderm that later invaginates to form the nares (Hansen and Zeiske 1993; Whitlock and Westerfield 2000).

At about 18 hpf, the first time point that we examined, anserine-LI was visible in the olfactory placodes and appeared as a cluster of loosely arranged cells (Fig. 5a). By 24 hpf, the number of positive cells and the intensity of the immunostaining was significantly augmented (Fig. 5b). As observed in the adult, immunostaining for anserine-LI mostly appeared to be confined to the outlines of some cells (Fig. 5b). However, diffuse cytoplasmic staining was also evident (Fig. 5b). In addition, rare labeled processes were observed at the base of the olfactory placode lateral to the telencephalon (arrow in Fig. 5b). Between 24 hpf and 48 hpf, olfactory placodes progressively differentiate into the olfactory epithelium, and olfactory neurons send their axons in the olfactory bulb where they organize into glomeruli (Hansen and Zeiske 1993; Byrd and Brunjes 1995; Dynes and Ngai 1998; Whitlock and Westerfield 2000). As illustrated in Fig. 5c, the number of anserine-LI cells had noticeably increased by 48 hpf, so that robustly stained fibers could be now seen entering and defasciculating in the olfactory bulb. Around 72 hpf, strong immunoreactivity for anserine was still evident throughout the olfactory epithelium, although it appeared to be more heterogeneous (Fig. 5d). Specifically, the intensity of the labeling seemed to intensify at the nasal border of the olfactory epithelium, suggesting a dendritic distribution (Fig. 5d). Anserine-LI was still intense at 7 dpf in the olfactory epithelium (Fig. 5e), and numerous positive fibers could be seen in the telencephalon (Fig. 5f).

To determine the identity of anserine-LI cells and fibers, co-labeling experiments were performed between anserineLI and HuC/D, a cytoplasmic marker of post-mitotic neurons, or acetylated tubulin, an axonal marker. As shown at the 48 hpf time point, some of the immunopositive profiles corresponded to neurons (Fig. 6a–c) and their processes (Fig. 6d–f). No association was found between anserine-LI and glial staining as assessed by using GFAP or the radial glial marker *zrf1* (data not shown).

Developing eye—As shown in Fig. 7, the distribution pattern of anserine-LI in the developing eye was similar to that observed in the adult and was also restricted to the outer nuclear layer of the retina and the cornea.

At 24 hpf, the retina corresponds to an actively dividing retinal epithelium. Between 24 hpf and 48hpf, the retinal lamination is progressively set into place accompanied by cell differentiation, and by 72hpf, retinal differentiation and synaptogenesis are basically complete (Malicki 1999).

The earliest developmental age at which anserine-LI could be detected in the retina was at 7 dpf (Fig. 7a). Discrete bright puncta were observed in the outer layer at the inner segments of the cone photoreceptors, although at a much lower density than that observed in the adult retina. No other staining was apparent in the rest of the retina.

Analysis of the occurrence of the labeling for anserine-LI was also conducted in the cornea. During development, the corneal epithelium originates from the surface ectoderm, while the corneal endothelium develops from the peri-ocular mesenchyme (Soules and Link 2005; Zhao et al. 2006). By 24 hpf, first time examined, strong anserine-LI was observed in the corneal epithelium, which was progressively detaching from the adjacent lens (Fig. 7b). This staining however was not confined to the corneal areas but could also be seen in much of the surface ectoderm (data not shown). By 48 hpf, the stroma and endothelium were visible. Staining for anserine-LI slightly intensified in the epithelium (Fig. 7c) and was associated with the epithelial marker β gH3 (Fig. 7d). At 72 h, labeling was maintained in the corneal layers that continued to thicken (Fig. 7e), and by 7 dpf, a brightly stained corneal epithelium could be observed (Fig. 7f).

Specificity of carnosine and anserine antibodies in zebrafish tissue

Although the carnosine and anserine antisera have been extensively characterized in several species including teleost fish (Margolis and Grillo 1984; Biffo et al. 1990; Panzanelli et al. 1997; Lamas et al. 2007), the contrast of our zebrafish data with those described for another teleost fish (Lamas et al. 2007) prompted us to assess the specificity of these antibodies further in the zebrafish tissue. As mentioned earlier, Western blots indicated that no zebrafish protein was specifically recognized by the antisera. Both antibodies directed against carnosine and anserine were preabsorbed respectively with BSA-carnosine and BSA-anserine conjugates before use. As illustrated in Fig. 8 for BSA-carnosine, preabsorption of the anti-carnosine antibody with BSA-carnosine or BSA-anserine did not abolish staining on zebrafish tissue, suggesting that the peptide recognized in zebrafish may be another carnosine-related peptide. Preabsorption of the anserine antiserum with BSA-anserine slightly diminished, but did not eliminate, immunostaining (Fig. 8). To validate our preabsorption results in the zebrafish, we conducted similar experiments on mouse tissue. Preabsorption of the carnosine antiserum with BSA-carnosine completely abolished the staining in mouse brain (Fig. 8). The preabsorbed anserine antibody was then assessed on mouse muscle tissue, since anserine is almost completely absent from the mouse brain. Preabsorption of the anserine antiserum resulted in the elimination of the immunostaining in mouse muscle (Fig. 8).

Effects of exogenous carnosine on embryonic development

A number of studies, including ours, have shown that carnosine has substantial neuroprotective properties in animal models of cerebral ischemia (Rajanikant et al. 2007; Tang et al. 2007). In contrast, anserine does not exert significant neuroprotection against permanent focal cerebral ischemia (Min et al. 2008). Therefore, carnosine appears to be a promising candidate for the treatment of fetal hypoxia/ischemia. No data, however, is yet

available on the influence that exogenously administered carnosine may have on normal development. Thus, we monitored the possible morphological changes in zebrafish embryos exposed to various concentrations of carnosine from 4–6 hpf to 7 dpf.

In an initial experiment, we tested six different concentrations of carnosine ranging from 0.01 μ M to 1000 μ M. As illustrated for the 1 mM carnosine concentration, gross morphological examination of the embryos revealed no significant developmental abnormalities between control (Fig. 9a, c) and carnosine-treated embryos (Fig. 9b, d). CNS structures such as the forebrain, midbrain, hindbrain, and eye were readily visible at around 22 hpf (Fig. 9a, b). In addition, melanogenesis proceeded normally between experimental groups as shown at 48 hpf (Fig. 9c, d). Carnosine treatment did not affect embryo/larvae survival, since no statistically significant differences in the survival rate were observed at 7 days post-treatment between control and treated embryos ($P=0.710$; Fig. 9e). In addition, exposure to carnosine had no effect on the hatching time between experimental groups ($P=0.875$).

To determine the maximal concentration of carnosine tolerated, we then exposed embryos to 0.1, 1, 10, and 100 mM carnosine for 7 days. None of the embryos/larvae maintained in 0.1, 1, and 10 mM concentrations showed any significant adverse effects in their development (Fig. 10b, e, h, k), hatching frequencies (Fig. 11a), or survival rates (Fig. 11b) compared with the controls (Fig. 10a, d, g, j). In contrast, all embryos exposed to the highest concentration of 100 mM carnosine exhibited delays in their developmental progression; these were visible as early as 24 h following the initiation of treatment (Fig. 10c). Whereas controls and embryos treated with 0.1, 1, or 10 mM carnosine displayed morphologies characteristic of the 24-hpf time point, embryos exposed to 100 mM carnosine exhibited morphologies corresponding to embryos aged 13–16 hpf (Fig. 10a–c). Two days following exposure, embryos maintained in 100 mM carnosine were notably smaller and thinner and exhibited delayed or paler pigmentation (Fig. 10f) as compared with the other experimental groups (Fig. 10d, e). In addition, a statistically significant difference ($P<0.001$) was noted in the time of onset of hatching in the group treated with 100 mM carnosine compared with the other experimental embryos (Fig. 11a). The morphological differences observed at 48 h following the initiation of carnosine exposure were maintained up to day 4 (Fig. 10g–i) at which time 85% of the larvae treated with 100 mM carnosine died (Fig. 11b). By day 5, no survivors could be found in the group treated with 100 mM carnosine (Fig. 11b).

To determine whether the effects observed following treatment with 100 mM carnosine were attributable to an osmolarity effect, embryos were also exposed to 100 mM or 200 mM mannitol. In contrast, to the results with 100 mM carnosine (Fig. 11d), no differences in morphology, time of onset of hatching ($P=0.634$), or survival rate ($P=0.531$) were observed between controls (Fig. 11c) and mannitol-treated embryos (Fig. 11e, f).

Discussion

Using two well-characterized carnosine and anserine antisera, we have established the presence and distribution of carnosine-LP in the olfactory system and the eye of the developing and adult zebrafish. Furthermore, we also show that exposure to log-spaced concentrations of 0.01 μ M to 10 mM carnosine do not exhibit significant toxic effects on embryonic developmental parameters and survival rates, thus providing a basis for further investigation of the role of carnosine in the developing zebrafish.

Specificity of the antibodies

The carnosine and anserine antisera used in this study have been shown to label carnosine and anserine successfully in the nervous system of a variety of species including mammals,

reptiles, amphibians, and, more recently, fish (Biffo et al. 1990; Artero et al. 1991; De Marchis et al. 2000b; Lamas et al. 2007). Our observation that labeling in the zebrafish is not eliminated following pre-absorption raises the possibility that the antibodies recognize a carnosine-LP that is different from anserine and carnosine. Several observations support the likelihood that carnosine-related dipeptides are detected. First, both carnosine and anserine antibodies have been extensively characterized and do not recognize other amino acids, such as histidine or glycine (Biffo et al. 1990). Second, even though the presence of N-acetylhistidine has been reported in the zebrafish (Baslow and Ruggieri 1966; Yamada et al. 2008), no significant reduction in immunolabeling has been observed following the pre-absorption of both antisera with a BSA-N-acetylhistidine conjugate (data not shown), suggesting that the immunodetection signal does not correspond to this peptide. Third, the observation that robust immunoreactivity is present in the olfactory pathway is similar to that described for carnosine-LP in other species (Margolis 1974; Bonfanti et al. 1990; Artero et al. 1991). One possibility is that the dipeptide identified in the zebrafish is homocarnosine, since the carnosine antiserum recognizes carnosine, anserine, and homocarnosine (Biffo et al. 1990). Furthermore, the presence of homocarnosine has been reported in the retina of the goldfish, a cypriniforme like the zebrafish (Margolis and Grillo 1984). The anti-anserine antibody, although pre-absorbed with a BSA-carnosine conjugate, might retain some affinity for homocarnosine detectable by immunofluorescence. Since homocarnosine is not commercially available at present, we have been unable to verify this hypothesis. A second possibility is that the antibodies recognize the N-acetylated forms of carnosine and anserine or another carnosine derivative such as balenine/ophidine (Crush 1970; O'Dowd et al. 1990; Abe 2000). Finally, we cannot exclude that a novel carnosine-LP specific to zebrafish is being detected.

Tissue distribution of carnosine-LP in the zebrafish

Few data are currently available concerning the distribution of carnosine-LP in the fish nervous system. In a recent study involving the use of the same carnosine antiserum, cells immunoreactive for carnosine have been reported in the brain of the teleost gray mullet (Lamas et al. 2007); interestingly, the carnosine is mostly localized in neurons distributed throughout the brain. In contrast to these observations, our data show that, in the zebrafish, carnosine-LI and anserine-LI are concentrated in the olfactory pathway and to a lesser degree in the retina, whereas it is almost absent from the brain and spinal cord. We cannot exclude the possibility that carnosine and anserine are indeed present in the zebrafish brain but at concentrations too low to be detected by our immunofluorescence techniques. Nevertheless, our data reveal variations in the brain content of carnosine-LP between one cypriniform (zebrafish) and one perciform (mullet) teleost fish; this might reflect different behavioral capabilities and/or represent adaptive environmental responses unique to both species.

Carnosine-LP in developing and adult zebrafish olfactory pathway

The presence of elevated levels of carnosine-LI and anserineLI in the olfactory epithelium and bulb observed in the zebrafish is reminiscent of previous descriptions in birds and mammals (Margolis 1974; Burd et al. 1982; Biffo et al. 1990; Bonfanti et al. 1999). In the adult zebrafish, carnosine-LP staining has been observed in sensory neurons, suggesting a possible role in odor processing. However, as a departure from what has been described in other species (Bonfanti et al. 1999), carnosine-LP is also present in the non-sensory portion of the olfactory epithelium in zebrafish. Little is known about the role played by cells from the non-sensory epithelium in fish, and the function that carnosine-LP might play in these cells is unclear. Our data also show that fibers positive for carnosine-LP terminate in glomerular structures. In the zebrafish, olfactory glomeruli are organized in a map-like manner so that sensory neurons expressing similar subfamilies of olfactory receptors

converge onto topographically determined glomeruli in the olfactory bulb (Baier et al. 1994; Laberge and Hara 2001; Sato et al. 2007). Our data show that carnosine-LI and anserine-LI fibers terminate in a majority of the glomeruli, suggesting that carnosine-LP are contained in olfactory sensory neurons, independently of the receptor genes that they express. Since no immunoreactivity has been observed in olfactory bulb cells, the positive fibers observed in the medial lateral tract and the ventral medial telencephalon probably originate from the olfactory epithelium. Indeed, olfactory neurons in the fish have been shown to project not only to the olfactory glomeruli, but also to more caudal targets (Becerra et al. 1994).

Earlier immunohistochemical studies on the ontogeny of carnosine-LP in the rat nervous system have demonstrated the occurrence of carnosine in the rat olfactory epithelium as early as embryonic days 13–14 (Margolis et al. 1985; Biffo et al. 1992; De Marchis et al. 2000a). As in mammals, our study indicates that anserine-LI and carnosine-LI are present in the developing olfactory epithelium as early as 18 hpf, the intensity of immunostaining increasing as the olfactory placode progressively differentiates into the olfactory epithelium. As observed in the adult zebrafish olfactory epithelium, labeling is concentrated in both neuronal and non-neuronal cells of the olfactory placode. This is in accordance with previous reports demonstrating that, in the zebrafish, receptor neurons and supporting cells derive from placodal cells (Hansen and Zeiske 1993; Hansen and Zielinski 2005). Transient pioneer neurons have been shown to form the initial connections between the olfactory placode and the developing olfactory bulb in the zebrafish (Whitlock and Westerfield 1998). These neurons observed at 24 hpf have large cell soma and are located basally in the olfactory placode. We have occasionally observed carnosine-LI and anserine-LI fibers at the 24-hpf developmental stage. However, the earliest time point at which a significant number of immunoreactive fibers emerges from the olfactory placode is approximately 48 hpf; this suggests that olfactory pioneer neurons probably do not contribute to many of the carnosine-LI and anserine-LI cells.

Carnosine-LP in developing and adult zebrafish eye

Our study has also shown that carnosine-LP is present in the zebrafish eye, where it is localized in the retina and the cornea. Previous studies have examined the presence of carnosine-LP in the retina and revealed that the retina of fish and mammals contains low levels of homocarnosine, that the amphibian retina is rich in carnosine, and that the avian retina contains anserine (Margolis and Grillo 1984; see references in Bonfanti et al. 1999). In some of these species, the cellular localization of carnosine-LP is variable with carnosine-LP being found in photoreceptors, bipolar neurons, and Müller glial cells (Margolis and Grillo 1984; Bonfanti et al. 1999). In the zebrafish, carnosine-LI and anserine-LI are restricted to the outer nuclear layer at the inner segments of photoreceptors but have not been detected in any other retinal cell type. Immunostaining in the outer nuclear layer appears at about 7 days of development, concomitant with the differentiation of the photoreceptor cells (Malicki 1999). In frogs, the co-localization of carnosine with glutamate has been previously demonstrated in photoreceptors and bipolar neurons (Panzanelli et al. 1997) and has led to the hypothesis that carnosine plays the role of a glutamate modulator in the retina. Glutamate is present in the outer nuclear layer of the zebrafish retina (Connaughton et al. 1999), but whether such a similar role is assumed in the fish eye has yet to be demonstrated. Interestingly, a previous study indicates that zinc plays the role of a neuromodulator in the zebrafish outer retina, and that exogenous application of histidine increases the retinal electroretinogram b-wave (Redenti and Chappell 2002). Carnosine-LP have been shown to chelate various elements such as copper and zinc (Trombley et al. 2000). Therefore, carnosine-LP might play a role in visual information processing via their chelating properties in the zebrafish.

We have also observed intense immunostaining for carnosine-LP in the corneal epithelium; this has not been described in other species. Whether the presence of carnosine in the cornea is specific to the zebrafish or can be generalized to other vertebrates remains to be determined. In fish, the corneal epithelium acts as a barrier to the aquatic environment regulating ion exchange. Carnosine-LP have been shown to display cytosolic buffering capabilities in fish (Abe 2000); these might be crucial at the interface between the eye and surrounding water. Furthermore, different healing rates have also been reported for the cornea between fish and mammals (Ubels and Ebelhauser 1982). Carnosine-LP are known for their wound healing properties, and the presence of high levels of carnosine-LP in the corneal epithelium might be responsible for such a difference. Interestingly, histidine-related peptides have previously been shown to have beneficial effects on cataracts both in fish (Breck et al. 2005) and in humans (Babizhayev et al. 2001).

Effects of carnosine chronic exposure on embryonic development

The proposed neuroprotective role of carnosine in the damaged brain following cerebral ischemia has prompted us to investigate whether carnosine could also promote neuroprotection during development. Utero-placental vascular diseases result in a reduction of placental blood flow that may severely compromise the fetus (Kaplan 2007). Furthermore, low plasma levels of carnosine in diabetic pregnant rats have recently been reported to compromise fetal development (Aerts and Van Assche 2001), suggesting that carnosine also exerts disease-ameliorating properties during embryogenesis. No side effects have been reported in adult rodents following exogenous administration of carnosine (Rajanikant et al. 2007). However, although *in utero* exposure to such a dipeptide might be of clinical relevance, its possible toxic effects on embryonic development have first to be carefully evaluated.

Because of its transparency and fast development, the zebrafish embryo has become a tool widely used to investigate the teratogenic effects of a variety of compounds (Zon and Peterson 2005). In this study, we have tested a wide range of carnosine concentrations, including physiologically relevant doses. An assessment of the exact amount of carnosine taken up by the embryos is difficult, since this dipeptide has been directly supplemented to the embryo water. Zebrafish embryos are able to absorb small molecules; additionally, by 5 dpf, a fully functional gastrointestinal tract facilitates an oral route of absorption (Wallace and Pack 2003). Our data show that exposure to carnosine concentrations ranging from 10 mM to 0.01 μ M during the first developmental week does not elicit any toxic response. Conversely, 100 mM carnosine treatment delays development, accelerates hatching timing, and ultimately results in larval death. This effect is not attributable to a change in osmolarity since treatment with 100 mM or even 200 mM osmotic controls has no significant effect on embryonic survival and hatching time. The mechanisms underlying the earlier onset of hatching observed following exposure to excessive carnosine levels are unclear. At the time of hatching, zebrafish embryos secrete hatching enzymes to digest the enveloping chorion (Inohaya et al. 1997). Our data suggest that, at high concentrations, carnosine induces the expression or activation of such hatching enzymes. Similar deleterious effects have been observed when using 100 mM carnosine from a different manufacturer (Hamari Chemicals), although it took the zebrafish larvae 2–4 days longer to exhibit developmental delay and subsequently die. Such differences in the time course of carnosine effects between the two manufacturers may be attributable to the presence of contaminants.

Further studies aimed at dissecting the effects of carnosine on embryonic development need to be undertaken to pinpoint the mechanisms of the developmental delay and cell death induced by 100 mM carnosine. The teratogenic potentials of metal chelators have previously been reported (Domingo 1998), and carnosine at high levels might exert its detrimental effects through its metal-chelating properties, which might interfere with enzyme function

and mineral metabolism. These effects of carnosine on embryonic development are of interest in the light of studies suggesting that a large excess of carnosine can be detrimental. Carnosinemia is a rare autosomal recessive metabolic disorder characterized by a deficiency in serum carnosinase, the degradative enzyme for carnosine. Patients affected with carnosinemia display excessive carnosine levels in the blood, urine, and cerebrospinal fluid associated with neurological symptoms, including developmental delays, mental retardation, and seizures (Perry et al. 1967; Wisniewski et al. 1981; Willi et al. 1997).

Concluding remarks

The present study analyzes the distribution of carnosine-LI and anserine-LI in the zebrafish nervous system during development and adulthood. Our results demonstrate that carnosine-LP are developmentally regulated and are mainly concentrated in the olfactory pathway and, to a lesser degree, in the retina, where they are sustained throughout adulthood. Furthermore, we also show that exposure to carnosine during development has concentration-dependent effects on embryonic development. A definition of the role that carnosine-LP play in the nervous system in the developing zebrafish and in zebrafish models of diseases should contribute significantly to the design of future therapeutic approaches.

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Fig. 1.

Microscopic analysis of immunofluorescence staining for carnosine-like immunoreactivity (*car-LI*; **b**, **c**) and anserine-like immunoreactivity (*ans-LI*; **d**) in the adult zebrafish olfactory epithelium. **a** DAPI staining illustrating the olfactory epithelium cytoarchitecture (*ns* non-sensory epithelium, *r* raphe, *s* sensory epithelium). **b** Immunofluorescence detection of *car-LI* illustrating positive staining in the non-sensory and sensory portions of the epithelium (*arrow* *car-LI*-positive fibers coursing in the raphe). **c** Higher magnification of the *boxed area* in **b**. Cells positive for *car-LI* localize in the middle (*arrows*) and apical (*arrowheads*) portions of the sensory olfactory epithelium. **d** Immunofluorescence detection of *ans-LI* illustrating positive staining in both non-sensory and sensory regions of the epithelium. Bars 90 μ m (**a**, **b**), 20 μ m (**c**), 58 μ m (**d**)

Fig. 2.

Micrographs of transverse sections through the olfactory epithelium and olfactory bulb from the adult zebrafish. Immunofluorescence detection of anserine-like immunoreactivity (*ans-LI*). **a–c** The merged image illustrates the association between *ans-LI* (blue) and the early neuronal marker HuC/D (red) in the olfactory epithelium (arrows). **d–f** The merged image illustrates the association between *ans-LI* (red) and the synaptic marker SV2 (blue) in the olfactory bulb. Bars 10 μ m (**a–c**), 50 μ m (**d–f**)

Fig. 3. Microscopic analysis of immunofluorescence staining for anserine-like immunoreactivity (*ans-LI*) in sagittal sections through the adult zebrafish brain. **a** DAPI staining (*dapi*) illustrating the olfactory bulb cytoarchitecture (*gl* glomerular layer, *icl* internal cell layer, *onl* olfactory nerve layer). **b** Immunofluorescence detection of *ans-LI* in the olfactory bulb. Immunopositive fibers are present in the olfactory nerve and glomerular layers. **c** Bundles of *ans-LI* fibers are observed in the ventral medial telencephalic area. **d** Meningeal cells robustly positive for *ans-LI* (*arrows*) are found in the cerebellum (*cer*). Bars 100 μm (**a**, **b**), 30 μm (**c**, **d**)

Fig. 4.

Confocal micrographs of transverse sections through the adult zebrafish retina.

Immunofluorescence staining for anserine-like immunoreactivity (*ans-LI*). **a** DAPI nuclear staining (*dapi*) illustrating the retinal cytoarchitecture (*INL* retinal inner nuclear layer, *IPL* retinal inner plexiform layer, *ONL* retinal outer nuclear layer, *OPL* retinal outer plexiform layer, *GCL* retinal ganglion cell layer). **b** Immunofluorescence detection of *ans-LI* (green) and the double cone marker *zpr1* (red) showing positive puncta in the inner segments of the cone photoreceptors. **c** Immunofluorescence detection of *ans-LI* and the rod photoreceptor marker *zpr3*. **d–f** Merged image showing the association between *ans-LI* and the corneal epithelial marker β igH3 (yellow). Nuclei are stained blue with DAPI (*ce* corneal epithelium). **g–i** Merged image illustrating the absence of association between *ans-LI* and the stromal marker keratan sulfate (*KS*). Nuclei are stained blue with DAPI (*cs* corneal stroma). Bars 11 μ m (**a–c**), 8 μ m (**d–i**)

Fig. 5.

Micrographs of horizontal sections through the olfactory placode/epithelium from zebrafish embryo/larva at 18 hpf (**a**), 24 hpf (**b**), 48 hpf (**c**), 72 hpf (**d**), and 7 dpf (**e**).

Immunofluorescence detection of anserine-like immunoreactivity (anserine-LI). **a**

Immunopositivity for anserine-LI was detected as early as 18 hpf in the zebrafish embryo. **b**

At 24 hpf, numerous cells positive for anserine-LI could be observed in the olfactory placode (*Tel*/telencephalon). A few positive fibers could be detected at the base of the placode (*arrowhead*).

c At 48 hpf, strong immunolabeling was present in the olfactory placode, and a thick fiber bundle (*arrowheads*) could be seen exiting from the placode and heading toward the telencephalon. **d** By 72 hpf, positive cells were observed at the outer portions of the olfactory epithelium. Note the intense dendritic staining close to the nasal cavity (*n*).

e, f Intense anserine-LI was observed in the olfactory epithelium at 7 dpf (**e**), and positive fibers were readily observed in the adjacent telencephalon (*arrows* in **f**).

Bars 15 μ m (**a–c**), 20 μ m (**d, e**), 40 μ m (**f**)

Fig. 6. Confocal micrographs of horizontal sections through the olfactory placode from a 48 hpf zebrafish embryo. Immunofluorescence detection of anserine-like immunoreactivity (*ans-LI*). **a–c** Immunofluorescence co-staining for *ans-LI* (green in **a**) and the neuronal marker HuC/D (red in **b**) demonstrates that a subset of cells positive for *ans-LI* are neurons (arrowheads in **c**). **d–f** Immunofluorescence co-staining for *ans-LI* (green in **d**) and acetylated tubulin (red in **e**) shows the association of these two markers (yellow-orange in **f**) in the olfactory placode (*OP* olfactory placode, *Tel* telencephalon). Bars 15 μ m (**a–c**), 40 μ m (**d–f**)

Fig. 7.

Micrographs of horizontal sections through the retina (**a**) and the cornea (**b–f**) from zebrafish embryo/larva, at 24 hpf (**b**), 48 hpf (**c**, **d**), 72 hpf (**e**), and 7 dpf (**a**, **f**).

Immunofluorescence detection for anserine-like immunoreactivity (*ans-LI*). **a** *Ans-LI* in the retina at 7 dpf (*INL* inner nuclear layer, *ONL* outer nuclear layer). Note the presence of a discrete punctate immunolabeling in the outer nuclear layer (*arrows*). **b–f** Confocal micrographs illustrate *ans-LI* staining (*green*) in the cornea at various developmental stages. DAPI nuclear staining (*blue*) illustrates the cytoarchitecture of the eye. **d** Note the association of *ans-LI* with the epithelial marker β igH3 (*yellow*). Bars 23 μ m (**a**), 20 μ m (**b**, **c**, **e**, **f**), 4.5 μ m (**d**)

Fig. 8.

Immunofluorescence staining analysis of the specificity of the carnosine and anserine antibodies in adult and mouse zebrafish tissue. Carnosine and anserine antibodies resulted in robust immunostaining in the zebrafish olfactory bulb (*OB*). Whereas the blocking of the carnosine and anserine antibodies with, respectively, BSA-carnosine and BSA-anserine did not eliminate the labeling in the zebrafish tissue, it completely abolished the immunostaining in the mouse brain (*Carn-blocking*) and muscle (*Ans-blocking*). Bars 65 μm (*top row*), 70 μm (*bottom row*)

Fig. 9. Effects of exposure to various concentrations of carnosine on zebrafish embryo development and survival. **a–d** Lateral views of live embryos (*right* anterior, *top* dorsal). Carnosine treatment was initiated at 6 hpf. **a, b** Bright-field micrographs of a control and an embryo treated with 1 mM carnosine (1 mM CAR), respectively, at about 22 hpf (*hb* hindbrain, *mb* midbrain, *ov* otic vesicle). **c, d** Control and an embryo treated with 1 mM carnosine (1 mM CAR), respectively, at 48 hpf. Bar 160 μm. **e** Survival rates of zebrafish embryos chronically exposed to 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, and 1000 μM for 7 days. No effect on survival was observed following carnosine treatment between treated and control embryos. Histogram values represent means ± SEM

Fig. 10.

Bright-field micrographs of zebrafish control embryos/larvae (**a, d, g, j**) and zebrafish embryos/larvae exposed to 10 mM (**b, e, h, k**) or 100 mM carnosine (**c, f, i, l**) at 24 hpf (**a–c**), 48 hpf (**d–f**), 72 hpf (**g–i**), and 96 hpf (**j–l**). Whereas controls and embryos treated with 10 mM carnosine exhibit normal development, embryos exposed to 100 mM show signs of delays in their development as characterized by their smaller size and paler pigmentation (*right anterior, top dorsal*). Bars 250 μm (**a–f**), 110 μm (**g–l**)

Fig. 11.

Effects of exposure to 0.1 mM, 1 mM, 10 mM, and 100 mM carnosine on zebrafish embryos/larvae. **a** Quantitative analysis of the rate of hatching success in control and carnosine-exposed embryos. Histogram values represent means $\pm$ SEM. ***Statistically significant difference ($P<0.001$) between embryos exposed to 100 mM carnosine and controls. **b** Quantitative analysis of survival rates for control embryos and embryos chronically exposed to 0.1, 1, 10, and 100 mM carnosine for 7 days. Daily survival rates are expressed as percentages. Histogram values represent means $\pm$ SEM. ***Statistically significant difference ($P<0.001$) between embryos exposed to 100 mM carnosine and

controls. (c–f) Bright-field micrographs of 24 hpf embryos exposed to egg water (*CONTROL*), 100 mM carnosine (*100 mM CAR*), 100 mM mannitol (*100 mM Mann*), or 200 mM mannitol (*200 mM Mann*). Mannitol-treated embryos exhibit developmental features similar to control embryos, whereas carnosine-treated embryos exhibit developmental delay. Bar 230 μm (c–f)