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Distribution and chronotropic effects of serotonin in the zebrafish heart

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ABSTRACT

Several lines of evidence suggest that serotonin (5-HT) has a regulatory role in cardiovascular function from embryogenesis through adulthood. However, the reported actions of 5-HT are often contradictory and include bradycardia or tachycardia, hypotension or hypertension, and vasodilation or vasoconstriction. Clarifying such cardiac effects requires further research and may benefit from utilizing a model simpler than the mammalian hearts traditionally used in these studies. In the present study, we describe the cardiac distribution and chronotropic responses of 5-HT in the zebrafish heart. A combined anatomical, electrophysiological, and pharmacological approach was used to investigate the involvement of 5-HT pathways, and to compare neural and direct myocardial pathways of biological action. Immunohistochemical methods revealed 5-HT in endocardial cells, glial-like cells, and intracardiac neurons in the atrium. Electrocardiogram (ECG) recordings combined with the administration of pharmacological agents demonstrated that 5-HT acted predominantly through direct myocardial pathways resulting in a reduction of heart rate. Overall, the results of this study contribute significant advances in the establishment of the zebrafish as a new model for studies of the role of 5-HT in autonomic cardiac control.

1. Introduction

Serotonin (5-hydroxytryptamine; 5-HT) was first isolated from blood and defined as a vasoconstrictor (Nebigil and Maroteaux, 2001), but has also been shown more recently to have diverse cardiovascular effects (Frishman and Grewall, 2000). Described effects include bradycardia or tachycardia, hypotension or hypertension, and vasodilation or vasoconstriction, and these effects vary greatly depending on route of administration and experimental parameters (see review by Nebigil and Maroteaux, 2001). Clarification of these complex and seemingly conflicting effects requires further research and may benefit from the employment of simpler models than the mammalian hearts traditionally used in these studies.

The zebrafish heart has been proposed as a powerful tool for studying cardiac electrophysiology, with the potential to provide broad insights into cardiovascular function (Briggs, 2002; Chi et al., 2008; Dvornikov et al., 2014; Nemtsas et al., 2010; Rider et al., 2012; Tessadori et al., 2012). Stoyek et al. (2015) previously described the basic structure of the intracardiac nervous system in zebrafish, which is consistent with that of humans and other mammalian models (Irisawa, 1978; Mangoni and Nargeot, 2008; Pauza et al., 2013; Pauza et al., 2014; Li et al., 2015). In addition, the zebrafish appears to have genes encoding for autonomic receptors, including 5-HT receptors, (5-HT: Airhart et al., 2007; Prieto et al., 2012; Maximino et al., 2013; Stewart et al., 2013), that are homologous to those that affect heart rate (HR) in mammals (β-adrenergic/catecholaminergic: Steele et al., 2011; nicotinic, muscarinic and β-adrenergic; Stoyek et al., 2016).

The study of serotonergic processes in zebrafish is well established. Previous reports have described behavioral responses (predominantly a decrease in spontaneous locomotor activity) to 5-HT exposure that are similar to those observed in mammalian studies, indicating the potential validity of the zebrafish model in such research (Airhart et al., 2007; Prieto et al., 2012; Maximino et al., 2013; Stewart et al., 2013). To date investigations of the organ-specific physiological effects of 5-HT in zebrafish have been limited, focusing primarily on the role of pathways involved in release of 5-HT from chromaffin cells in the head kidney (Steele et al., 2011) or chemosensory neuroepithelial cells in the gills (Jonz et al., 2004). While 5-HT is known to be present in the circulatory system of the zebrafish (Steele et al., 2011), information on the cardiac effects of 5-HT is lacking, with only a single report alluding to chronotropic effects of high doses of fluoxetine, a commonly used 5-HT selective reuptake inhibitor (SSRI; Airhart et al., 2007).

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The aim of the present study was to investigate the distribution of 5-HT and determine the chronotropic responses to 5-HT application in isolated zebrafish hearts. A combined anatomical, electrophysiological, and pharmacological approach was used to investigate the role of 5-HT, and to define the neural- and tissue-level actions of this agent. We used immunohistochemical methods to characterize the distribution of 5-HT and tryptophan hydroxylase (TPH, the rate-limiting enzyme in the production of 5-HT; Levy, 2006) within intracardiac neurons (ICNs) and myocardial cells. Electrocardiogram (ECG) recordings combined with bath-applied pharmacological agents were used to demonstrate in the isolated zebrafish heart that 5-HT acted predominantly through direct myocardial pathways to modulate heart rate (HR). The results of this study establish this preparation as a tractable model to investigate serotonergic effects on the heart.

2. Methods

2.1. Animals

A total of 35 AB strain adult zebrafish (12–18 month post fertilization; 33 ± 5 mm standard body length) was used in this study. Procedures for animal care and use followed the "Guidelines on the Care and Use of Fish in Research, Teaching and Testing" document issued by the Canadian Council of Animal Care. Institutional approval (Protocol #15-006) for animal use in this study was obtained from the Dalhousie University Committee on Laboratory Animals.

2.2. Animal husbandry

Animals were acquired from breeding stocks in the Faculty of Medicine Zebrafish Facility at Dalhousie University. Fish were maintained in standard 3–10 L tanks (Aquatic Habitats, Apopka, FL, USA) at 28.5 °C, supplied continuously with conditioned water from a recirculating water system, and subjected to a 14 hour light:10 hour dark illumination cycle. Zebrafish were fed commercial, dry fish food (Golden Pearl pellets, Brine Shrimp Direct, Ogden, UT, USA) and live *Artemia* (raised in-house) twice a day.

2.3. Heart isolation

Zebrafish hearts were isolated following procedures described in Stoyek et al. (2016). Briefly, fish were anaesthetised in a buffered solution (pH 7.2) of tricaine (MS-222; 1.5 mM; Sigma–Aldrich, Oakville, ON, Canada) in tank water (28.5 °C) until opercular movements ceased and the animals lacked response to a fin pinch with forceps. A ventral midline incision was made through the body wall to expose the heart, and a block of tissue encompassing the ventral aorta, ventricle, atrium, sinus venosus and ducts of Cuvier was then removed for whole-mount immunohistochemistry or *in vitro* recordings.

2.4. Tissue preparation for immunohistochemistry

Tissues were fixed overnight in 4% paraformaldehyde (PFA; RT-15710, Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate-buffered saline (PBS, composition in mM: 50 Na_2HPO_4, 140 NaCl, pH 7.2). To visualize the overall spatial relationship of serotonergic elements within the heart of the zebrafish, the whole-mount procedures used for immunohistochemistry in this study were similar to those described in previous publications (Newton et al., 2014; Stoyek et al., 2015; Stoyek et al., 2016). Briefly, fixed tissues were rinsed in PBS and then transferred to a PBS solution containing 2% Triton X-100 (X100, Sigma Aldrich), 1% bovine serum albumin (BSA; A9576, Sigma Aldrich) and 1% normal goat serum (NGS; G9023, Sigma Aldrich) for 48 h at 4 °C with gentle agitation. Tissues were then incubated with primary antibodies (see Section 2.5), which were diluted in a solution containing 0.25% Triton X-100, 1% BSA and 1% NGS in PBS (designated PBS-T). After incubation in primary antibodies for 3–5 d with agitation at 4 °C, the tissues were rinsed in PBS-T, and then transferred to a solution of PBS-T containing the appropriate secondary antibodies, raised in mouse or rabbit, and conjugated to AlexaFluor 488, 555, or 647 fluorophores (Life Technologies, Burlington, ON, Canada). Incubation time with secondary antibodies was also 3–5 d with agitation at 4 °C. Final rinsing was done in PBS, and then specimens were placed in Scale CUBIC-1 clearing solution (Susaki et al., 2014) overnight at room temperature with gentle agitation. Tissues were mounted on glass slides in CUBIC-1 for confocal microscopy.

2.5. Antibodies

Serotonergic elements were detected with an anti-5-HT antibody (dilution 1:100; 20080, Immunostar). This antibody has been used in previous studies in zebrafish (Uyttebroek et al., 2010; Jackson et al., 2013). Antibodies against tryptophan hydroxylase (TPH; dilution 1:100; P21961, Life Technologies), the rate-limiting enzyme in the synthesis of 5-HT, and tyrosine hydroxylase, the rate limiting enzyme in the synthesis of norepeinephrine (TH; dilution 1:100; 22941; Immunostar) were used to investigate potential synthetic pathways for monoamines. As the TPH antibody was raised in the same host as the 5-HT antibody, it was not possible to co-label for 5-HT in the same heart. To compare 5-HT elements to the general innervation of the heart, antibodies against acetylated tubulin (AcT, axons; dilution 1:250; T6793, Sigma Aldrich) and human neuronal protein C/D (Hu, neuronal somata; dilution 1:250; A21271, Life Technologies) were combined as previously described (Stoyek et al., 2015). In a separate trial (n = 8) hearts were processed as outlined above, except that either the primary or secondary antibody was omitted, both of which eliminated detection of histofluorescence in all specimens.

2.6. Myocardial labelling

In order to determine how immunohistochemically labelled neuronal elements were related to the regional structure of the myocardium, some specimens were double-labelled either with AcT-Hu or 5-HT antibodies in combination with the F-actin marker phalloidin (77418, Sigma Aldrich; Stoyek et al., 2015), conjugated with tetramethyl rhodamine isothiocyanate to show cardiac myocytes.

2.7. Imaging

Tissues were viewed using a Zeiss LSM510 confocal microscope operating with Zen2009 software (Zeiss Canada, Mississauga, ON). Preparations were epi-illuminated with a 488 nm argon laser, and 543 nm and 605 nm helium-neon lasers, reflected by a 488/543/633 nm dichroic mirror (HFT 488/543/633; Carl Zeiss AG). Emitted fluorescence was collected from the specimens, using 480–520 nm and 500–615 nm band-pass filters (BP565-615; Carl Zeiss AG) and a 565–615 nm long pass filter (LP565-615; Carl Zeiss AG) through a 10 ×, 0.45 NA objective (Plan-Apochromat SF25; Carl Zeiss AG), a 25 ×, 0.80 NA objective (LCI Plan-Neofluar; Carl Zeiss AG), or a 40 ×, 0.95 NA objective (Plan-Apochromat M27; Carl Zeiss AG). Z-stacks were taken from regions of interest surrounding immunoreactive tissues, and ranged from 20 to 100 μm in depth. Z-stack scans also encompassed 5–25 μm above and below the regions of interest to ensure that all structures were captured, while limiting issues of light scattering associated with deeper tissue scans. Image stacks were processed with Zeiss Zen2009 software. Figure plates were constructed from images processed with Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA). Brightness and contrast of some images were adjusted to ensure panel-to-panel consistency in each figure.